# SUSCEPTIBILITY OF

# MUCOSITIS-ASSOCIATED GRAM-NEGATIVE BACTERIA

# TO PHOTODYNAMIC ACTION

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by

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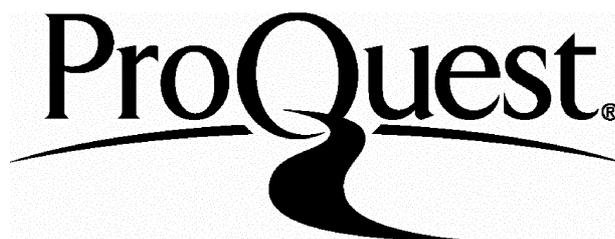Eastman Dental Institute for Oral Health Care Sciences

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*To the memory of my parents*

# ABSTRACT

Opportunistic pathogens such as enteric Gram-negative bacilli in the oral cavity of cancer patients undergoing radio/chemotherapy may contribute to the mucosal lesions (mucositis) caused by these cytotoxic therapies and also constitute a source of infection. What is more, resistance of organisms to conventional antibacterial regimes is a growing cause of concern. Photodynamic action (PDA), elimination of target cells by use of a photosensitising compound in combination with light, may be a potential alternative therapeutic regime for topical infections. This study evaluated the effectiveness of PDA on a number of potential pathogens associated with mucositis.

The PDA of toluidine blue O (TBO) with light from a HeNe gas laser on the viability of a range of Gram-negative bacilli considered to play a role in mucositis was assessed. The susceptibility of other opportunistic pathogens together with the indigenous oral flora was also studied. Gram-negative bacilli were shown to be killed by PDA the effectiveness of which was dependent on both the TBO concentration and light dose. All the organisms tested were susceptible to PDA, indicating its broad spectrum effect.

Studies of the effects of physiological factors on the efficacy of PDA showed that bacteria could be killed under all the physiological conditions tested. However, PDA in saliva and serum was not as effective as it was in saline, and greater kills were obtained at increased pHs.

Mechanistic studies demonstrated that TBO was an effective photosensitiser when outside the bacteria and indicated that the outer membrane may be the site where initial photo-damage takes place through the production of singlet oxygen and, to a lesser extent, hydroxyl radicals.

PDA was also shown to impair some virulence factors (lipopolysaccharide and proteolytic enzyme activity) of bacteria.

An animal study was conducted to elucidate any possible adverse effects on host tissues and this demonstrated that at all doses tested, PDA had no adverse effect on the buccal mucosa of rats. Furthermore, fluorescence microscopy studies demonstrated a high level of TBO in the epithelium but no detectable levels in the underlying connective tissue.

Although it was shown that killing by PDA of both laboratory and clinical strains of organisms is possible, further animal studies are needed to establish the effectiveness of this novel technique for the treatment of topical, localised infections.

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## DECLARATION

The findings reported in this thesis result entirely from my own work. Colleagues who helped me in the various aspects of the work are listed in the Acknowledgements. This work has not previously been submitted, in part or in full, for a degree or diploma of this or any other University or examination board.

# ACKNOWLEDGEMENTS

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# ABBREVIATIONS

| | |
|---|---|
| AlSPc | aluminium sulphonated phthalocyanine |
| 5-ALA | 5-aminolaevulinic acid |
| *C. albicans* | *Candida albicans* |
| CCD | charge couple device |
| cfu/ml | colony forming units per millilitre |
| DHE | dihaematoporphyrin ether |
| $D_2O$ | deuterium oxide |
| *E. coli* | *Escherichia coli* |
| GaAlAs | gallium aluminium arsenide |
| H&E | haematoxylin and eosin |
| HeNe | helium neon |
| HpD | haematoporphyrin derivative |
| HSV | herpes simplex virus |
| $^3$H-TBO | tritiated-toluidine blue O |
| IL | interleukin |
| LAL | limulus amoebocyte lysate |
| LED | light emitting diode |
| $\log_{10}$ | logarithmic |
| LPS | lipopolysaccharide |
| *K. pneumoniae* | *Klebsiella pneumoniae* |
| KTP | potassium titanium phosphate |
| MB | methylene blue |
| m-THPC | Meta-tetrahydroxyl tetraphenyl chlorin |
| Nd:YAG | neodymium:yttrium aluminium garnet |
| nm | nanometer |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PBMC | peripheral blood mononuclear cells |
| PDA | photodynamic action |
| PDT | photodynamic therapy |
| PFW | pyrogen-free water |
| PIT | pre-irradiation time |
| PTA | polymyxin, tobramycin, amphotericin B |
| *S. aureus* | *Staphylococcus aureus* |
| TBO | toluidine blue O |
| TEM | transmission electron microscopy |

**Chapter 1**

**GENERAL INTRODUCTION**

## 1.1   INFECTION IN THE ORAL CAVITY OF CANCER PATIENTS

The cytotoxic effects of radiotherapy and chemotherapy used in the management of malignant diseases are not specific for cancerous tissue and damage to normal tissue may also be induced.  The oral cavity is a frequent site of adverse effects following radiotherapy to the head and neck region and chemotherapy for both oral or distant malignancies, such as leukaemia (Toth et al., 1990; Singh et al., 1996; Scully and Epstein, 1996). These complications are more frequent and severe in children than in adults having similar treatment (Dreizen, 1990).  Oral morbidity may be so severe that suspension of the therapies may be required until such complications resolve.   Among the most common oral complications associated with cancer therapy are infections of bacterial, viral and fungal origin, mucositis (see 1.1.4), salivary gland dysfunction leading to xerostomia, haemorrhagia and dental and periodontal problems (Toth et al., 1990; Singh et al., 1996; Scully and Epstein, 1996).  The adverse effects of these cytotoxic therapies are inter-related and exacerbate the degree of discomfort and make the treatment more complicated.

Oral infections are of particular importance as they may lead to serious systemic infections via the lungs, the digestive tract and circulatory system, and can be life-threatening in cancer patients under cytotoxic therapies as many of them are immunocompromised.  The possibility of oral infection in such patient groups is mainly dependent on the microbial status of the oral cavity, damage to host defence systems and the grade of myelosuppression.

### 1.1.1   Oral Ecosystem in Health and Disease

The integrity of the oral cavity is maintained by both local and general defence mechanisms.  An intact epithelium is the first line of local defence which acts as a physical barrier to prevent penetration by micro-organisms or their antigens.  Intra-epithelial Langerhans cells and lymphocytes also serve as a cellular barrier to penetrating organisms.  Saliva in particular is an important component of oral defence, regulating the bacterial population in the mouth by a dual function.

Salivary flow provides mechanical cleansing by diluting and removing the micro-organisms (which prevents microbial adherence and their overgrowth) and debris. In addition, saliva contains non-specific (lysozyme, lactoferrin, lactoperoxidase, and histidine-rich polypeptides) and specific (immunoglobulins, especially secretory IgA) defence factors which provide an antimicrobial action. If the local defences are breached, the lymphocytes and myelocytes from the general defence mechanism then become active against invading micro-organisms (Marsh and Martin, 1999; Heimdahl and Nord, 1990; Epstein and Chow, 1999).

Despite having these extensive defence systems, the oral cavity has one of the most complex microbial ecosystems in the body due to its highly diverse and abundant microflora. It is a possible reservoir for both commensal and exogenous micro-organisms, of which bacteria are the primary constituents. The oral bacterial population of an adult is estimated to contain more than 200 different species, and these are dominated by obligate anaerobes (e.g., veillonella and fusobacteria) and facultative anaerobes (Midtvedt, 1990). Streptococci (particularly viridans group) are among the most commonly isolated organisms (Schuster, 1999). In contrast, staphylococci, enteric Gram-negative bacilli and Pseudomonas species are less frequently isolated from the mouths of the healthy population. These organisms are not generally regarded as being part of the normal oral microflora and will be referred to as non-indigenous organisms. Candida spp. on the other hand, can be isolated from 2-70% of individuals but usually in low numbers (Marsh and Martin, 1999). Although microbial carriage in the oral cavity of healthy people may vary depending on age, denture wearing, (Sedgley et al., 1995) diet and geographical distribution (Schuster, 1999; Sedgley and Samaranayake, 1994), the residential predominant microflora suppresses the overgrowth of exogenous bacteria and fungi thus providing a balance in the microflora of the oral cavity (van Saene, 1992).

In health, there is a balance between micro-organisms and the host with the colonising organisms seldom causing any problem. When the defence mechanisms are impaired by underlying diseases or therapies, organisms which would rarely be pathological in healthy individuals may be responsible for life-

threatening systemic infections (Marsh and Martin, 1999; McElroy, 1984; Heimdahl and Nord., 1990). Accumulation and multiplication of certain microbial species and infiltration of micro-organisms into tissue may cause opportunistic local and systemic infections.

The importance of the oral flora in immunocompromised patients who are less able to respond to any infection due to compromised defence systems is well established. Members of the oral flora may pose a threat for systemic infection in certain patient groups including patients with endocardial defects, patients with active renal disease or diabetics, leukemia, organ transplant patients and patients receiving therapeutic antimetabolites, irradiation and those on long term systemic steroid treatment and HIV disease (Ellen, 1978; Sowell, 1982; Hills-Smith and Schuman, 1983; Scully, 1992). Surgical procedures, or any manipulation in the oral cavity of such patients including both soft and hard tissues, may result in detectable levels of oral bacteria in the blood and systemic infections. For instance viridans streptococci may cause bacterial endocarditis or glomerulonephritis in renal transplant patients (Longman and Martin, 1999).

In the case of patients with a compromised defence system, an imbalance in the composition of the oral flora, with an increase in number and pathogenicity of the normal oral inhabitants and the introduction and proliferation of exogenous pathogenic organisms can be observed (Marsh and Martin, 1999; McElroy, 1984; Heimdahl and Nord., 1990). Commensal bacteria, e.g., streptococci, as well as non-commensal organisms, can be potentially pathogenic in these circumstances (Heimdahl et al., 1989; Donnelly et al., 1993; Beighton et al., 1994; Dreizen and Bodey, 1983; Dreizen et al., 1986; Greenberg et al., 1982; Bergmann, 1991; Galili et al., 1992).

An alteration in the oral ecosystem with increased colonisation of enteric Gram-negative bacteria, fungi and staphylococci in patients with compromised defence mechanisms has been reported (van Saene, 1992; Johnson and Yu, 1989; Sedgley et al., 1995; Pindborg, 1989; Schmidt-Westhausen et al.,1991). Changes in the ecology of the oral flora in patients undergoing anti-neoplastic treatments

(radio/chemotherapy) is particularly well established (Martin *et al.*, 1981; Greenberg *et al.*, 1982; Dreizen and Bodey, 1983; Main *et al.*, 1984; Dreizen *et al.*, 1986; Minah *et al.* 1986, Epstein and Gangbar, 1987; Makkonen *et al.*, 1989; Bergmann, 1991; Galili *et al.*, 1992; Sixou *et al.*, 1996). The disease-inducing ability of micro-organisms is generally controlled by a combination of normal host defence mechanisms and the balance of the oral flora. Either or both of these factors may be disturbed in cancer patients.

## 1.1.2 Factors Influencing the Oral Flora During Cytotoxic Therapies

The adverse effects of anti-neoplastic therapies on oral defence mechanisms may follow different pathways. In patients undergoing radiotherapy, the defects in defence mechanisms are local. These patients generally have a functioning systemic defence system. In contrast, myelosuppressed patients under chemotherapy have a significantly weakened general defence mechanism (impaired cell mediated immunity). In chemotherapy patients, particularly those treated for bone marrow transplantation (BMT), it is easier for the local infection to spread, and lead to septicaemia (Heimdahl *et al.*, 1989). Among chemotherapy patients, those with leukaemia may develop infections more frequently than those with solid tumours. This is due to a more aggressive treatment approach to already-impaired bone marrow cells causing granulocytopenia. During the period of chemotherapy-induced neutropenia and decreased neutrophil function, patients are most vulnerable to therapy-induced opportunistic infections (Bergmann, 1991). Cell mediated immunity is important against intracellular pathogens such as herpes virus, hence viral infections are also common in immunosuppressed patients (Epstein and Chow, 1999). In BMT patients with serological evidence of an earlier herpes simplex virus (HSV) infection, up to 80% are likely to have an HSV-reactivated infection if prophylaxis is not instituted (Meyers *et al.*, 1980). Infections caused by HSV, consequently, may cause disruption of the oral mucosa hence a breakdown in the first line of defence and so predispose to secondary infections by bacteria or fungi (Ringden *et al.*, 1984).

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Particularly in patients receiving radiotherapy, the local host response to infection may decrease due to alterations in the composition of saliva and its flow rate. Saliva has buffering, diluent, antibacterial, lubricant and cleansing properties, which are forfeited by the cytotoxic effects of cancer therapies. The impaired salivary flow in these patients will not be sufficient to dilute and remove micro-organisms together with food debris, which provides abundant nutrition for bacteria. The saliva of irradiated patients may also become acidic with a pH ranging between 4 and 6.9 (Ben-Aryeh *et al.*, 1975) leading to overgrowth of acidophilic bacteria. Increased proportions of *Streptococcus mutans* and lactobacilli were reported in radiotherapy patients with xerostomia (Joyston-Bechal *et al.*, 1992; Epstein *et al.*, 1998; Spijkervet *et al.*, 1989c). The composition of saliva is also affected; a reduction of lysosyme and IgA has been observed in these patient groups (Brown *et al.*, 1976; Courts and Meuller, 1983). Subsequently, saliva may fail to control certain bacteria present in the oral cavity due to the lack of such defence components (Main *et al.*, 1984).

Prolonged treatment with antibiotics and antimicrobial agents is very common in cancer patients undergoing cytotoxic therapies. A prolonged antibiotic regimen directed at particular organisms may cause emergence of species that are resistant to the antibiotic (Wade *et al.*, 1982; Bodey, 1984; Nord and Heimdahl, 1986). The use of broad spectrum antibiotics, in particular, may cause dramatic shifts in the normal flora. For instance, an increased population of *Enterobacteriaceae*, *Candida* spp. and staphylococci in the subgingival flora of periodontitis patients who received one or more broad spectrum antibiotic courses has been reported (Slot *et al.*,1988; Rams *et al.*, 1990). Bergmann (1991) observed oral acquisition of *Enterobacteriaceae*, *Enterococcus* spp. and *Candida* spp. in the saliva of acute leukaemia patients when antibiotic therapy was given during remission induction therapy. In contrast, Wahlin *et al.* (1991) reported no difference in the number of enterobacteria following antibiotic administration in the same patient group.

The ability of organisms to adhere to the mucosal surface may be related to alterations in the structure or number of receptor sites on the epithelium.

Changes in the microplication pattern of the irradiated epithelium have been reported during radiotherapy. Straight and parallel microplicae that are characteristic of normal mucosa were replaced by short, inconsistent microvilli during irradiation. Small areas with no surface projection were noted when irradiation was highest (Robertson *et al.*, 1987). Loss of fibronectin in the oral cavity of cancer patients, possibly due to the increased levels of proteolytic activity, has also been reported (van Saene, 1992). Colonisation of Gram-negative bacilli in the oral cavity of seriously-ill patients was suggested to be related to a reduction or loss of fibronectin, which may expose binding sites on epithelial cells for, and promote adherence of, Gram-negative bacilli (Hasty and Simpson, 1987). Fimbriae projecting from the cell surface of Gram-negative bacilli can recognise receptors on the surface of epithelial cells uncovered by loss of fibronectin (Beachey and Courtney, 1987).

The cytotoxic effects of ionising radiation and neoplastic agents impair the epithelial turnover rate, which results in atrophy and eventual ulceration of the oral epithelium (Squier, 1990). Mucosal inflammation and breakdown may promote an increased retention of microbial cells by the mucosa and provide a good source of nutrition for organisms. These sites may increase the prevalence of the opportunistic flora and may serve as a potential source of infection. Indeed, Dahlen *et al.* (1982) reported that 66% of 271 patients with oral mucous membrane lesions, which were not related to cytotoxic therapies, had a microflora (*Staphylococci aureus,* enterobacteria and *Candida albicans*) that was not commensal for the sampling location. In the majority of cases, micro-organisms were of pathogenic importance to the lesions.

Nosocomial introduction of the organisms to the mouth by contact with hospital personnel and other patients, and with equipment and food is also likely (Klastersky, 1989). In addition, generalised physical debilitation and inadequate hygiene may also contribute to alterations in the oral flora.

### 1.1.3 Oral Flora of Patients Receiving Cytotoxic Therapies

In this section, the oral flora of cancer patients undergoing radiotherapy or chemotherapy is described with a special emphasis on the carriage of non-resident species i.e., Gram-negative bacilli, candida, staphylococci and herpes virus.

### 1.1.3.1 Oral flora of radiotherapy patients

Colonisation and infection with candida is common in the oral cavity of patients receiving radiotherapy for head and neck cancer. Martin et al., (1981) reported that although many patients had yeast colonisation before the initiation of irradiation, there was an increased colonisation by yeast immediately after radiotherapy and this persisted for 6 months. C. albicans and C. tropicalis were the most commonly isolated species. Redding et al (1999) found that candida carriage in 73% of radiation patients (n=30) resulted in infections in 27% of these patients and these were predominantly due to C. albicans (78%).

Simultaneous colonisation of yeasts and Gram-negative bacilli has also been reported (Samaranayake et al., 1988; Makkonen et al. 1989). 23 of 25 patients who had squamous cell carcinoma in their mouths harboured both yeasts and Gram-negative bacilli on one or more occasions during a 6 week-period of radiotherapy. C. albicans was the most frequently isolated micro-organism in irradiated patients followed by Gram-negative bacilli including Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae, Klebsiella oxytoca and Escherichia coli (Samaranayake et al., 1988). While obtaining similar results, Makkonen et al. (1989) found an increase in the colonisation rate (from 20% to 80%) of the patients during therapy. A correlation between increased colonisation by yeast and Gram-negative bacilli and moderate and severe mucositis was also reported (Makkonen et al., 1989; Matthews and Ercal, 1996)

In contrast, Spijkervet et al. (1989a) demonstrated no difference between healthy subjects and post-operative head and neck cancer patients in the carriage or the

colonisation index of *Candida* spp. Furthermore, higher carriage of *Enterobacteriaceae, Pseudomonaceae, Acinetobacter* spp. and *Staphylococcus epidermidis* was found in cancer patients. Bernhoft and Skaug (1985) also reported a higher frequency of enteric bacteria than *C. albicans* in 11 radiotherapy patients.

An increase in acidogenic bacteria (including lactobacilli and mutans streptococci) has also been shown, and this was reduced after the introduction of an oral hygiene program (Joyston-Bechal *et al.*, 1992; Epstein *et al.*, 1998; Spijkervet *et al.*, 1989c). Redding *et al.* (1990) reported no positive cultures for HSV from the ulcerative lesions in patients receiving head and neck radiation and suggested that the critical factor for increased HSV reactivation is immunosuppression. Therefore, no acyclovir prophylaxis in this patient population was recommended.

### 1.1.3.2 Oral flora of chemotherapy patients

As discussed in 1.1.2, chemotherapy patients, particularly those with leukaemia, are more vulnerable to opportunistic infections (Bergmann, 1991). Dreizen *et al.* (1986) documented that 34% of leukaemia patients under chemotherapy (n=1500) developed oral infections during their period of hospitalisation. On the other hand, the oral infection rate was 9.7% in 1000 adult chemotherapy inpatients who had solid tumours including carcinoma, sarcoma and lymphoma (Dreizen and Bodey, 1983). *C. albicans* was the most common isolate in both studies (20% in the former and 67% in the latter group). HSV was diagnosed in 8.8% of leukaemia patients compared to 10.7% in patients with solid tumours. Gram-negative bacilli (including *Pseudomonas, Klebsiella, Escherichia, Enterobacter, Serratia* and *Proteus*) caused infections in 8.1% of leukaemia patients. These bacteria (specifically *P. aeruginosa* and *K. pneumoniae*) were also the causative agents of infections in 10.7% of patients with solid tumours. Higher numbers of Gram-positive bacteria were isolated in patients with solid tumours (9.7%, mainly *S. aureus* and *S. epidermidis*) compared to 4.3% (mainly streptococci and staphylococci) in the other group. In addition, Sixou *et al.* (1996) reported that

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bacillary infections were common in patients with lymphoma, while patients with carcinoma developed fungal, viral and coccal infections more frequently.

Enteric bacteria (including *K. pneumoniae, Ent. cloacae, P. aeruginosa, E. coli and Citrobacter freundii*) were isolated from the oral cavity of 62% of leukaemia patients compared to 28% in the control group (Galili *et al.*, 1992). *K. pneumoniae, Enterobacter aerogenes*, and *S. epidermidis* were the predominantly isolated bacteria from the oral cavity of patients who had developed septicaemia with the same infectious agents (Greenberg *et al.*, 1982). In another study, *P. aeruginosa* was found to be the most commonly isolated Gram-negative bacillus from bacteraemia, although *E. coli* was the most common isolate on admission and follow-up cultures. *K. pneumonia, E. coli* and *Pr. mirabilis* were also associated with bacteraemia but this was not as common. It was also found that those patients had a high risk of yeast infections (Kathryn *et al.*,1981).

Minah *et al.* (1986) reported a considerable shift towards Gram-negative bacilli at mid-chemotherapy in all different oral sites, which was correlated with a decreased indigenous flora. In contrast, Wahlin and Holm (1988) showed that the total numbers of salivary micro-organisms remained unchanged in leukaemia patients. However, all patients had staphylococci and lactobacilli in all samples. In addition, 80% of patients were carriers of yeast, 70% had enterobacteria and pseudomonads and 45% had enterococci. A correlation between *Staphylococcus* spp. and yeasts in the supragingival flora of acute leukaemia patients was observed at mid-chemotherapy (Peterson *et al.*, 1990). Reynolds *et al.* (1989) reported that *Veillonella* spp. were the highest in supra/sub gingival sites in leukaemia inpatients before chemotherapy, and these were isolated concomitantly with Gram-negative bacilli, *Staphylococcus* spp., *Lactobacillus* and yeasts during chemotherapy.

## 1.1.4 Mucositis

Mucositis is one of the most common and distressing complications of radiotherapy of the head and neck region and/or chemotherapy. It can be defined

as a reactive inflammation-like process of the oral and pharyngeal mucous membranes caused by the cytotoxic effects of such anti-cancer therapies. A panel of experts reported that 78% of chemotherapy patients and 64% of radiotherapy patients had clinically significant mucositis in a multi-centre study (Sonis *et al.*, 1999). Moreover, a higher degree of mucosal disruption is experienced in young patients (Dreizen, 1990).

The cytotoxic effects of radiotherapy and chemotherapy interfere with the proliferation of cells and are most potent on those cells with high mitotic activity and increased metabolic rate (Squier, 1990). The mitotic activity of the germinal layer of the epithelium in the oral cavity is amongst the most rapid in the body (Schubert *et al.*, 1992). Therefore, the mouth tends to be the most vulnerable to the adverse effects of both cytotoxic treatments. The most susceptible areas are the buccal surfaces, lips, soft palate, the ventral surface of the tongue and the floor of the mouth.

Clinical effects can be observed approximately 12 days after the initiation of radiotherapy. In contast, chemotherapeutic agents have more abrupt effects with lesions appearing 3-10 days after the administration of cytotoxic agents (Toth *et al.*, 1990). Hyperkeratinisation, which appears as white plaque, is due to the increased cell renewal rate of the epithelial cells as a non-specific defence response to the irritation. The renewal rate of the keratinocytes cannot keep pace with the cytotoxic effects of continued treatment, leading to erosive areas (Dudjak, 1987). Erythema, which is caused by vascular dilatation and congestion in the affected lamina propria, develops by the end of the second week when the irradiation dose reaches 20 Gray (Spijkervet *et al.*, 1989b). The mucosa becomes oedematous due to the hyperpermeability of vessels. The mucosal deterioration intensifies with the progression of the cytotoxic therapies. Ulceration covered by a thick, patchy, fibrinous exudate membrane arises with higher doses, generally at the end of the third or fourth week of therapy. A plateau is established after these maximum reactions and persists until the end of therapy. Healing is generally spontaneous, approximately 3 weeks after cessation of treatment. However, erythema may persist for up to 6 months (Al-Tikriti *et al.*, 1984).

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Pain is the primary symptom associated with these lesions. Pain increases as therapy progresses and can range from a mild discomfort to a debilitating pain that may require opioid analgesia or local anaesthetic blocks (Bavier, 1990; Epstein and Stewart, 1993). Difficulty in eating and swallowing lead to weight loss and malaise. Most patients can tolerate only soft diets and sometimes require percutaneous gastric feeding when they cannot tolerate oral nutrition. Patients avoid movements of the tongue and masticatory muscles because of the pain, and this leads to speech problems and stagnation of food debris. When the patients are unable to tolerate the effects of cytotoxic therapies, suspension of treatment may be necessary to alleviate the symptoms, which in turn may result in tumour regrowth.

The intensity of mucositis correlates with the total dose of ionising radiation and chemotherapeutic agents. Dose per treatment and interval between treatments are determinants of tissue damage in both radiotherapy and chemotherapy. The effect of radiation is site specific. For instance, patients who have radiotherapy to the laryngeal region have less mucosal reaction but more problems with swallowing than patients irradiated in the oral region (Al-Tikriti *et al.*, 1984). Chemotherapy has a non-specific inhibitory effect in which toxicity is related to the type of cytotoxic drug. The most toxic chemotherapeutic agents include methotrexate, 5-fluorouracil and doxorubicin (Dreizen *et al.*, 1986). When radiotherapy and chemotherapy are combined, the cytotoxic effects will be intensified (Joyston-Bechal, 1992).

Mucositis does not simply stem from the cytotoxic effects of the ionising radiation and chemotherapeutic agents, a variety of other factors contribute to the duration and severity of lesions. Changes in saliva are found concurrently with mucositis and these may exacerbate the problems associated with the lesions. A decreased salivary flow will deprive the soft tissues of lubrication and may increase the discomfort related to mucositis. It may also cause difficulty in swallowing which in turn could affect the food intake of the patients and increase stagnation of debris.

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Infection associated with mucositis can increase the severity of lesions (see 1.1.5.2). The individual immune defence of patients, granulocyte count (Ramirez-Amador *et al.*, 1996), oral hygiene and the presence of pre-existing periodontal disease can also be important factors. Furthermore, nutritional status, mechanical and chemical trauma, smoking and alcohol consumption are other factors which affect mucositis (Sonis and Clark, 1991).

## 1.1.4.1  Current management of mucositis

The management of mucositis is complex and requires a multi-factorial approach. Treatment of this condition is mainly palliative (Rothwell and Spektor, 1990) and is mainly directed to alleviate pain and discomfort, as well as to lubricate the mouth and lips. Maintenance of good oral hygiene by atraumatic removal of debris and dental plaque and by the elimination of potential sites of infection, is imperative. Greenberg *et al.* (1982) identified septicaemia in 25% of acute leukaemic patients who received oral care prior to chemotherapy, compared with 77% among patients without such oral care. Mouth rinses can be helpful in maintaining the moisture of the mouth, in removing debris and in preventing accumulation of dental plaque thereby reducing the likelihood of infection.

At present there is no effective measure accepted for the prevention or treatment of mucositis. The literature describing the management of mucositis is controversial. Numerous agents are under evaluation with varying results. The agents currently used and those under investigation are listed in table 1.1.

Table 1.1   Selected studies on various agents for the treatment of mucositis

| Agent | Function | Results | References |
|---|---|---|---|
| Sucralfate | forms protective barrier on the damaged mucosa | Good results in radiotherapy and chemotherapy-induced mucositis<br>Similar good results<br>No significant reduction in radiotherapy-induced mucositis<br>Mild adverse reactions were experienced<br>Blocking effect of bound sucralfate prevents the effect of other topically-applied agents | Franzen et al., 1995<br>Barker et al., 1991<br>Epstein and Wong, 1994<br>Meredith et al., 1997<br>Toth et al., 1990 |
| Prostaglandin E2 | cytotoprotective activities | Reduced the intensity of pain and radiotherapy and radio/chemotherapy-induced mucositis<br>No beneficial effect in chemotherapy patients | Porteder and Rausch, 1988<br>Labar et al., 1993 |
| Silver nitrate | stimulation of proliferation of surviving stem cells | Claimed to be effective in reducing mucositis<br>No effect | Maciejewski et al., 1991<br>Dorr et al., 1995 |
| Granulocyte colony stimulating factor | accelerates cell division | Reduced the severity and duration of mucositis | Chi et al., 1995 |
| Chlorhexidine | antibacterial | Less painful and fewer occurrences of mucositis in bone transplant patients after radiotherapy<br>Similar good results in chemotherapy patients but no effect on radiotherapy patients. Also increased colonisation of Gram-negative bacilli<br>No difference with use alone or in combination with benzydamine. Colonisation with candida and coliforms was noted.<br>No effect when used in combination with nystatin. Colonisation with candida and coliforms noted. 26% patients developed bacteremia originating from oral cavity.<br>Inactivation by saliva of antimicrobial properties of chlorhexidine | Rutkauskas and Davis, 1993<br>Ferretti et al., 1990<br><br>Samaranayake et al., 1988<br><br>Epstein et al. 1992<br><br>Spijkervet et al., 1990 |
| Benzydamine hydrochloride | analgesic and anti-inflammatory | Some satisfying results with radio/chemotherapy patients<br>Less pain in chemotherapy<br>Reduced pain in radiotherapy patients but patients required systemic analgesics later on. A burning sensation was experienced with use and this was related to alcohol content | Prada and Chiesa, 1987<br>Sonis et al., 1985<br>Epstein et al., 1989 |
| Polymyxin, tobramycin amphotericin B (PTA) lozenges | antimicrobial | Reduced the degree of mucositis and colonisation by yeast and Gram-negative bacilli<br>Similar good results | Spijkervet et al., 1991<br>McIlroy, 1996 |
| Cryotherapy | rinse with ice chips | Significantly lower mean mucositis scores obtained | Cascinu et al., 1994 |
| immunoglobulin | support mucosal immune response | No effect on radiotherapy patients but decreased the incidence of high degrees of mucositis in patients receiving a combination of chemotherapy and radiotherapy | Mose et al., 1997 |
| low level laser (Helium neon) | stimulation of healing | Reduced high dose chemotherapy-induced mucositis, pain, improved xerostomia | Cowen et al., 1997 |

## 1.1.5 Oral Flora in Relation to Mucositis

### 1.1.5.1 Oral infections leading to bacteraemia

For many years (during the 1970's and 1980's), Gram-negative bacilli were shown to be the most frequent cause of bacteraemia in cancer patients. The leading causes of bacteraemia in the granulocytopenic patients were found to be *E. coli*, *K. pneumoniae* and *P. aeruginosa*. This has led to the use of antimicrobial prophylactic regimens with activity against Gram-negative rods. The basic approach to infection prevention has been to suppress Gram-negative bacilli colonising the alimentary canal with antibiotics such as fluoroquinolones which had no effect on the anaerobic flora thereby maintaining colonisation resistance (Winston *et al.*, 1988). Combinations of antibiotics such as beta-lactams and aminoglycosides have also been used (Schimpff *et al.*, 1994). More recently, however, following the use of such antibiotics a reduction in Gram-negative bacteraemia has been noted to coincide with an increase in Gram-positive infections. In a French multi-centre study (Escande and Herbrecht, 1998) with a total of 403 patients with haematological malignancy or with solid tumours, coagulase-negative staphylococci were shown to be the leading cause of bacteraemia (50.6% and 44.9% respectively), followed by *E. coli* (11.2% and 12.2% respectively), *S. aureus* (6.3% and 7.5% respectively), *Streptococcus* spp. (4.8% and 5.4% respectively ) and *P. aeruginosa* (5.2% and 4.8% respectively).

An increased incidence of Gram-positive infections has been attributed to the good activity of the agents used against aerobic/facultative Gram-negative bacteria with their relatively poor activity against Gram-positive bacteria (Klastersky, 1989). 56% of bacteraemia episodes in 221 neutropenic cancer patients receiving chemotherapy under prophylactic norfloxacin were caused by Gram-positive bacteria compared to 35% caused by Gram-negative organisms. 9% were polymicrobial. The most frequently isolated organisms were coagulase negative staphylococci (25%), viridans streptococci (17%), *E. coli* (16%) and *P. aeruginosa* (13%) (Gonzalez-Barca *et al.*, 1996). In another study, 21 bone marrow transplant patients received either vancomycin/polymyxin/tobramycin

(n=12) or norfloxacin (n=9) and *Strep. mitis* sepsis was detected in 1 patient in the former group compared to 5 patients in the latter (Classen *et al.*, 1990). Nevertheless, newer members of the quinolone class e.g. clinafloxacin have been reported to be active against viridans streptococci (Kerr *et al.*, 1999).

Although several sites can be the sources of bacteraemia in cancer patients, (e.g., venous catheters, the lower respiratory tract and the digestive tract), the role of the oral cavity cannot be overlooked. The causative organisms of bacteraemia can be isolated from the mouth, highlighting the need to establish appropriate interventions for prevention, control and treatment of oral complications.

A shift in a certain microbial population, although this can be present independently with no mucosal disturbance, is often associated with mucositis. Ulcerative lesions of mucositis tend to be colonised by a mixture of organisms, both local or exogenous, which can lead to secondary infections. Infected lesions may provide a portal of entry through broken mucosa allowing micro-organisms to enter the blood stream to cause systemic infection and bacteraemia, especially in neutropenic patients.

Hospitalisation, in particular, is associated with a shift towards a more aerobic Gram-negative oral flora which may serve as a potential source of septicaemia (Greenberg *at al.*, 1982; Epstein and Gangbar, 1987; Heimdahl *et al.*, 1989). However, chemotherapy-induced mucositis has also been implicated as a significant portal of entry for Gram-positive bacteria especially viridans streptococci (Heimdahl *et al.*, 1989; Donnelly, 1993; Beighton *et al.*, 1994; Lucas *et al.*, 1998; Ruescher *et al.*, 1998). Among the viridans streptococci, *Strep. oralis, Strep. mitis* and *Strep. salivarius* are the most likely species to be isolated (Beighton *et al.*, 1994). Patients with ulcerative mucositis were shown to have developed alpha-haemolytic streptococcal bacteraemia three times more frequently than those without ulcerative mucositis (Ruescher *et al.*, 1998). Viridans streptococci from the oral cavity have been reported to be the leading cause of bacteraemia in 500 allogeneic bone marrow transplant patients. 89% of patients had viridans streptococci-positive blood cultures as opposed to only 4%

of cases with Gram-negative bacilli. However, death from Gram-negative bacteraemia was frequent. 3 out of 7 patients compared to 8 out of 164 patients died as a consequence of bacteraemia due to Gram-negative bacilli and viridans streptococci respectively (Sparrelid et al., 1998).

Fungal infections occur frequently in cancer patients undergoing radio/chemotherapy. Certain species of fungi are more virulent than others. For instance, C. albicans colonisation is less likely to cause infection than C. tropicalis (Pfaller et al., 1987). Similarly, certain species of aspergillus are more invasive (Schimpff, 1990). HSV is the most common viral infection in the mouth.

## 1.1.5.2 Association of organisms with the severity of mucositis

Colonisation by members of the oral microflora can not only disseminate but also may accelerate wound progression and thus exacerbate the mucositis lesions (Greenberg et al, 1982; Bergmann 1991; van Saene and Martin, 1990). Organisms accumulated on the ulcerated tissue may contribute to the tissue damage. The metabolic by-products of organisms can aggravate the inflammation process and amplify local tissue damage. Organisms invade tissues by releasing potent toxic products that may deteriorate the condition via a wide range of mechanisms including inactivating complement, killing fibroblasts, and degrading basement membrane laminin and immunoglobulin. This is especially important in cancer patients under radiotherapy since the ionising radiation has a direct effect on the connective tissue. Increased vascular permeability, with an inflammatory infiltrate and tissue oedema which lead to occlusion of capillaries and hypovascularity, has been reported in radiotherapy patients (Baker, 1982).

Gram-negative bacteria, in particular, can contribute to mucosal inflammation by release of lipopolysaccharide (LPS) (Sonis, 1998; Spijkervet and Sonis, 1998). The released LPS-binding protein complex binds to mononuclear cells through the CD14 receptor and subsequently stimulates the release of a wide range of inflammatory mediators (Wilson, 1995; Wilson et al., 1996). These cytokines (e.g., IL-1, TNF-$\alpha$ and IL-6), in addition to their pro-inflammatory effects, can also

increase subepithelial vascularity which may result in an enhanced delivery of chemotherapeutic agents to the affected mucosa.

LPS released from *E. coli* has been shown to be 1000 times more potent than the LPS of anaerobic Gram-negative bacilli usually found in the oral cavity (Leenstra *et al.*, 1996). Therefore, it may be postulated that coliforms may have an important impact on wound progression and subsequent tissue damage compared to the pathogenic effect produced by commensal anaerobic Gram-negative bacteria.

Bacteria produce a range of enzymes that have the potential to degrade host molecules. Viridans streptococci, especially *Strep. oralis* and *Strep. sanguis,* may have a detrimental effect on the connective tissue due to their proteolytic activity (Homer *et al.,* 1990). *Strep. oralis* produces a range of proteolytic and glycosidic enzymes which may degrade host-derived glycoproteins (Rafay *et al.,* 1996), while *Strep. intermedius* has been shown to degrade chondroitin sulphate (Shain *et al.,* 1996).

## 1.1.6 Antimicrobial Agents for Oral Infections and Mucositis

Oral infection (bacterial, fungal and viral) is one of the most common complications of cytotoxic therapies for cancer patients. Management of contributing factors such as the prevention of mucosal breakdown, suppression of microbial colonisation, control of viral reactivation, and management of xerostomia are necessary steps for preventing such infections. ( McElroy, 1984; Toth *et al.,* 1990; Scully and Epstein, 1996; Epstein and Chow, 1999). Proper antimicrobial prophylaxis in patients at high risk, i.e., granulocytopaenic patients, has been commonly instituted. When infection is diagnosed, treatment can be achieved by effective use of topical and/or systemic antimicrobial agents. Clinically, the mucosal wound pattern may not be typical and bacterial, fungal or viral infections may mimic each other in the form of white, erythematous , erosive or ulcerated areas. Systemic broad spectrum antibiotic therapy is employed empirically for the treatment of bacterial infections (covering facultative Gram-negative bacilli and

*Pseudomonas* spp. as well as streptococci and oral anaerobes) until the identity of the causative agents and their susceptibility to certain antibiotics have been confirmed. Agents such as the new fluoroquinolones with activity against Gram-positive bacteria and aerobes (e.g., trovafloxacin, clinafloxacin), a third generation cephalosporin or a carbapenem are the drugs of choice. Less serious oral infections may be treated with an aminopenicillin (with or without a beta-lactamase inhibitor), or penicillin in combination with metronidazole. Clindamycin may be given to penicillin-allergic patients. Topical agents (e.g., tetracycline, vancomycin, neomycin, bacitracin, polymyxin B, povidone-iodine, chlorhexidine) can also be used (Epstein and Chow, 1999). Nystatin is commonly used for the local treatment of fungal infections whereas amphotericin B and fluconazole are preferred for systemic fungal infections. For viral infections, particularly HSV, acyclovir has been used successfully (Saral, 1990). Ganciclovir or foscarnet, on the other hand, can be used for cytomegalovirus infections.

Although it may be present on its own, infection may further complicate mucositis lesions. Dreizen *et al.* (1986) documented that 78% of leukaemia patients suffering from mucositis had simultaneous oral infections. Therefore, the presence of ulcerative lesions, especially in granulocytopaenic patients, should be identified. These should then be assessed by culture to ascertain whether mucosal lesions are due to infection or are therapy-toxicity related and appropriate control measures should then be employed. Suppression of the density of organisms from the oropharyngeal flora in these patients is of importance for two reasons. When potential sources of infection are considered in the patients receiving cytotoxic therapies for cancer, the mouth provides ideal conditions for microbial growth and is the portal of entry for contamination of the lungs, the digestive tract and the circulatory system. Therefore, suppression of the colonising flora will decrease the risk of septicaemia through the infected lesions. Secondly, a reduction in the microbial load (the existing normal flora together with exogenous species) of the oral cavity appears to have some benefit in the treatment of mucositis. The inflammation potential can be minimised by reducing the number of the potential pathogens flourishing on the ulcerated lesions, hence decreasing the concentration of their toxic products.

A number of therapeutic and prophylactic modalities that aim at reducing the microbial burden on the mucosal membranes have been evaluated for their potential to reduce the level of oral mucositis. Oral antiseptic rinses, immunoglobulins and granulocyte colony stimulating factor have been tried, with promising results (see table 1.1).

The effectiveness of chlorhexidine rinses in the control mucositis is controversial. There is apparently little effect on the degree of mucositis, although several studies reported some good results (Rutkauskas and Davis, 1993). Although the use of chlorhexidine was shown to be effective against cariogenic bacteria, i.e., mutans streptococci and lactobacilli (Epstein et al., 1991; Simons et al., 1997), no effect (or an increase) in the number of yeasts or Gram-negative bacteria has been shown (Ferretti et al., 1990; Samaranayake et al., 1988; Epstein et al., 1992). Furthermore, a reduction in the bactericidal activity of chlorhexidine when it is mixed with whole saliva has been found (Spijkervet et al., 1990a).

The incidence, severity and duration of mucositis in cancer patients (n=20) receiving radio/chemotherapy were shown to be reduced by oral rinsing with an antimicrobial solution of povidone-iodine compared to a control group rinsed with sterile water (n=20). Both groups had standard prophylaxis with nystatin, rutosides, panthenol and immunoglobulin. The success of the treatment was attributed to a decrease in the density of organisms in the test group (Adamietz et al., 1998).

The prophylactic use of sucralfate, with either ciprofloxacin or ampicillin and clotrimazole troches, has been shown to ameliorate the degree of mucositis in head and neck cancer patients undergoing radiotherapy (Matthews and Ercal, 1996).

An association between oral colonisation of Gram-negative bacilli and yeast with mucositis has been reported (Samaranayake et al, 1988; Makkonen et al., 1989; van Saene, 1992; Martin and van Saene, 1992; Spijkervet et al. 1990b; Symonds

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*et al.*, 1996). Selective elimination of increased concentrations of such micro-organisms from the oral flora as part of a prevention and treatment protocol of the severe form of mucositis was suggested (Jansma *et al.*, 1992). Eradication of Gram-negative bacilli and yeast overpopulation while retaining the normal flora (hence maintaining colonisation resistance) can be helpful in reducing the severe form of mucositis. Beneficial results have been obtained with agents selectively targeting Gram-negative bacilli and yeasts with lozenges containing polymyxin E 2 mg, tobramycin 1.8 mg and amphotericin B 10 mg. A reduction in the mucositis distribution, area, and the severity of lesions have been reported (Spijkervet *et al.* 1990b; Symonds *et al.*, 1996).

Emergence of bacterial drug resistance is a growing cause of concern. Many micro-organisms are now resistant to commonly-used antibiotics and they continuously develop resistance strategies to newly-developed antibiotic agents which are mainly modifications of those already in existance. Gram-negative bacteria in general exhibit resistance to many antibacterial agents and enteric rods and *P. aeruginosa* are particularly difficult to eradicate. The growing resistance of *S. aureus* to antibiotics is another concern. Therefore, novel approaches such as photodynamic therapy (PDT) for improved treatment of infections are being investigated. If it is shown to be an effective alternative to antibiotics, PDT may also eliminate the side effects associated with many antibiotics (e.g., gastrointestinal disturbance or skin reactions) due to its localised nature. PDT may eventually replace the need for combinations of antimicrobial agents since it has been shown that bacteria, yeast and viruses are all susceptible to killing by photodynamic action (PDA).

## 1.2  AN INTRODUCTION TO PDA

### 1.2.1  Definition of PDA

PDA can be defined as damage to biological systems caused by visible light in the presence of a chemical (termed the photosensitiser) and oxygen. The photosensitiser absorbs light energy of a certain wavelength inducing a series of photochemical reactions. Cytotoxic species, which are the end-products of the photochemical reaction cascade, result in the destruction of biomolecules and subcellular organelles (Fisher, 1995).

PDT is a new treatment modality which is based on eradicating target cells by PDA (Carruth, 1986). PDT may be non-invasive, is non-ionising, and is a repeatable technique with no cumulative effect. Although the primary application of PDT has been for the local treatment of solid tumours, there are a number of other applications (including antimicrobial applications) under investigation aimed at exploiting the technique both preclinically and clinically.

### 1.2.2  History of PDT

At the beginning of this century, PDA was discovered by scientists working in Germany. Raab (1900), a medical student working with Professor von Tappeiner, discovered accidentally that the dye, acridine orange, had a greater effect on protozoa during a thunderstorm. He confirmed this observation using sunlight, which dramatically increased the effect of acridine orange. Raab showed that paramecia were sensitive to light in the presence of the sensitiser and anticipated that this occurred due to the conversion of light energy to chemical energy. The term "photodynamic action" was introduced by von Tappeiner and Jodlbauer (1904) to distinguish it from the photosensitisation of photographic plates which was popular at that time. This series of experiments also led to the conclusion that the process required the presence of oxygen.

Jesionek and von Tappeiner (1905) were probably the first to use PDT in oncology. They used eosin as a photosensitiser and exposed basal cell carcinomas to sunlight and to light from an arc lamp for several weeks, experiencing some success. No significant subsequent studies on the photodynamic effect on tumours were reported until 1942 by Auler and Banzer, who injected tumour-bearing animals with haematoporphyrin (a substance processed from human blood sources) and exposed them to a quartz lamp, observing necrosis and fluorescence of tumours. The photodynamic action of haematoporphyrin was subsequently improved by purification to form a haematoporphyrin derivative (HpD) (Lipson and Baldes, 1960). This was first used by Lipson *et al.* for diagnostic purposes using its fluorescence properties and in the treatment of tumours with good effects (1966). The development of PDT for the treatment of malignancy was accelerated by this discovery.

After the advent of the laser by Maiman in 1960, more widespread therapeutic applications of PDT were developed, due to the superior properties of laser light over ordinary light. In 1978, Dougherty *et al.* demonstrated a partial or complete response in all tumours in humans when HpD and light at 630 nm were used together. No evidence of systemic toxicity, other than local skin reactions, were seen. In the early days of clinical PDT, many malignant lesions accessible to light exposure were treated successfully. Later, endoscopic devices were developed which offered a new opportunity for the treatment of cancers in less accessible areas such as the bladder, lung and oesophagus, Hayata *et al.* being the first to use fiberoptic laser exposure to treat early bronchial cancer with PDT in 1982.

Although studies of PDA commenced with observations on prokaryotic cells, less attention has subsequently been paid to PDA on micro-organisms. This was probably due to the urgent need for an effective treatment for malignancy (as antibiotics were widely and effectively used for the treatment of infections at that time).

In 1933, the first demonstration of the photodynamic inactivation of viruses using methylene blue (MB) was reported by Perdrau and Todd. Mathews and Sistrom

reported in 1960 that *Sarcina lutea* could be killed with toluidine blue O (TBO) and visible light, which prompted further bacterial studies. The discovery of lasers and the optimisation of the light and sensitiser dose resulted in a more effective bacterial kill. Macmillan *et al.* (1966) reported that a series of bacteria, along with candida, were sensitive to TBO in conjunction with continuous wave laser light tuned to 633 nm.

PDT was first used clinically to treat HSV infection in the early 1970's, using neutral red with good effect. Unfortunately, the oncogenic potential of the virus (Duff and Rapp reported in 1973 that the photo-inactivated virions were still capable of transforming hamster embryo fibroblasts) led to controversies over the safety of the therapy and further clinical applications were discouraged. Recently, a light-activated benzoporphyrin-derived monoacid ring was shown to render HIV uninfectious in whole blood from individuals infected with HIV, without damaging the red blood cells (North *et al.*, 1994).

## 1.2.3 Principles of PDT

PDT is based on the interaction between a photosensitising compound and light in the presence of oxygen. This interaction induces photochemical reactions, ultimately producing singlet oxygen and hydroxyl free radicals which kill cells. The majority of work on this subject has been conducted on mammalian cells and tumour tissue. However, there is now an increasing interest in the use of PDA against micro-organisms (Wilson *et al.*, 1992).

This section gives a general review of light, photosensitisers and the mechanism of PDA with a particular emphasis on PDA on micro-organisms.

## 1.2.3.1 Light

Any light source can be used for PDT as long as it has the required spectral characteristics. The light source must emit light of an appropriate wavelength, corresponding to the absorption maximum of the sensitiser, and must have sufficient enery to activate it. Before the advent of the laser, sunlight and artificial

light sources such as tungsten, quartz, xenon, mercury and fluorescent lamps were employed for PDT. Appropriate wavelengths were obtained by filtration of the emitted spectrum. The disadvantages associated with these light sources include their broad emission spectrum, and their low power output after filtering the unwanted wavelength. Since their development, lasers have become the standard light source for most PDT applications, owing to their superior properties over ordinary light -mainly their high energy content, their monochromacity and the fact that the light emitted can be targetted via optical fibres.

LASER is an acronym for Light Amplification by the Stimulated Emission of Radiation. Laser light differs from ordinary light in three main ways:

*Coherence*: Coherent light is of a defined wavelength and exhibits no phase differences (all troughs and peaks are synchronised). Ordinary light is incoherent with light radiating in all directions without phase conservation.

*Collimation:* A collimated laser beam is parallel and does not diverge significantly in contrast to ordinary light, which spreads out as it travels away from the source. The advantage of a collimated beam is that there is minimum loss of power along the beam and that it can be focused to intensify its effect, coupling into single optical fibers, thus enabling manipulation of light to the desired areas.

*Monochromacity:* The light is emitted at a single wavelength.

Coherence and collimation of laser light are rapidly degraded by scattering in tissue. Therefore, these properties do not appear to be fundamentally essential to PDA. However, the technical advantages of producing a monochromatic and directed beam make lasers attractive for many applications, especially where fibre-optic guidance is essential. Red light in the 600-800 nm range is generally used for PDT since tissue transmission is most effective in this part of the spectrum.

All laser sources have similar fundamental elements: A lasing medium, energy source and optical resonator. Laser tubes contain the lasing medium which exhibits lasing action and gives its name to the laser. The lasing medium can be liquid (dyes of different types which allow emission of various wavelengths depending on the dye), solid (e.g., Nd:YAG, neodymium:yttrium aluminium garnet) or gas (e.g., carbon dioxide, argon, helium neon).

Atoms (or molecules) of the lasing medium in their ground state (lowest energy level) are raised to one of several distinct higher energy levels when excited through the process of absorption. The excited atoms decay back to their low energy ground state releasing the absorbed energy (spontaneous emission) in the form of a photon, which corresponds to the difference between the ground and excited states. All particles making the transition between the same two energy levels will emit light of identical energy and wavelength. If a photon of emitted light interacts with an atom at a high energy level, it stimulates the release of an identical photon and decays back to its ground state. Subsequently, these two photons interact with additional atoms in excited states. Succession of this reaction will yield an amplifying cascade of photons. This process is called stimulated emission. The released photons reflect the lasing medium through parallel mirrors (one fully, the other partially reflective) situated on each side of a tube, which produce a cascade effect with rapid build up of light energy within the tube. The light can then be released through the partially reflective mirror.

The lasing medium and the design of the mirrors determine the wavelength of the coherent light. Most lasers, other than tunable dye lasers, are fixed to one wavelength. This means that the laser can be used optimally only with certain photosensitisers, which have an absorption maximum at the particular laser wavelength.

The desired properties of a light generating system for PDA are:
* Generation of light with a wavelength above 600 nm
* Adjustable for generation of various wavelengths
* High power output capable of inducing effective PDA

- Portable

- Inexpensive

- Easy to operate

- Suitable for different delivery modes

## 1.2.3.1.a  Light sources used for PDT

### i) Dye Lasers

### Argon-pumped dye laser

The tunable argon dye laser produces a continuous wave output that can be tuned over a significant range from 500-700 nm.  The power output is low due to a 25% reduction of total power output of the driving argon laser.  A 20 W power produced in an argon tube decreases to only 5W in the dye laser.  The limiting factors for argon pumped dye lasers are that they are expensive, complicated, require a medical physicist to adjust wavelengths required for each use and also require a significant flow of water for cooling.

### Copper vapour-pumped dye laser

This laser system generates a pulsed beam.  It has high pulse repetition frequency and high average pulse power and can also be tuned to give a light output from 600-800 nm.  It has a large beam divergence that requires a large diameter fiber for light delivery.

### Excimer-pumped dye laser

The excimer pumped dye laser is produced by ultra violet light and the excitation of xenon/chlorine gas mixtures or xenon/fluorine gas, which is used for pumping rhodamine or DCM dye to generate light from 400-700 nm.

## ii) Gold vapour laser

The gold vapour laser produces a pulsed output at a fixed wavelength of 628 nm and cannot be tuned.  However, it is relatively easy to change the metal in the laser to copper, which can be used to drive a dye laser.  The gold vapour laser has advantages over dye lasers, such as greater power and wavelength stability due to elimination of the complex dye system.  The gold vapour laser can generate power up to 10 W (Carruth and McKenzie, 1985).

## iii) Tunable solid state lasers

This group includes tunable Nd:YAG lasers and potassium titanium phosphate (KTP) lasers.  The pulsed KTP laser produces 532 nm light, while the Nd:YAG laser can generate far-red and near infra-red light (1060 nm), which is a great advantage since greater tissue penetration can be achieved although the wavelength is too long to excite currently-used sensitisers.

Light-generating devices required for PDT have been moving into the field of cheaper, less complicated and more user-friendly light sources such as diode lasers and non-coherent light sources.

## iv) Diode laser

Diode lasers are an alternative to bulky dye lasers and are the most efficient and stable lasers presently available.  These laser sources are small, portable, require no water cooling and are becoming less expensive.  The gallium aluminium arsenide (GaAlAs) diode laser has a fixed wavelength in the 630-850 nm range with 1-5 W power.  This wavelength is suitable for second generation sensitisers such as bacteriochlorin A.  The lasers operate with a continuous or pulsed output and the light can be delivered through an optical fiber.  The light intensity is relatively low but the power output is increasing with the development of these light sources.  Some diode lasers have a power output of nearly 25 W.

## v) Light emitting diode (LED)

LEDs are semiconductor light sources which emit light over a 20-30 nm wavelength band in the 400-800 nm range. Output beams are generated by multiple low power diodes to make a single, sufficiently high power, non-coherent beam. The quality of the output beam is relatively divergent compared to laser systems, which makes it difficult to couple to fiber optics.

## vi) Helium Neon (HeNe) gas laser

The strongest wavelength of the HeNe gas laser is 632.8 nm and can be directed toward the tissue via a fiberoptic. Low power HeNe lasers have been widely exploited in biostimulation with beneficial results. HeNe lasers have a limited application in PDT for tumour destruction due to the low power output (35 mW). However, many studies showed that a HeNe gas laser can be effectively used for PDA on micro-organisms. The wavelength of the HeNe laser corresponds to the absorption maxima of thiazine dyes such as TBO and MB, as well as of porphyrins.

### 1.2.3.1.b  Light-tissue interactions

The interaction of light with biological tissue can be described in terms of reflection, transmission, scattering or absorption.

*Reflection:* Light is reflected from the surface of a tissue, and has no effect.

*Transmission:* Light is transmitted through a tissue, and has no effect.

*Scattering:* Light is scattered by a tissue and diffused over a large area, weakening its effective intensity. Back-scattered light near the surface however can undergo internal reflection which enhances the light intensity just below the surface.

*Absorption:* Light is absorbed by a small volume of tissue, and exerts its effects (thermal or photochemical) within this volume.

Reflected          Transmitted          Absorbed          Scattered
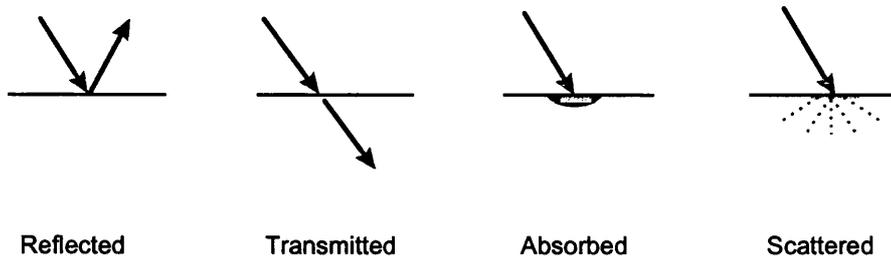
Figure 1.1   Forms of light-tissue interactions

For the initiation of a photochemical process, absorption and transmission of light throughout the tissue is essential. However, the optical properties of each tissue will differ due to different light scattering and absorption coefficients. Generally, for a parallel beam of light incident on a tissue surface, the intensity decreases exponentially with depth. The intensity 'half-life' (i.e., intensity reduced to 37% of its value at the surface) defines the 'penetration depth' of the light at that wavelength. Typically, for red wavelengths near 630 nm, the penetration depth is about 3 mm. Thus, at a depth of 1cm the intensity is reduced by a factor of ten compared to the intensity at the surface. The absorption of laser light by different tissue elements is wavelength-dependent and tissue penetration is greater at longer wavelengths. Moreover, longer wavelengths are scattered less than shorter ones. Penetration is almost doubled when near infrared light (700-850 nm) is used, thus new photosensitisers which absorb in this range are under development.

There are several factors that affect the photodynamic effect on tissues: photosensitiser absorption coefficient, total light dose, the delivery system, optical properties of the field where the light is absorbed, tissue vascularity and oxygenation. To obtain a photodynamic effect, sufficient amounts of sensitiser, light and oxygen must be present throughout the tissue. However, absorption of light by the photosensitiser itself can also limit the absorption of light by tissues if the concentration is too high. Tissues with high haemoglobin content have high light absorption coefficients and thus lower light penetration depths (van Hillegersberg et al., 1994).

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**1.2.3.1.c  Light dosimetry**

The effect of PDT is determined by light energy absorbed per second and per unit volume by the photosensitiser. The activation rate of a photosensitiser at a certain point in a tissue is proportional to the energy fluence rate (power density, $W/cm^2$), multiplied by the absorption coefficient of the photosensitiser. The energy dose (also known as the fluence, $J/cm^2$ ) is the total amount of light energy delivered per unit area of the applied laser beam. It is the product of fluence rate and duration of laser application (the basic equations are given in 2.1.1.4)

**1.2.3.1.d  Light delivery**

The type of light delivery system used will vary depending on the particular application. A simple lens and aperture optic can be used to produce uniform illumination for superficial areas such as the skin and oral cavity. Laser light can be directed via flexible silicon quartz optical fibres. However, more uniform irradiation is achieved by fitting a microlens to a fiber to produce a focused beam, or by using a spherically-tipped fibre to produce a diffuse halo of light. Interstitial laser systems are used for deeper targets. Insertion of the optical fiber into the desired area can be achieved by point-insertion (through a needle, trochar or cannula) of a flat-cut fiber tip under ultrasound guidance or by insertion of a spherical or cylindrical diffusing tip. Translucent nylon catheters can be surgically implanted for subsequent laser treatments. For intraluminal or intracavitary illumination of the bronchus, oesophagus or bladder, the flexible quartz fibre can be placed through an endoscope. Spatial distribution can be achieved by modifying the fiber end or by placing the fiber in a light-diffusing medium.

Surface illumination          Interstitial illumination          Intracavitary illumination
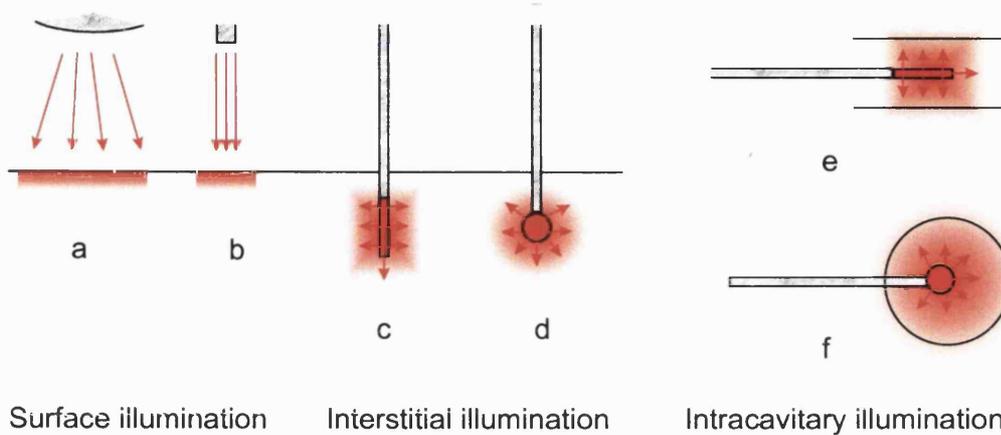
Figure 1.2    Various light delivery systems.    Surface illumination: with a) conventional lamp and b) laser light guided through an optical fibre; Interstitial illumination with: c) cylindrical diffuser and d) spherical diffuser; Intraluminal illumination with: e) cylindrical diffuser and intracavitary illumination: with f) spherical diffuser.

### 1.2.3.2  Photosensitisers

The ideal properties of a sensitiser for the treatment of neoplastic tissues can be identified as (MacRobert *et al.*, 1989)

• Chemically pure, water soluble and stable in solutions at a physiologically neutral pH to allow transport through the circulation

• High quantum yields (high probability of triplet state formation per photon absorbed) for the generation of type I and type II photochemical reactions

• Strong photoactivation at longer wavelengths; the ideal wavelength is near the infra red region of the spectrum (above 600 nm), which has relatively higher tissue absorption. To avoid a potential mutagenic effect, the photosensitisers should not have an absorption band below 400 nm.

• No dark toxicity

• Rapid plasma and tissue clearance

• Minimal skin sensitivity

• Selective uptake by the target tissues

• Fluorescence during excitation for diagnostic visualisation

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Similar properties are desirable for photosensitising drugs for treatment of infectious diseases. In addition, an ideal photosensitiser for PDA of micro-organisms should;

• provide an efficient and non-recovering killing effect when irradiated

• be independent of the antibiotic-susceptibility of the organism

• be active on both Gram-negative and Gram-positive pathogenic bacteria

• be non-toxic to the host. It is particularly essential that surrounding healthy tissue remains unaffected when target bacteria are eradicated by PDT.

At the present time, there are no photosensitisers which meet all of the desired properties listed above.

## 1.2.3.2.a Photosensitisers used for PDT

There are many sensitisers which have been developed and are currently under investigation. Porphyrins, chlorins and phthalocyanines are the most commonly used sensitisers for the PDT of tumours. For the photodynamic inactivation of micro-organisms, histologic dyes such as MB, TB, acridine dyes, rose bengal and neutral red have also been used. In the following discussion, the most widely used sensitisers in addition to TBO will be described in detail (Bonnett, 1994; Bonnett,1999).

i) Haematoporphyrin Derivative (HpD)

HpD is the purified form of haematoporphyrin and is considered to be a "first generation" sensitiser. HpD is excited most effectively by a light wavelength around 400 nm with a weak absorption peaking at the red end of the spectrum. Hence, it is not a very active photosensitiser and requires a long illumination time (20-30 min). HpD has no systemic toxicity other than prolonged photosensitivity (3 months). It may cause severe sunburn-type reactions as a result of exposure to direct sunlight (Schuitmaker et al., 1996). The purified active component of HpD is enriched in dihaematoporphyrin ether (DHE), and this has become available under the commercial name of Photofrin (which is the first substance to receive

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regulatory approval for PDT applications). Currently, it is the most commonly used sensitiser and is activated by light with a 630 nm wavelength. Although Photofrin has proved to be an effective sensitiser, it is far from being an ideal one. More recently, so called "second generation" sensitisers are being investigated to improve the effectiveness of PDT. The most commonly studied compounds will be described in this section.

ii) 5-Aminolaevulinic acid (5-ALA)

5-ALA is the metabolic precursor of protoporphyrin IX (PpIX) in the biosynthetic pathway to haem. After administration, 5-ALA metabolises into PpIX which can act as an endogenous sensitiser. The depth of necrosis produced by 5-ALA-induced PDT is generally not more than 1.5 mm (Fan et al., 1996) and therefore it is used for the treatment of superficial skin cancers, especially basal cell carcinoma (Cairnduff et al., 1994) and dysplasias in the oral cavity, but is not suitable for invasive cancers (Grant et al., 1993). The advantage of the sensitiser over HpD is that 5-ALA-induced PpIX is cleared from the body more rapidly, so duration of the period of skin sensitivity beyond 2 days is avoided.

iii) Meto-tetrahydroxyl tetraphenyl chlorin (m-THPC)

mTHPC is a pure compound and can be activated at a number of wavelengths (e.g., 514 and 652 nm). Depending on the desired penetration depth, the wavelength of illumination can be selected, i.e., 514 nm for a more superficial effect and 652 nm for deeper tissue effects. It is effective at low drug and light doses. m-THPC-induced PDT offers a deeper tumour destruction (1 cm) and larger tumours (up to stage T3 tumours) can be effectively treated (Fan et al., 1997). Furthermore, treatment duration with mTHPC is much shorter. Cutaneous photosensitivity is around 6 weeks at the most and some scarring on healing can be expected.

## iv) Phthalocyanines

Phthalocyanines are synthetic porphyrin analogues. Although they have similar properties to HpD, they have a maximum absorption band at 675 nm, and so have greater tissue penetration than HpD. This wavelength can be generated by diode lasers, which gives additional benefits since such lasers are simple to use, portable and cheap. Phthalocyanines investigated for PDA are metallated since metal-free phthalocyanine has a lower photodynamic effect. The most commonly studied complex of the sensitiser is water-soluble aluminium phthalocyanine, particularly the sulphonated form (AlSPc). Phthalocyanines are not retained in the skin so long as porphyrins therefore less cutaneous sensitivity is experienced (Tralau *et al.*, 1989). Although there are many *in vitro* and animal studies with phthalocyanines, clinical trials in humans (although there are some anecdotal reports) are yet to be conducted.

## v) Toluidine blue O (TBO)

TBO is a member of the thiazine dye family. TBO together with azure A, B and C, and thionin are the derivatives of MB. Thiazine dyes have quinone-amine groups and have a triple ring structure. All thiazine dyes, although having a low quantum yield (the probability of triplet state formation per photon absorbed, see 1.2.3.3.a), are reported to be effective at killing micro-organisms (Schafer *et al.*, 1979).

MB in particular has been shown to have good antimicrobial properties when exposed to light. It absorbs strongly in the optimal wavelength region (around 680 nm). Application of MB and light has also been shown to be toxic to tumour cell lines (Bellin *et al.*, 1961). MB intercalates with bacterial DNA causing both genetic mutation and photo-damage. Gutter *et al.* in 1977 reported that MB, when DNA damage is not complete, can cause mutagenicity in bacteria and viruses implying that mutagenic effects may occur in human cells when the cytotoxicity is not complete.

TBO is a metachromatic nucleus stain, and stains basophilic substances in tissue specimens. It is a positively-charged, cationic dye and has an affinity to negatively charged molecules. It has an absorption band of 620-660 nm (the absorption maximum is 633 nm). TBO has been shown to be an active photosensitiser of many micro-organisms and it has been reported to be a membrane-active photosensitiser (Ito, 1983).
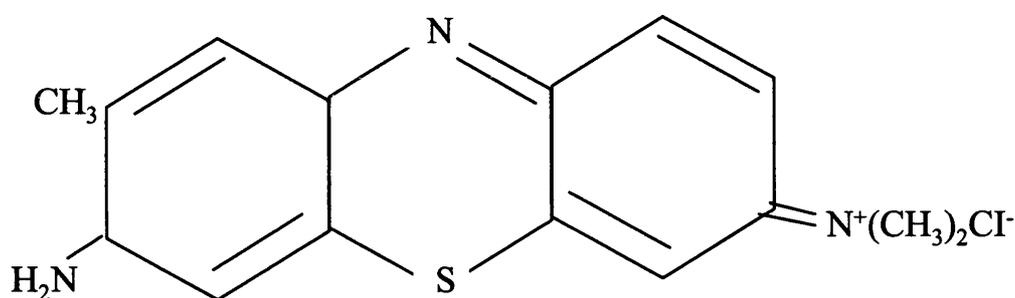


Figure 1.3   The molecular structure of TBO ($C_{15}H_{16}CIN_3S$)

Since the 1960s, many studies have been performed on the diagnostic use of topical 1% TBO for precancerous and cancerous lesions in the mouth (Mashberg, 1983; Warnakulasuriya et al., 1996). There have been no reported reactions or side effects following its topical application. However, Dunipace et al. (1992) reported a mutagenic effect of TBO with the Ames test on salmonella in vitro. The authors also stated that the mutagenicity could not be reliably determined by any single genotoxic test procedure. Ashby et al. (1989) ratified that the oncogenic capacity of a chemical can not be reliably predicted from a positive mutagenicity test. Furthermore, no carcinogenic effect was manifested when TBO was tested on the hamster cheek pouch, which is more representative of oral tissue exposure to TBO (Redman et al., 1992).

Table 1.2   Potential photosensitisers and their therapeutic absorption maxima

| Photosensitisers | absorption maximum (nm) |
|---|---|
| Photofrin | 630 |
| 5-ALA-induced PpIX | 635 |
| m-THPC | 652 |
| Phthalocyanine | 675 |
| MB | 680 |
| TBO | 630 |

## 1.1.3.3   Mechanism of PDA

### 1.2.3.3.a   Photochemistry

When a photosensitive dye absorbs light energy of an appropriate wavelength in the presence of oxygen, a cascade of events occurs. Following the absorption of light, the dye molecule transfers from the ground state to the singlet state. The excited singlet state of the dye molecule has high energy but an extremely short lifetime (less than 1 $\mu$sec). From this excited singlet state, the molecule can then follow two pathways; it can either decay back to its ground state emitting light (fluorescence), or form a triplet state by electron spin conversion in a process called "intersystem cross over". Although molecules in the triplet state have a lower energy, they have a longer lifetime than when in the singlet state (1 $\mu$sec-10s). Therefore, the excited triplet state has a high chance of interacting with ground state oxygen and/or biomolecules. The fluorescence properties of some sensitisers have been used diagnostically for detecting the sensitiser in tissues and for visualising tumour localisation. For a photodynamic effect, however, the photosensitiser should generate an excited triplet state which then undergoes a type I or type II reaction, or a combination of both. As a result of these reactions,

highly reactive cytotoxic species are produced which are responsible for cell death.

## Type II reaction:

It is believed that tissue damage induced by PDT occurs primarily via a type II reaction pathway which involves singlet oxygen formation. Singlet oxygen is generated by energy transfer from the sensitiser molecule in its triplet state to a free oxygen molecule in the tissue. Although the lifetime of singlet oxygen is quite short ($4 \times 10^{-6}$ sec in water, $50\text{-}100 \times 10^{-6}$ sec in lipid and $0.6 \times 10^{-6}$ sec in a cellular environment) (Moan and Berg, 1991) due to its electrophilic nature, it is highly reactive to biological systems and causes cellular damage and tissue destruction by oxidation of biomolecules. After inducing the formation of singlet oxygen, the sensitiser returns to its ground state. The same sensitiser molecule can subsequently absorb another photon of light and thereby generate another singlet oxygen -this can continue indefinitely.

## Type I reaction:

The excited triplet photosensitiser can also interact with a biomolecule or solvent molecule by electron or hydrogen atom transfer. This interaction produces radical forms of the substrate or photosensitiser, which can then react directly with oxygen to produce active species such as hydroxyl radicals, hydrogen peroxide and superoxide anion. The minor role of type I interactions in PDT is believed to be due to competition between substrates and oxygen for triplet state photosensitiser molecules (Malik *et al.*, 1990).
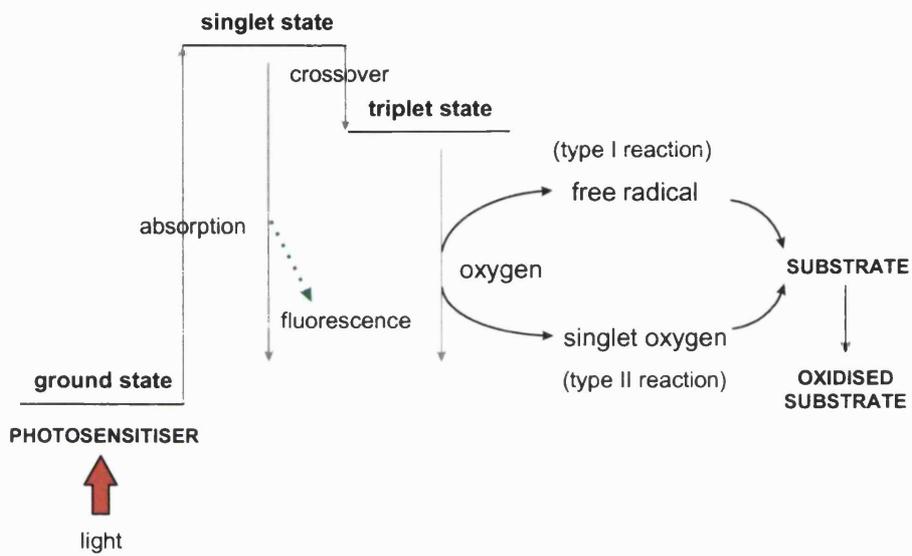
Figure 1.4   Schematic diagram showing the excitation of a sensitiser by light.  The type I and II mediated pathways are via the formation of free radicals and singlet oxygen respectively.

## 1.2.3.3.b Site of action

The mechanism of cell destruction by PDA has been exhaustively investigated in a range of mammalian tissue types, in particular, tumour tissue. There is comparatively limited data available on the mechanisms involved in micro-organism photosensitisation.

### i) Site of action on mammalian tissue

PDT, using currently available sensitising agents, has been shown to cause a massive inflammatory response with superficial necrosis of both the malignant tissue and surrounding normal tissues with loss of endothelial cells and damage to the micro-vasculature. However, adjacent normal tissues regenerate satisfactorily with minimal tissue loss and preservation of function (Grant et al., 1993).

The mechanism of cell damage induced by lipophilic sensitisers (e.g., zinc-phthalocyanine) is different from that of hydrophilic sensitisers (e.g., AlSPc). In general, hydrophobic sensitising agents which accumulate intracellularly induce direct tumour damage whereas hydrophilic ones (which accumulate within the interstitial space and the vascular stroma) cause indirect damage, through blood vessels (Ochsner, 1997).

*Cellular damage*

Singlet oxygen can penetrate tissues only for a distance of 0.01-0.02 μm, therefore the sites of initial cell damage are closely related to the localisation of the sensitiser (Moan and Berg, 1991). There are three major target sites in the cell; the cytoplasmic membrane, DNA and organelles such as lysosomes and mitochondria. Sensitisers that are localised in the membrane are likely to cause lipid peroxidation and protein damage. This damage can lead to an increased permeability and an inhibition of cell nutrient transport. Disruption of mitochondrial enzymes such as cytochorome c oxidase, malate dehydrogenase and succinate dehydrogenase are thought to be responsible for mitochondrial damage. Increased levels of DNA-DNA and DNA-protein cross-links, degradation of nucleic

acids have been observed in response to cytotoxic damage. DNA repair enzymes are also extremely sensitive to reactive species. Sensitisers localised in lysosomes will cause release of hydrolytic enzymes upon light exposure (Gomer *et al.*, 1988; Dougherty *et al.*, 1998). In addition, cell lines exposed to PDA have been shown to exhibit cell death with features of apoptosis (Ketabchi *et al.*, 1998).

*Vascular targets*

The most significant destructive effect on tumour tissue appears to be mediated by vascular damage rather than direct tumour cell damage. Arterial endothelial cells are particularly susceptible to photodynamic destruction because of the high local oxygen tension. It has been shown that poorly vascularised tissue is not as affected by PDT (White *et al.*, 1988).

ii) Site of action in micro-organisms

The mechanism of action may vary according to the type of organism, the chemical characteristics of the photosensitiser and its localisation properties. Exogenous sensitisers are mainly effective on the cell membrane whereas endogenous sensitisers target DNA and the other structures in the cytoplasm.

*Membrane damage*

A number of studies have shown that the cell membrane is a possible target for PDA in both bacteria and yeasts. Membrane damage produced by PDA has been shown to be via both lipid peroxidation and protein damage. Disturbances in the synthesis of the cell membrane and mesosomes in deuteroporphyrin-treated *S. aureus* were demonstrated by Nitzan *et al.* in 1992. Crosslinking of cytoplasmic membrane proteins of zinc sulphonated phthalocyanine-sensitised *Strep. faecium* has also been observed. Similar membrane damage was shown in yeast cells (Bertoloni *et al.*, 1992). Fast ionic fluxes in *S. aureus* with total K loss as well as marked Na efflux was noted by Malik *et al.* (1993). When treated with $CaCl_2$ or Tris EDTA to remove the outer membrane, *E. coli* was rendered sensitive to PDA with zinc phthalocyanine and deuteroporphyrin (Bertoloni *et al.*, 1990).

*DNA damage*

Porphyrin-mediated PDA on purified DNA has been shown to result in cleavage of plasmid supercoiled DNA to relaxed and linear DNA. A modification in guanine residues was also observed (Aft and Mueller, 1983). Treatment of *S. aureus* with photo-activated deuteroporphyrin caused random breaking of supercoiled plasmid DNA (Nir *et al.*, 1991). Similar structural changes were shown in *E. coli* DNA. MB has been reported to result in mutations in the DNA of several strains of *Salmonella typhimurium* (Eldar *et al.*, 1989).

## 1.2.4 Current and Possible Future Applications of PDT

PDT has been widely investigated for the treatment of cancer in nearly all disciplines including dermatologic, pulmonary, oesophageal, head and neck, and genitourinary systems, with varying success (Carruth, 1998). PDT has also been used in combination with surgery, chemotherapy and radiotherapy or intra-operatively. However, best results were obtained in superficial small tumours i.e., "field cancerization" in the oral cavity (Grant *et al.*, 1993) and cutaneous malignancies such as basal cell carcinoma and Bowen's disease (Robinson *et al.*, 1988). This is mainly due to the limited penetration of light. At present the deepest photodynamic effect achieved is around 1cm. However, deeper effects can be achieved by fiber implantation and interstitial light applications. HpD-mediated PDT has also been shown to be effective in the treatment of psoriasis (Berns *et al.*, 1984), but skin photosensitivity limited the application.

Further possible applications of PDT were implied by animal studies. Atheromatous plaques were demonstrated to selectively retain haematoporphyrins and copper phthalocyanines in animal models (Litvak *et al.*, 1985). AlSPc was shown to effectively inhibit intimal hyperplasia in rats (Ortu *et al.*, 1992). Manyak *et al.* (1989) used photofrin-mediated PDT for the treatment of endometriosis in rabbits and achieved complete endometrial epithelial destruction.

There is also a growing interest in the microbiological applications of PDT. In this section, a general review of PDA on micro-organisms will be given. A more detailed discussion on PDA on bacteria is given in the experimental chapters.

## 1.2.5 PDT and Micro-organisms

### 1.2.5.1 Photodynamic inactivation of viruses

Viruses were first shown to be photosensitive in the 1930's by Perdrau and Todd. In the presence of light and oxygen, a range of sensitisers including MB, TBO, proflavin and neutral red were reported to inactivate HSV (Wallis and Melnick, 1966). Following these *in vitro* observations, PDA was demonstrated to resolve HSV-induced keratitis in rabbits. In 1973, the first clinical study was performed by Felber *et al.* Repeated application of neutral red and irradiation was shown to be effective on primary and recurrent genital HSV infections. The frequency of recurrence was decreased and complete healing of lesions was achieved. PDT was mainly employed in skin and genital herpes lesions with similar good results. The virus was demonstrated to be most sensitive in its rapid replication stage. However, a controversy over the use of PDT (particularly with polycyclic hydrocarbon dyes) as being potentially carcinogenic discouraged further clinical studies (Bockstahler *et al.*, 1979). It was proposed that the possible carcinogenesis associated with PDA occurs through unmasking the oncogenic potential of HSV particles and transformation-susceptible viruses induced by PDT. Moreover, activation of latent virus may also occur.

These observations have led to the exploitation of other sensitisers. Application of haematoporphyrin with visible light has been shown to inhibit the replication of influenza A and HSV *in vitro*. Embryonated eggs and mice infected with influenza A virus have been shown to be protected by photodynamic application. On the other hand, HSV-induced keratitis in rabbits and dermatitis in mice was found to be not responsive to treatment (Perlin *et al.*, 1987). Light activation of phenothiazine dyes such as MB, TBO and azure B, has been shown to decrease or eliminate the infectivity of a number of different viruses and bacteriophages.

Viral and DNA damage were found to be dependent upon dye concentration, light dose and oxygen. DNA damage was not correlated with loss of phage infectivity (Specht, 1994).

Sterilisation of blood plasma has been employed in German and Swiss centres since 1992. Numerous blood born viruses such as hepatitis B virus, hepatitis C virus, HSV type 1, suid herpes virus type 1, HIV-1 and also non enveloped porvovirus B19, have been shown to be sensitive to MB/light treatment. Although, human plasma can quench singlet oxygen and subsequently suppress the photodynamic inactivation of viruses, intense light for a short time was demonstrated to be more detrimental to viruses than lower doses over a longer period (Muller-Breitkreutz et al., 1995; Mohr et al., 1997). Phthalocyanines have also been studied for virus inactivation of erythrocytes. Interestingly, red blood cell damage produced by photosensitisation was decreased by increasing the rate of irradiation and the dose. This is favourable as erythrocyte damage could be minimised by higher energy doses (Ben-Hur et al., 1995).

## 1.2.5.2 Photodynamic inactivation of yeasts

Susceptibility of yeasts to photodynamic inactivation has been reported with several sensitisers in vitro. In 1966, Macmillan et al. demonstrated that Saccharomyces cerevisiae was susceptible to irradiation with light from a continuous wave gas laser in TBO suspensions. In addition to TBO, other sensitisers including acridine orange, haematoporphyrin (Ito, 1980) and chloroaluminium-phthalocyanine (Paardekooper et al., 1992) were reported to be effective sensitisers of yeast cells. Interestingly, an antifungal drug, amphotericin B, was reported to protect Kluyveromyces fragilis against photodynamic-induced cell damage by a variety of sensitisers. This unexpected effect was attributed to scavenger action against species produced by the drug (Lazarova and Tashiro, 1995). Wilson and Mia (1993) reported that C. albicans was susceptible to lethal photosensitisation by a series of dyes including TBO, thionin and crystal violet, but dihaematoporphyrin and $AlS_2Pc$ had no effect on the viability of the yeast.

## 1.2.5.3   Photodynamic inactivation of bacteria

In 1960, Mathews and Sistrom reported that *Sarcina lutea* could be killed when exposed to visible light in the presence of TBO. This finding accelerated studies of PDA on bacterial cells. Macmillan *et al.* (1966) demonstrated the aerobic kill of several other bacteria, including *E. coli, Chromobacterium violaceum, Arthrobacter atrocyanus, and P. aeruginosa,* when they were irradiated with a continuous-wave gas laser at 632.8 nm in TBO solutions. MB and neutral red were also found to induce mutation in addition to photolysis of *E. coli* (Bellin *et al.*, 1969).

After the development of haematoporphyrins, many studies evaluated their effect on Gram-negative and Gram-positive bacteria. Porphyrins appeared to be highly effective against Gram-positive bacteria including *Staphylococcus aureus, Streptococcus faecalis* and *Bacillus cereus,* (Ehrenberg *et al.*, 1985). However, Gram-negative bacteria displayed significant resistance to porphyrin-induced photosensitisation (Bertoloni *et al.*, 1984). Similar results were reported with the use of water and lipid-soluble phthalocyanines. Lipopolysaccharide in the outer membrane of Gram-negative bacteria was thought to prevent access of sensitising agents. Permeabilizing agents such as polymyxin nonapeptide or EDTA were used to render Gram-negative bacteria susceptible to photosensitisation by altering the outer membrane of the bacteria (Nitzan *et al.*, 1992; Bertoloni *et al.*, 1990). Moreover, meso-substituted cationic porphyrins (Merchat *et al.*, 1996a) and cationic water-soluble zinc phthalocyanines (Minnock *et al.*, 1996), in addition to rose bengal, induced some photodynamic inactivation of Gram-negative bacteria (Banks *et al.*, 1985).

Susceptibility of oral bacteria to lethal photosensitisation has been widely investigated by Wilson *et al* (1992). Treatment of periodontal diseases and dental caries was suggested to be a potential application of PDT as periodontal and plaque-related bacteria were reported to be susceptible to PDA.

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## 1.3 OBJECTIVES

Colonisation of organisms (including enteric Gram-negative bacilli) in the oral cavity of cancer patients undergoing cytotoxic therapies creates a potential source of systemic infection and may also be associated with mucositis lesions. Although numerous approaches have been investigated, treatment of mucositis is still unsatisfactory. Some antimicrobial approaches have been reported to be beneficial in reducing the severity of mucositis. However, there are several side-effects related to antibiotic-use and problems with resistance have now reached a crisis point. Efforts to design new antimicrobial regimes are, therefore, in progress. PDT may be an alternative approach for the treatment of mucositis by killing the associated organisms. Resistance to PDT is less likely to occur. Antibiotics damage specific target sites or organelles, on the other hand, singlet oxygen and free radicals generated by PDA cause damage to a range of biomolecules. Therefore, cytotoxicity would be maintained even if alteration to one structure occurred. In addition, side effects of such regimes may be avoided by PDT due to the non-invasive nature of this approach. Infections in the oral cavity could be one of the possible uses of PDT because of the relatively localised and topical nature of the infections.

PDT is better known for its application in the field of oncology. In spite of the fact that the discovery of this technique involved micro-organisms, studies on its clinical use in the field of microbiology have been somewhat neglected. The possible exploitation of this approach for the treatment of infections is, however, now required even more as the problems due to antibiotic resistance increase. Although promising results dating back to 1966 reported by Macmillan *et al.* showed that TBO-mediated PDA was effective in killing Gram-negative (including *P. aeruginosa and E. coli*) as well as Gram-positive cells, subsequent studies have mainly focused on the use of haematoporphyrins and phthalocyanines which were effective against Gram-positive but not Gram-negative cells. Recently, studies have demonstrated that TBO is a very effective photosensitising agent for a wide range of oral bacteria (both Gram-negative and Gram-positive) and candida (Wilson *et al.*, 1992; Bhatti *et al.*, 1997; Wilson and Mia, 1993).

The main objective of this study was to study the effect of PDA on the three most commonly-isolated Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa* and *E. coli*) which may be associated with mucositis. Following basic studies determining the optimal light, photosensitiser and TBO-light interval, studies were performed on the effects of environmental factors, mechanistic aspects and on virulence factors. Although a special emphasis was placed on Gram-negative enteric bacteria, the effect of PDA on the viability of other organisms which may inhabit the oral cavity was also investigated. In addition, in order to determine the likelihood of host tissue damage and to establish a safe light dose and photosensitiser concentration for future clinical application, the effect of PDA with various doses of light energy and TBO on the buccal mucosa of rats have been evaluated.

Chapter 2

MATERIALS AND METHODS

## 2.1 EVALUATION OF PDA *IN VITRO*

### 2.1.1 General Method for PDA

#### 2.1.1.1 Organisms

The organisms used in the PDA studies were Gram-negative bacilli including *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Klebsiella pneumoniae* NCTC 9633.

A number of bacteria and yeasts in mixed suspensions were also evaluated for their susceptibility to PDA. These were *Staphylococcus aureus* NCTC 6571, *Candida albicans* NCTC 3091A, *Streptococcus mutans* NCTC 10449, *Veillonella dispar* NCTC 11831, *Neisseria subflava* ATCC 1078 and *Actinomyces viscosus* NCTC 10951. All the strains used for the experiments were obtained from the National Collection of Type Cultures, Colindale, UK.

In addition, clinical isolates of enteric Gram-negative bacilli, candida and streptococci from the oral cavity of radiotherapy patients were assessed for their susceptibility to PDA (described in more detail in 3.2.4).

#### 2.1.1.2 Growth conditions

All the microbial strains were maintained by overnight subculture on Wilkins-Chalgren agar supplemented with 10% defibrinated horse blood. In addition, selective media were used to cultivate specific organisms (table 2.1).

Table 2. 1   Selective media used for the isolation of specific organisms

| Medium | Selected organisms |
| --- | --- |
| MacConkey | enteric Gram-negative bacilli |
| Mitis salivarius | *Streptococcus* spp. |
| Sabouraud | *Candida* spp. |
| Baird Parker | *Staphylococcus* spp. |
| Veillonella | *Veillonella* spp. |
| Cadmium fluoride acriflavin telluride | *Actinomyces* spp. |
| Thayer Martin | *Neisseria* spp. |

Most of the nutrient media were purchased from Oxoid Ltd., Basingstoke, UK with the exception of mitis salivarius and veillonella agar which were obtained from Difco Laboratories, Detroit, USA.

Cadmium fluoride acriflavin telluride agar was prepared by the addition of tryptone soya broth powder (30 g), agar technical powder (15 g), glucose (5 g), sheep blood (50 ml), cadmium sulphate (13 mg), sodium fluoride (80 mg), neutral acriflavin (1.2 mg), potassium telluride (2.5 mg) and basic fuschin (0.25 mg) to 1 litre of distilled water.

For the purpose of the experiments, a few colonies of each organism were inoculated into Wilkins-Chalgren broth, except *Candida* spp. which were inoculated into sabouraud broth.   Cultures of enteric Gram-negative bacteria, *Staphylococcus spp., Candida spp.* and *N. subflava* were grown aerobically at 37°C overnight.   Cultures of anaerobic and facultatively-anaerobic bacteria, including *Strep. mutans*, *V. dispar*, *A. viscosus*, were grown overnight in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK) at 37°C.

### 2.1.1.3  Photosensitiser

The photosensitiser used in this study was TBO and this was obtained from Sigma Chemicals Ltd., Poole, UK.   TBO solutions of varying concentrations were

prepared by dissolving TBO powder in 0.85% NaCl (w/v) which was then filter-sterilised. TBO solutions were stored in the dark to avoid bleaching by light. See 1.2.3.2.a for the molecular structure and properties of TBO.

## 2.1.1.4 Laser source

The laser light source used for the *in vitro* studies was a helium neon (HeNe) gas laser (Spectra Physics, Tokyo, Japan). The HeNe gas laser is a low power class 3b laser which emits light with a wavelength of 632.8 nm. The power output of the machine was 35 mW and the beam diameter of the light emitted was 2 mm. However, power density and energy doses were calculated on the assumption that light was reflected over the total area of the wells of the microtitre plates used for the *in vitro* experiments. Therefore, the diameter of the wells (6mm) was used for calculation of the spot area (0.283 $cm^2$) so that the power density was 124 $mW/cm^2$.

The basic equations used for the calculations are given below:

energy (J) = power (W) x exposure time (sec)

Power density ($W/cm^2$) = power (W) / per unit area ($cm^2$)

Energy dose ($J/cm^2$) = power density ($W/cm^2$) x irradiation time (sec)

## 2.1.1.5 PDA on microbial suspensions

Overnight (stationary phase) cultures of bacteria were centrifuged at 5000 *g* for 15 min, and resuspended in 10 ml of sterile 0.85% NaCl (w/v). Throughout the experiment, microtitre plates containing 4 mm micro-stirring bars (Camlab Ltd., Cambridge, UK) were positioned on a magnetic stirrer to allow continuous stirring so that each bacterial cell would have equal light exposure. Equal volumes of bacterial suspensions and TBO (100 μl unless otherwise stated) were placed into duplicate wells of a 96-well microtitre plate (Sterilin Ltd., Stone, UK). After a certain pre-irradiation time, PIT (the time required for the incubation of bacteria with TBO prior to irradiation), each suspension was exposed to laser light for a further period. These samples were designated as S+/L+.

Further duplicate control wells were prepared in order to determine the original number of viable bacteria. 100μl of bacterial suspension received 100 μl of 0.85% NaCl (w/v) and was not exposed to laser light, this was designated as S-/L-. Additionally, the effects of laser light and photosensitiser on their own were assessed. To observe the effect of light alone, 100 μl of 0.85% NaCl (w/v) was added to 100 μl of the bacterial suspension, and after a fixed PIT, exposed to laser light for an identical period of time to that of the test suspension (S-/L+). The effect of sensitiser alone was assessed by the addition of photosensitiser to the bacterial suspension. This was left for a period of time corresponding to PIT+irradiation time of each energy dose, but was not exposed to light from the laser (S+/L-). All experiments were repeated to assess the reproducibility of the results.

At the end of the photo-inactivation procedure, 100 μl of each of the well contents were pipetted into bijoux containing 900 μl of Wilkins-Chalgren broth. 10-fold serial dilutions were prepared from $10^{-1}$ to $10^{-8}$. 50 μl of each dilution was pipetted out and plated on Wilkins-Chalgren agar. The plates were incubated overnight and survivors were scored on their ability to form colonies.

## 2.1.2 Electron Microscopy

To visualise the effect of lethal photosensitisation at the ultrastructural level, bacteria were examined by transmission electron microscopy (TEM). Bacterial cell cultures, both before and after TBO and/or laser light exposure, were centrifuged at 5000 *g* for 15 min, and the supernatant was removed. The cells were resuspended in 2.5% EM grade gluteraldehyde in 0.1 M sodium cacodylate buffer and fixed for 1 hour at room temperature. After further centrifugation, the fixative was removed. During the remaining processing, the bacteria were kept as a pellet. Bacterial pellets were dehydrated in a graded series of concentrations of ethanol (up to 90%) and were embedded in LR White resin (London Resin Co., London, UK) at 0°C. Ultrathin sections (90-100 nm) were cut and collected on 200 mesh Cu grids. The sections were stained with uranyl acetate and lead citrate.

Specimens were viewed in a JOEL 100CX transmission electron microscope (JOEL UK , Herts, UK).

## 2.1.3 Autoradiography

Autoradiography was performed using sections prepared as for conventional TEM except that they were collected on 200 mesh carbon formvar-coated Cu grids. The grids were coated with a thin film of Ilford L4 nuclear emulsion (Ilford Ltd., Cheshire, UK) and attached to clean glass slides. The slides were stored in light-tight boxes containing silica gel at 4°C for 2 days, 1 week, 3 weeks and 2 months. Development of the emulsion was performed in a water bath at 19-20°C. The glass slides with the specimens were placed in D17 developer (Kodak, UK) for 2 min. After a 5 sec rinse in distilled water, the emulsion was fixed in Gb (Agfa, UK) for 5 min. The slides were washed in several changes of distilled water for 10-15 min and allowed to dry. The specimens were post-stained in uranyl acetate and lead citrate, and viewed in a JOEL 100CX TEM.

## 2.1.4 Limulus Amoebocyte Lysate (LAL) Assay

Aseptic techniques were employed throughout this assay to avoid exogenous endotoxin contamination and all materials used in the test were pyrogen-free.

### i) Preparation of LAL

The lyophilised LAL was reconstituted with sterile pyrogen-free water, PFW (Associates of Cape Cod, Liverpool, UK) and left to dissolve for 5 min. The solution was aliquoted into small sterile pyrogen-free tubes (one tube of lysate, 1 ml, for each 96-well plate) and stored at -20°C. Immediately before use, the frozen suspension was thawed at room temperature and kept on ice during the assay.

## ii) Preparation of *E. coli* LPS

The experimental *E. coli* endotoxin suspensions were thawed at room temperature and mixed vigorously for 5 min using a vortex mixer. Duplicate twofold dilutions of each test group were prepared starting with the concentration of 10 ng/ml down to 0.0015 ng/ml. A standard *E. coli* LPS with a same concentrations was also used. Test groups and standards were diluted in the wells of a sterile 96-well cell culture plate. The top of the plate was kept aside in its original wrapper and used to perform the LAL assay.

## iii) The assay procedure

10 μl of LAL was pipetted out using a micropipette with a pyrogen-free tip (Associates of Cape Cod) on to the labelled sterile 96-well plate cover, forming a droplet of liquid in the middle of each of the rimmed well covers. Then 10 μl of each of the experimental suspensions and standard was added on top of each lysate spot, starting with the most dilute test and standard and progressing to the most concentrated. Care was taken not to create any air bubbles. All test groups were treated in the same manner. The 96-well plate lid containing the test groups was covered with another sterile plate lid and placed in a humidified metal box to prevent evaporation and stored in a non-$CO_2$ incubator ($CO_2$ interferes with the lysate formation) at 37°C for an hour. After the incubation time, the formation of gels was checked by adding one drop (approximately 2μl ) of 0.2% MB in 70% ethanol onto each spot, again going up the concentration series and in the same order in which the test groups and standard were prepared. The results were recorded within 5 min of the addition of MB. When gelation was present, a star-like appearance was formed by the addition of MB on the surface of the firm gel, and the test was scored as positive. A negative test was revealed by the absence of gelation and the homogenous blend of MB in the soluble spot.

## iv) Calculation of the endotoxin concentration

The sensitivity level of the lysate was stated by the company as being 0.003 ng/ml. The endotoxin concentrations in the experimental groups were calculated by multiplying the label claim by the dilution factor of the most dilute positive dilution to give a value ≥ the endotoxin content, then multiplying the label claim by the dilution factor of the most concentrated negative dilution to give a value ≤ the endotoxin content, this gave a range of values rather than a single value.

## 2.1.5  Preparation of Human Peripheral Blood Mononuclear Cells (PBMC)

PBMC were prepared by Ficoll density gradient centrifugation as described by Bristow *et al.* (1991). Heparinised blood was obtained from healthy donors (North London Blood Transfusion Centre). 25 ml of blood was diluted with an equal volume of RPMI 1640 media (Sigma) in a sterile falcon tube (Sterilin). 35 ml of blood-RPMI media mixture was layered gently onto 15 ml of Ficoll-Histopaque 1077 density gradient (Sigma) so that the blood would float on top. Following centrifugation at 400 $g$ for 30 min at room temperature, the interface layer containing a mononuclear cell layer was collected. The cells were washed twice with RPMI 1640 medium, being centrifuged at 500 $g$ for 15 min. Cells were then resuspended in RPMI medium containing 2% foetal calf serum, 100 U/ml streptomycin/penicillin mixture (Gibco Life Technologies Ltd., Paisley, Scotland) and 2 mM glutamin (Sigma). 1 ml of medium containing 2 x10$^6$ of mononuclear cells per ml was added into each well of 24 well plates. Plates were incubated for an hour at 37°C in 5% $CO_2$/air. Subsequently non-adherent cells were removed by washing twice with RPMI 1640 and incubated with 8 μl (final volume of 0.08 ng/ml) of each test solution of LPS for stimulation of monocytes in 1 ml of RPMI medium for 24 h at 37°C in 5% $CO_2$/air. Control cultures were incubated with RPMI medium alone. Each plate also had a standard LPS preparation. At the end of the incubation period, the plates were centrifuged. Supernatants were carefully transferred either into eppendorfs to be stored at -20°C or directly into ELISA plates for the cytokine assay.

## 2.1.6  Cytokine Assay

The media from the stimulated PBMC were assayed for the presence of pro-inflammatory cytokines. Cytokines were assessed using enzyme linked immunosorbent assay (ELISA). All the coating and detection antibodies and cytokine standards used for the assay were kindly provided by Dr. Steve. Poole, Dept. of Endocrinology, NIBSC, Potters Bar, UK.

## i) ELISA for interleukin-6 (IL-6)

Microtitre plates (Nunc, Fisher Scientific, Loughborough, UK) were coated with 100 μl/well of immunoaffinity-purified polyclonal goat anti-rh IL-6 serum G150/BM (Taktak et al., 1991) at 1 μg/ml, diluted in PBS coating buffer (pH 7.2). Plates were incubated overnight at 4°C. Unbound antibody was removed by washing with wash/dilution buffer (0.01 M phosphate/0.05 M saline buffer containing 0.1% v/v Tween 20, pH 7.2). 100 μl/well of human recombinant standards of IL-6 (over the concentration range of 6000 to 4 pg/ml) were added to the wells of microtitre plates and the supernatants to be tested were added into the remaining wells. Plates were then incubated for 2 h at room temperature and washed three times with wash/dilution buffer. 100 μl of biotinylated affinity-purified polyclonal goat anti-rh IL-6 antibody G150/BM, diluted to1:4000, was added to each well and incubated for 1 h at room temperature. The plates were washed three times and 100 μl of 1:4000 avidin horseradish peroxidase (Avidin-HRP, Dako Ltd., Glostrup, Denmark) was added to each well. Plates were then incubated for 15 min at room temperature before washing three times with wash dilution buffer. The wells were then developed with 100 μl of colour reagent OPD containing 0.2 mg/ml orthophenylenediamine (Sigma) in 0.1 M citric acid-phosphate buffer (pH 5.0) with 0.4 μl/ml 30% $H_2O_2$ (Sigma) and incubated in the dark for 15-30 min at room temperature. The reaction was terminated by the addition of 150 μl of 1 M sulphuric acid ($H_2SO_4$) and the absorbance was measured at 492 nm on a Titertek Multiscan spectrophotometer. A standard curve was plotted of the absorbance (optical density) versus the concentration of standards. The activity of each

preparation of LPS was compared on three occasions with each cell population, with reproducible relative potency and efficacy data being recorded.

## ii) ELISA for interleukin-8 (IL-8)

The assay of IL-8 used a similar protocol to the IL-6 assay described above with the following modifications. Microtitre plates were coated with an immuno-affinity purified goat anti-human IL-8 antibody S333/BM at 0.5 µg/ml. The IL-8 standard used was the international standard for IL-8 at a concentration range of 10000-20 pg/ml. The detecting antibody was biotinylated immuno-affinity purified sheep anti-human IL-8 antibody diluted to 1:2000. A curve was plotted of the absorbance versus the concentration of IL-8.

## 2.1.7 Azocasein Assay

The total proteolytic activity of the bacterial cultures was measured by assay of the azocasein hydrolysing activity. 250 µl of the substrate azocasein (Sigma), a 6% solution in 0.5 M tris buffer (Sigma) at a pH of 7.0, was added to each experimental suspension, and these were incubated aerobically at 37°C for 2.5 h. (Control group consisted of 500 µl 0.85% NaCl (w/v) plus 250 µl azocasein solution.) After this incubation time, 750 µl of 20% acetic acid was added to halt the enzymatic reaction. The eppendorfs were then centrifuged at 5000 $g$ for 15 min. 1ml of supernatant was removed from each sample and placed into a cuvette. The absorbance was read at 420 nm.

As a blank, a sample containing 250 µl 0.85% NaCl (w/v) and 250 µl broth (instead of supernatant) was prepared. 250 µl azocasein was added. To observe the interference of TBO to the readings, 250 µl TBO was mixed with 250 µl broth, and 250 µl azocasein was added. These suspensions were incubated in the same way as the rest and the same steps were followed thereafter.

One unit of activity was defined as that which caused a change in absorbance of 0.001 in an hour at 420 nm.

## 2.2 ANIMAL STUDIES

### 2.2.1 Animals

Female Wistar rats, weighing 250-300 g, provided by the Imperial Cancer Research Fund (London, UK), were housed in groups of five in accordance with Home Office regulations. 6 groups of animals including 3 animals in each group were used (listed in table 2.2).

### 2.2.2 Laser Source

A copper vapour-pumped dye laser (Oxford lasers, Oxford, UK) was used for the animal experiments. The laser machine was tuned to a wavelength of 633 nm. The power output of this laser source was 100 mW. A 6 mm diameter area was irradiated so giving a power density of 350 mW/cm$^2$.

### 2.2.3 Experimental Procedure

Throughout the experiments, the animals were kept under inhaled anaesthesia (Fluothane, FCI Pharmaceuticals, Cheshire, UK). In order to gain easy access, the mouth was retracted using a mouth retractor and any fold in the buccal mucosa was eliminated with tweezers. The head was positioned to the left side so that topically-applied TBO solution would stay in place. Following topical application of 60 μl TBO, a microlens optical fibre was fixed to illuminate a 6 mm diameter area of the buccal mucosa. The opposite, untreated buccal mucosa served as a control.

The treated area was observed daily by visual examination for 3 days for any adverse effects. 72 h after the treatment, the animals were sacrificed by carbon dioxide asphyxiation.

In order to determine if a certain drug/light interval was required prior to light exposure, 5 sec and 15 min intervals were allowed between the application of 25

μg/ml TBO and laser irradiation of 111 J/cm² (5 min). After demonstrating that there was no difference between these 2 groups, drug/light intervals of 5 sec were used throughout the remainder of the study, where 50 μg/ml TBO with 169 J/cm² (8 min) light irradiation and 200 μg/ml of TBO with 338 J/cm² (16 min) of light exposure were compared respectively.

Table 2.2 Parameters used for PDT of rat buccal mucosa

|            | TBO (μg/ml) | Light dose (J/cm²) | TBO/light interval |
|------------|-------------|--------------------|--------------------|
| Protocol 1 | 25          | 111                | 5 sec              |
| Protocol 2 | 25          | 111                | 15 min             |
| Protocol 3 | 50          | 169                | 5 sec              |
| Protocol 4 | 200         | 338                | 5 sec              |
| Dye alone  | 200         | no light           | -                  |
| Light alone| no dye      | 338                | -                  |

The effects of laser light exposure alone and TBO alone were also studied in control groups. Animals either received no sensitiser but were subjected to 338 J/cm² laser light irradiation (light controls) or received 200 μg/ml TBO without irradiation (sensitiser controls). A complete control group which received neither TBO nor light exposure was also used.

## 2.2.4 Histopathologic Examination

The specimens were cut cross-sectionally including the whole buccal mucosa intraorally. The extraoral surface of the section contained dermis. Specimens were fixed immediately in 10 % formalin. 24 h later, the mucosa samples were processed and embedded in paraffin. Serial sections were prepared and stained with haematoxylin and eosin (H&E) for histologic evaluation.

## 2.2.5  Digital Fluorescence Imaging

Fluorescence microscopy allows the detection and localisation of a fluorescent photosensitiser in different regions of tissue at a magnification of x40.  The fluorescence microscope (Oympus IMT-2, Hamburg, Germany), which is an inverted microscope with epifluorescence and phase contrast, was attached to a charged-coupled device (CCD) camera system (Wright Instruments Ltd., model 1, Cambridge, UK).

Fluorescence was excited using an 8 mW helium neon laser operating at 632.8 nm, the output of which was directed on to the section using a liquid light-guide and dichroic mirrors after removal of the extraneous light with a 10 nm bandpass filter.  Fluorescence of TBO was detected in the range of 620-660 nm, using a combination of bandpass (Omega Optical Inc.) and longpass (Schott RG665) filters. The fluorescence signal was detected by a highly-sensitive, cryogenically-cooled CCD camera.

Image operations and processing were carried out using an IBM personal computer which generated false colour-coded images that depict the fluorescence intensity as counts per pixel in arbitrary units.

The slides were subsequently fixed in formalin and stained with H&E.  All specimens were viewed by phase-contrast microscopy to facilitate the orientation of specimens.

## 2.3 STATISTICAL ANALYSIS

A regression analysis was carried out to demonstrate whether there was a dose-response relationship between energy doses or TBO concentrations (either alone or when used in conjunction) and bacterial kill.

One way analysis of variance (ANOVA) was performed to determine whether there were significant differences between the different test conditions. If a significant difference was found between the groups as a whole, further analyses were performed to determine where these differences occurred. A Bonferroni correction was applied to allow for the effect of multiple testing.

Chapter 3

**PDA ON MICRO-ORGANISMS *IN VITRO***

## 3.1 INTRODUCTION

TBO has been shown to be an efficient photosensitising agent of a wide range of micro-organisms. A very early study reported the killing of eleven strains of micro-organisms (including both Gram-positive and Gram-negative bacteria, yeasts and algae) following sensitisation with TBO and irradiation with light (Macmillan *et al.*, 1966). Ito (1977) showed that TBO was one of the most effective sensitisers of yeast cells. Wilson *et al.* have recently shown that a wide range of oral bacteria, including *Strep. sanguis, Por. gingivalis, A. actinomycetemcomitans* and *F. nucleatum* (Wilson *et al.*, 1992), *S. aureus* (Wilson and Yianni, 1995) and yeast cells (Wilson and Mia, 1993), were sensitive to photosensitisation using TBO and HeNe laser light. The aim of this part of the study was to determine the effectiveness of TBO at killing Gram-negative bacteria (*P.aeruginosa, E. coli* and *K. pneumoniae*) when used in conjunction with light from a HeNe gas laser. Quantitative assays were performed to determine the effects of light energy dose, TBO concentration and PIT on the killing of the three target bacteria. Various strains of one of the three target organisms (*E. coli* ) were also tested for their susceptibility to PDA.

The next stage of the investigation was to evaluate and compare the susceptibility of a number of organisms (which may be present in the oral cavity of cancer patients) in mixed cultures as this is closer to the situation *in vivo* i.e., bacteria are rarely present in pure culture in the oral cavity.

The effect of PDA on strains of the enteric Gram-negative bacilli, candida and staphylococci isolated from mucositis lesions was also determined as the susceptibilities of these may differ from those of the laboratory strains.

In summary, the aims of this part of the study were to determine:

i) effect of primary parameters (TBO concentration, light energy dose, PIT) on the killing of the three target bacteria

ii) effect of PDA on different strains of *E. coli*

iii) effect of PDA on a suspension containing a number of laboratory strains

iv) effect of PDA on clinical isolates of organisms isolated from mucositis patients

## 3.2 MATERIALS AND METHODS

### 3.2.1 Dosimetric Studies of the PDA on Gram-negative bacilli

#### 3.2.1.1 Assessment of photosensitiser concentration

The first parameter studied was the effect of TBO concentration on the kills attained. The experimental procedure described in 2.1.1.5 was followed, using various TBO concentrations (50, 25, 12.5, 6.3 and 3.1 µg/ml) with a constant light energy dose (22.3 J/cm$^2$) and a PIT (60 sec). Control groups were composed of the bacterial suspensions in identical concentrations of the photosensitiser, with no light exposure.

#### 3.2.1.2 Assessment of energy dose

The second parameter studied was the energy dose required to achieve substantial kills. The experimental method was identical to that described in 2.1.1.5. After a fixed (60 sec) PIT, bacterial suspensions in TBO (12.5 µg/ml) were exposed to laser light of varying energy doses, which were obtained by increasing the irradiation times. The control suspension received the same light energy doses but in the presence of 0.85% NaCl (w/v) rather than TBO. The energy doses used, corresponding to the various exposure times, were as follows:

Table 3.1   Energy doses together with corresponding exposure periods

| Irradiation time | Energy Dose (J/cm$^2$) |
| --- | --- |
| 30 sec | 3.7 |
| 1 min | 7.4 |
| 3 min | 22.3 |
| 5 min | 37.2 |
| 10 min | 74.4 |
| 15 min | 111.6 |

## 3.2.1.3  Assessment of PIT

The final parameter was the effect of PIT. The same experimental method as above was followed, except that various PITs were tested using a fixed concentration of TBO and a constant light dose. After addition of 25 µg/ml of TBO, suspensions were incubated for 0 sec, 3 min and 15 min and exposed to 22.3 J/cm$^2$ of laser light. Zero incubation time was taken as 5 sec PIT, as the time between the addition of TBO into the bacterial suspension and initiation of the exposure of laser light was 5 sec. Control groups received the same concentration of TBO and were incubated following various PITs but were not exposed to laser light.

All the above-mentioned experiments also had control groups (S-/L-).

## 3.2.2  Evaluation of Photosusceptibility of Different Strains of *E. coli*

Overnight cultures of three strains of *E. coli* (JM 109, XCF, PHSa) were tested for their susceptibility to PDA. Suspensions of each strain were examined as described in 2.1.1.5 except that only conditions as in S+/L+ and S-/L-groups, using 25 µg/ml TBO and 22.3 J/cm$^2$ light dose, were tested.

## 3.2.3  Comparison of Photosensitivity of a Mixture of Organisms

### 3.2.3.1  Comparison of photosensitivity of Gram-negative bacilli

Overnight suspensions of *E. coli*, *P. aeruginosa* and *K. pneumoniae* were centrifuged and resuspended in 10 ml 0.85% NaCl (w/v). The approximate number of bacteria in each suspension was equalised by dilution of the samples to an optical density of 1.0 at 560 nm. An equal amount (1 ml) of each bacterial suspension was collected and mixed. A homogenous mixture was obtained by vigorous shaking with a vortex mixer. 100 µl of the suspension was added into the wells of a 96 well-microtitre plate. After the addition of 100 µl of 25 µg/ml TBO,

the suspensions were irradiated for 1 min, 3 min and 10 min. The control group consisted of bacterial suspensions with 0.85% NaCl (w/v) and no laser irradiation.

MacConkey agar was used for the isolation of each microbial species. A viable count of each organism was performed to allow comparison of their susceptibility to PDA.

### 3.2.3.2 Comparison of photosensitivity of various organisms

Representatives of Gram-negative/positive and aerobic/anaerobic bacteria were selected and assessed for their susceptibility to PDA.

Table 3.2   List of organisms used in a comparison of their response to PDA

| Specific micro-organism | Representative group |
| --- | --- |
| A. viscosus | Gram-positive, microaerophilic |
| Strep. mutans | Gram-positive, facultative |
| N. subflava | Gram-negative, aerobic |
| V. dispar | Gram-negative, anaerobic |
| E. coli | Gram-negative, facultative |
| S. aureus | Gram-positive, facultative |
| C. albicans | Gram-positive, facultative |

Cultures of micro-organisms were incubated overnight at 37°C. Facultative and anaerobic bacteria were incubated under anaerobic conditions while the aerobic bacteria and candida were incubated under aerobic conditions. Cultures were then centrifuged and washed. Micro-organisms were suspended in 0.85% NaCl (w/v) with an optical density (1.0 for bacteria and. 2.0 for C. albicans) which would give a similar viable count of each.

The remainder of the experiment was conducted as described before (using parameters of; TBO concentration: 25 μg/ml, light energy doses: 3.7, 22.3, 74.4 J/cm$^2$, and PIT: 60 sec), except that suspensions were plated onto several selective media for the isolation of each microbial species (table 2.1).

### 3.2.4  Evaluation of Photosensitivity of Clinical Isolates

10 patients with head and neck cancer who were to receive radiotherapy in the Department of Radiotherapy at the UCL hospitals were identified. Patients were selected from those who had no antibiotic intake for at least 2 weeks and were asked whether they were willing to take part in the study. Routine oral hygiene and pain control continued for all the selected patients and they were all monitored regularly.

Rinse samples were collected just before the first radiotherapy session, half way through the radiotherapy course and at the final radiotherapy session. Patients were supplied with 10 ml of sterile saline solution (Baxter, Healthcare Limited, Norfolk, UK) and were asked to rinse their mouth thoroughly for 1 min. The patients returned the mouth rinse to the container, which was then transported to the laboratory for microbiological analysis (Samaranayake et al., 1986). In addition, swab samples were taken by rubbing a sterile cotton-tipped swab gently on the lesions of the patients who developed mucositis. No intake of food was allowed for one hour before the collection of samples.

The samples were vortexed for 10 sec to obtain a homogenous suspension. Serial ten-fold dilutions were performed, vortexing thoroughly, until a 10$^{-7}$ dilution. 25 μl of each dilution was then plated onto selective media (listed in table 2.1) for isolation of candida, Gram-negative bacilli and streptococci. Processing of the samples was completed within 2 hours of sample collection. Following 1-3 days incubation under aerobic conditions, the organisms were enumerated by viable counting.

The identity of the organisms was established on the basis of the following criteria:

- Ability to grow in certain media

- Atmospheric growth requirements

- Gram-staining for determination of the Gram-positive or Gram-negative characteristics and shape on microscopic examination

- Macroscopic features, i.e., characteristic morphology and colonial appearance

- Biochemical tests (API 20 E biochemical kit, Bio Merieux UK Ltd., Basingstoke, UK) were performed for the identification to genus level of aerobic and facultative Gram-negative bacilli. Tables established for the specific characteristics of each bacteria were the reference for biochemical identification. Yeasts were identified as *C. albicans* by the germ tube test. The slide coagulase test was used to identify *S. aureus*.

Following their identification, pure clinical isolates of candida, Gram-negative bacteria and staphylococci were examined to establish if they were as susceptible as their laboratory counterparts using the method described in 2.1.1.5.

## 3.3 RESULTS

### 3.3.1 Effect of Different Parameters

The results from the preliminary quantitative assay demonstrated that killing of all three bacteria with low power light from a HeNe gas laser was possible following their sensitisation with TBO. Neither the laser light alone nor the TBO alone had any significant effect on the viability of bacteria with any of the doses tested.

#### 3.3.1.1 Effect of TBO concentration

Figure 3.1 shows the effect of various TBO concentrations on the viability of a) *P. aeruginosa,* b) *E. coli* and c) *K. pneumoniae.* There was a dose-dependent relationship between TBO concentration and kill for all three bacteria (p<0.001). Increasing the TBO concentration from 1.6-6.3 $\mu$g/ml increased the $\log_{10}$ reduction in viable counts from 1.2, or 94% kill, (*E coli* and *K. pneumoniae*) and 1, or 90% kill, (*P. aeruginosa*) to 5.5, 5.2 and 4.3 (*E. coli, K. pneumoniae* and *P. aeruginosa* respectively). However, successive increase in TBO concentrations up to 25 $\mu$g/ml, did not significantly further increase the reduction in viable counts. Irradiation of suspensions sensitised by 12.5-25 $\mu$g/ml TBO resulted in approximately 5 $\log_{10}$ reduction in the viable count of *E. coli* and *K. pneumoniae* and 4 $\log_{10}$ reduction for *P. aeruginosa.* Therefore, 12.5 $\mu$g/ml TBO concentration was regarded as the optimal concentration.

#### 3.3.1.2 Effect of light energy dose

The effects of light energy dose on each organism are shown in figure 3.2. There was a highly significant dose-dependent decrease in the number of viable cells of all three bacteria with increasing light energy dose (p<0.001). The lowest dose of 3.7 J/cm$^2$ light (exposure period of 30 sec) reduced the colony counts of *E. coli* and *K. pneumoniae* by 0.9 $\log_{10}$ (86%) but no statistically significant effect was observed on *P. aeruginosa.* Reductions of more than 99% in the viable counts

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were obtained by increasing the energy dose to 7.4 $J/cm^2$ (60 sec). Further increasing the dose to 22.3 $J/cm^2$ (3 min) resulted in more than 99.99% reduction in the viable counts of all three bacteria. (giving 5.5 $log_{10}$, 5.1 $log_{10}$ and 4.8 $log_{10}$ reductions for *E. coli*, *K. pneumoniae* and *P. aeruginosa* respectively). The highest dose of 111.6 $J/cm^2$ (exposure period of 15 min) light led to 8.3 $log_{10}$, 8 $log_{10}$, and 5.8 $log_{10}$ reductions of viable counts of *E. coli*, *K. pneumoniae* and *P. aeruginosa* respectively.

### 3.3.1.3 Effect of PIT

The effects of variations in PIT on the viability of bacteria are shown in figure 3.3. Increasing the PIT from 5 sec to 300 sec did not result in significantly greater reductions in the viability of any of the three organisms (p=0.8, 0.2 and 0.6 for *E. coli*, *K. pneumoniae* and *P. aeruginosa* respectively). Similar results were obtained at each PIT; approximately 6 $log_{10}$ reduction for *E coli*, 5.5 $log_{10}$ reduction for *K. pneumoniae* and 3 $log_{10}$ reduction for *P. aeruginosa*.

a)
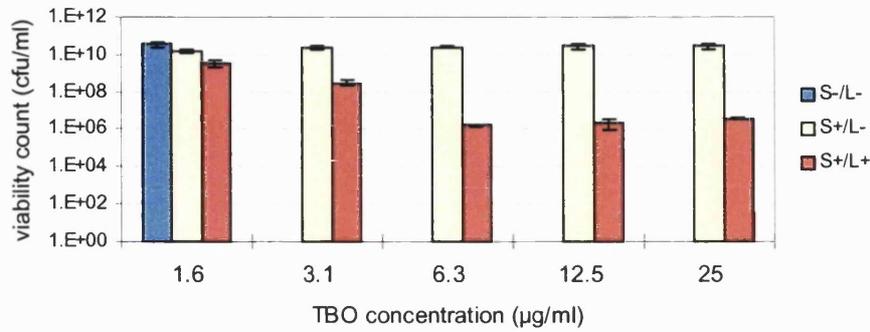


b)
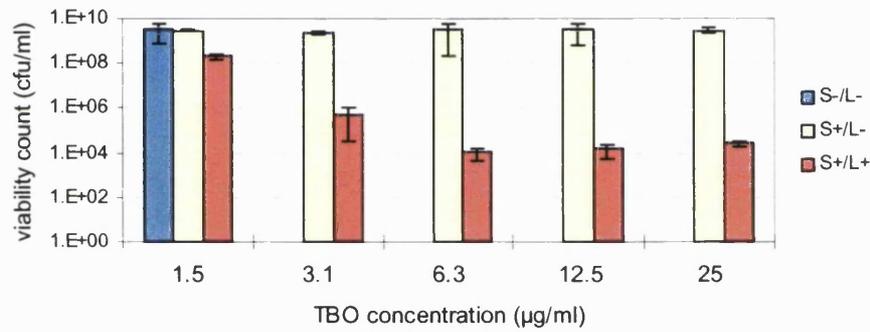


c)
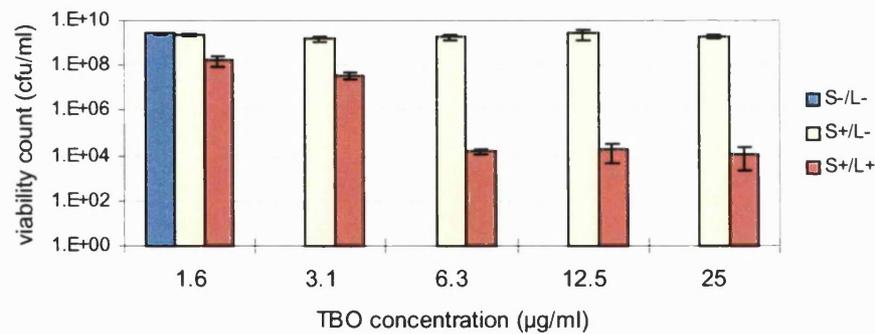


Figure 3.1    Effect of various concentrations of TBO on the photodynamic inactivation of a) *P. aeruginosa,* b) *E. coli* and c) *K. pneumoniae* with 22.3 J/cm$^2$ laser light.   S-/L: exposure to neither sensitiser nor light, S+/L-: exposure to sensitiser but not light, S+/L+: exposure to both sensitiser and light.   Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).   Bars represent the mean viable count (n=4) and error bars represent standard deviations.

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a)
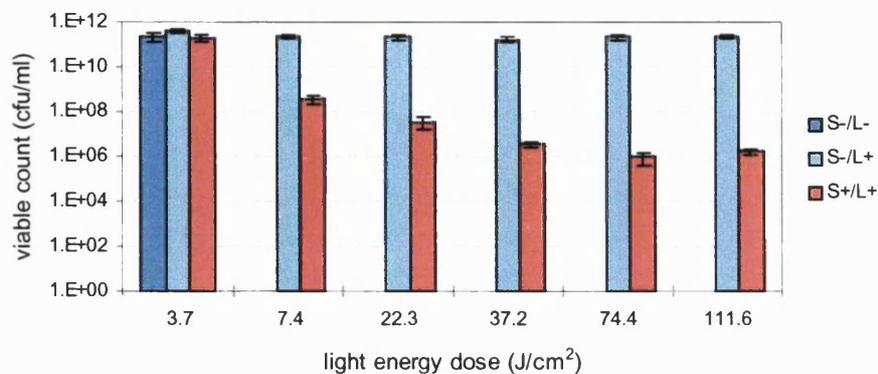


b)
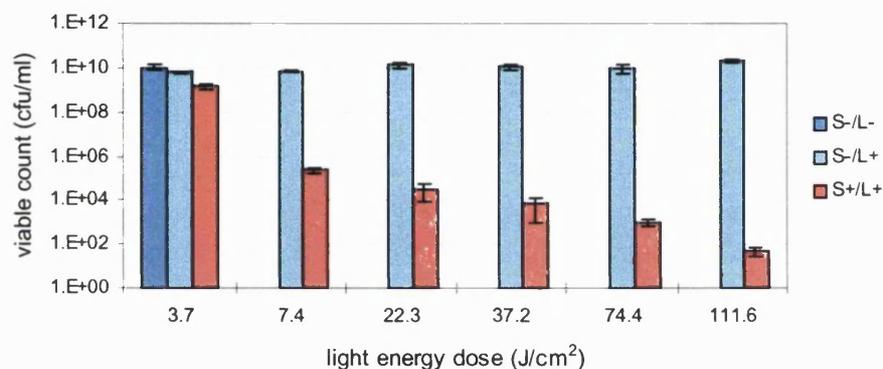


c)
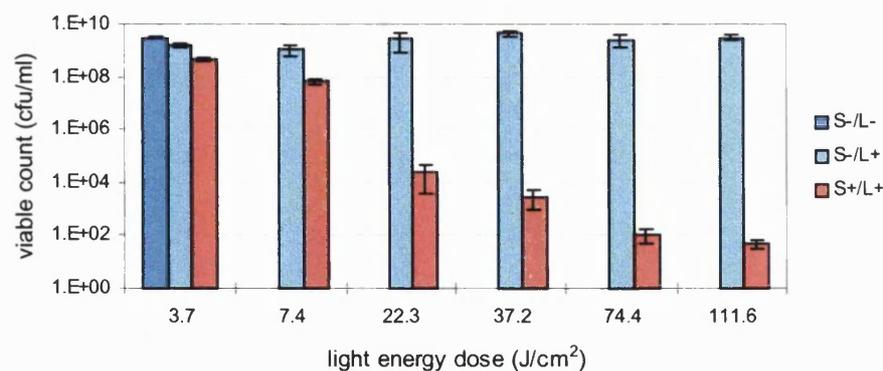


Figure 3.2   Effect of HeNe laser light on the viability of a) *P. aeruginosa,* b) *E. coli* and c) *K. pneumoniae* in the presence of 12.5 µg/ml TBO.   S-/L-:exposure to neither sensitiser nor light, S-/L+: exposure to light but not sensitiser, S+/L+: exposure to both sensitiser and light.   Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml). Bars represent the mean viable count (n=4) and error bars represent standard deviations.

a)
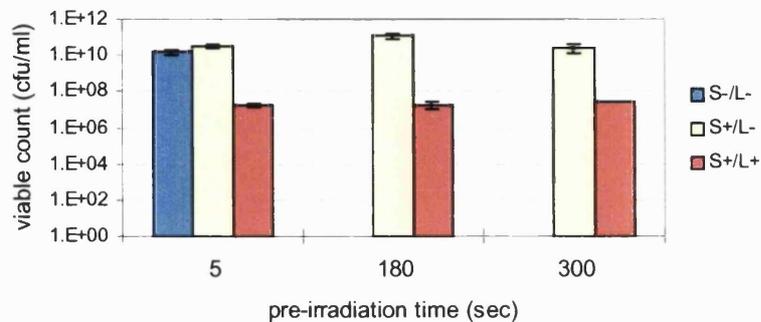


b)
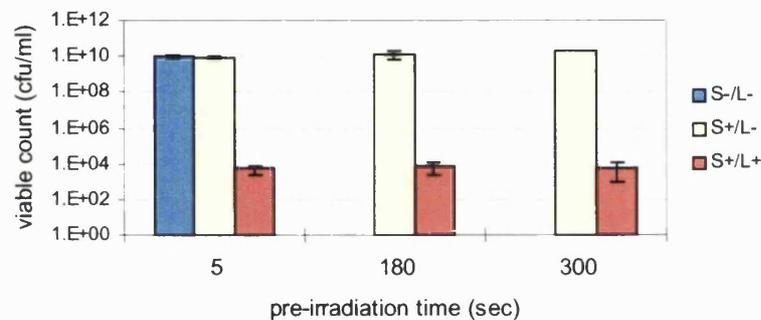


c)
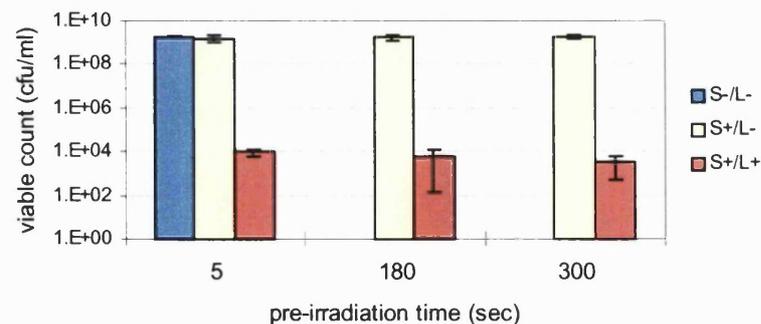


Figure 3.3    Effect of various PITs on the photodynamic inactivation of a) *P. aeruginosa*, b) *E. coli* and c) *K. pneumoniae* in the presence of 12.5 µg/ml TBO and 22.3 J/cm$^2$ laser light. S-/L-:exposure to neither sensitiser nor light, S+/L-: exposure to sensitiser but not light, S+/L+: exposure to both sensitiser and light.    Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).  Bars represent the mean viable count (n=4) and error bars represent standard deviations.

### 3.3.2 Photosensitivity of Various Strains of *E. coli*

Figure 3.4 shows the response of various strains of *E. coli* to exposure to TBO/light. Each of four strains of *E. coli* was found to be susceptible to PDA. Furthermore, there was no evidence of any statistically significant difference between the strains, and similar reductions in bacterial viability were achieved (approximately 6.5 $\log_{10}$ reductions in viable counts) following exposure to 22.3 $J/cm^2$ laser light of the four strains sensitised with TBO.
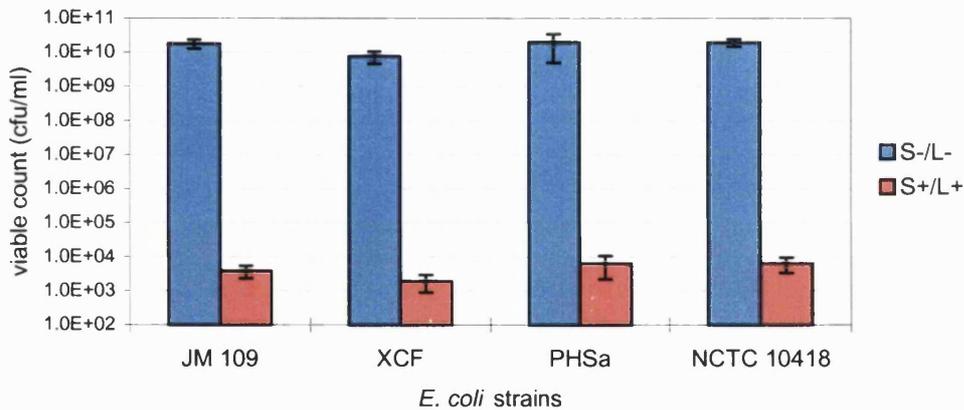


Figure 3.4    Viable counts of various strains of *E. coli* before (S-/L-) and after (S+/L+) photodynamic inactivation.  Suspensions were treated with 25 μg/ml TBO for 60 sec prior to 22.3 $J/cm^2$ light exposure.  Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).  Bars represent the mean viable count (n=4) and error bars represent standard deviations.

### 3.3.3  Comparison of Photosensitivity of Three Target Bacteria

Figure 3.5 shows the susceptibility of the three target bacteria to photodynamic inactivation in mixed cultures. Irradiation of TBO-treated suspensions containing approximately $10^{10}$ colony forming units per millilitre of each organism led to similar reductions. Approximately 3.5 $\log_{10}$, 5 $\log_{10}$ and 5.5 $\log_{10}$ reductions in colony counts of each species were found with light doses of 7.4, 22.3 and 37.2 $J/cm^2$ respectively. There was a statistically significant difference between the kill obtained for *E. coli* and *P. aeruginosa* ($p=0.02$ and $p<0.001$ respectively for 22.3 and 37.2 $J/cm^2$), however the magnitude of this difference was relatively small.
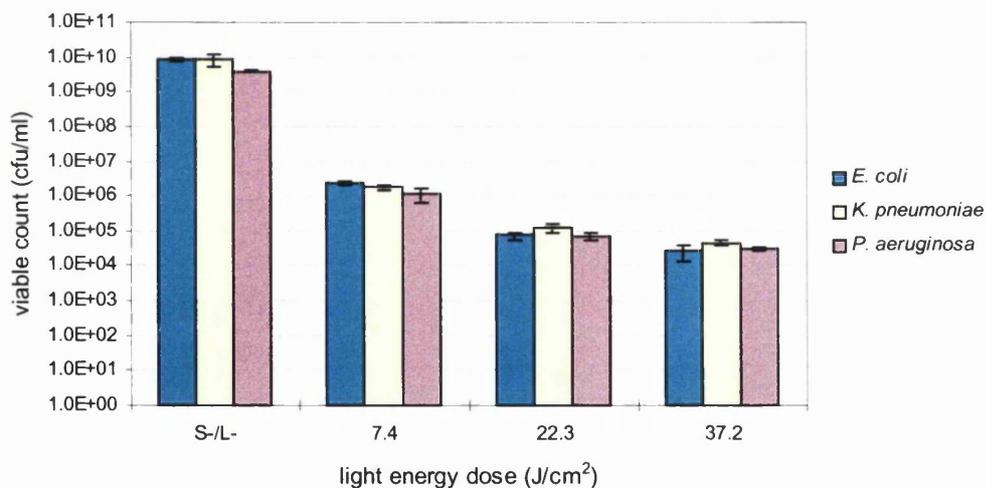


Figure 3.5   Viable counts of suspensions of the three facultative Gram-negative bacilli before (S-/L-) and after exposure to 7.4-37.2 $J/cm^2$ of light in the presence of 12.5 µg/ml TBO. Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml). Bars represent the mean viable count (n=4) and error bars represent standard deviations.

### 3.3.4  Photosensitivity of Various Organisms

The results of the susceptibility to PDA of various micro-organisms likely to be encountered in mucositis lesions can be seen in figure 3.6.   When various organisms were treated in a mixed suspension, all the organisms tested were found to be susceptible.  The Gram-negative and the Gram-positive bacteria were shown to be similarly affected.  However, when a 3.7 $J/cm^2$ light dose was used, the kill rate obtained for *N. subflava* was significantly different from that of the other bacteria tested ($p<0.001$), no evidence of any statistically significant difference was found with higher light doses.  However, the kills obtained with *C. albicans* were significantly different from those of any of the bacteria studied on all occasions ($p\leq0.004$).  Increasing the energy dose led to similar death curves for each of the bacterial species.  Over 90%, 99.99% 99.999% reductions in the viable counts of each TBO-treated species were obtained by irradiation with 3.7 $J/cm^2$, 22.3 $J/cm^2$ and 74.4 $J/cm^2$ light respectively.  The same light doses resulted in 54%, 88% and 98% reductions in the viable counts of *C. albicans*.
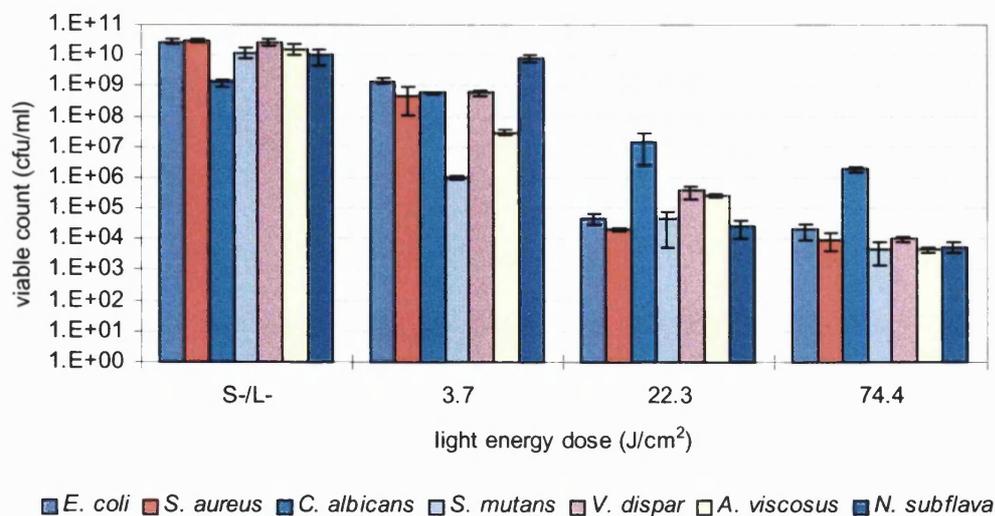


Figure 3.6    Response of individual species (non-oral and oral) to various periods of exposure to light in the presence of 12.5 µg/ml TBO.  S-/L-: exposure to neither sensitiser nor light.  Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).  Bars represent the mean viable count (n=4) and error bars represent standard deviations.

### 3.3.5 Susceptibility of Clinical Isolates to PDA

A list of candida, Gram-negative bacilli and streptococci isolated from the saliva and mucosal lesions of patients undergoing radiotherapy is given in table 3.3. The saliva samples taken prior to the radiotherapy course showed no growth of Gram-negative bacilli except in one patient who was colonised with *P. aeruginosa* and *K. pneumoniae*. 3 patients were colonised with *C. albicans* and 1 patient with *S. aureus*. Mid-radiotherapy samples showed an increase in the number of patients who were colonised with these organisms. 3 patients were colonised with a single species of these organisms whereas 4 patients were colonised with two species concomitantly. Consecutive samples at the end of the radiotherapy course showed colonisation patterns similar to those observed in the mid-therapy samples. 8 out of 10 patients were colonised (either alone or concomitantly) with at least one of the three organisms at some period during the radiotherapy course.

The swab samples from the lesions gave a similar colonisation pattern to the rinse samples. During radiotherapy, 7 out of 10 patients developed clinical evidence of mucositis to varying degrees and 2 out of 3 patients who showed no sign of mucositis were colonised with *C. albicans*. On the other hand, one patient, who was not colonised with any of the three organisms, developed mucositis. Samples from lesions in 5 patients who developed mucositis showed simultaneous colonisation by 2 of the 7 organisms isolated. In 4 patients *Candida* spp. co-existed either with Gram-negative bacilli or with *Staphylococci* spp. While in one case, colonisation by *Staphylococci* spp. and *E. coli* was observed.

Table 3.3    List of organisms isolated from saliva and mucosal lesions of patients undergoing radiotherapy.

| | Rinse samples | | | Swab samples |
|---|---|---|---|---|
| Patients | pre-radiotherapy | mid-radiotherapy | end-radiotherapy | |
| 1 | - | - | - | no mucositis |
| 2 | C. albicans (192000±7244) | C. albicans (85000±7225) | C. albicans (210000±4850) | no mucositis |
| 3 | C. albicans (600±516) | - | C. albicans (2200±148) | no mucositis |
| 4 | - | - | - | - |
| 5 | | Ent. aerogenes (17000±5033) C. albicans (4500±1361) | Ent. aerogenes (41000±1438) C. albicans (1800±1366) | Ent. aerogenes (1500±887) C. albicans (3000±2643) |
| 6 | K. pneumoniae (98000±1327) P. aeruginosa (63000±2295) | K. pneumoniae (2400±730) C. albicans (10100±6600) | C. albicans (68000±13856) | K. pneumoniae (20000±5657) Candida spp. (18000±5164) |
| 7 | C. albicans (83000±1740) | C. albicans (5700±2720) S. aureus (8100± 3814) | C. albicans (6700±2600) S. aureus (7770±2891) | Candida spp. (1100±383) S. aureus (4100±2559) |
| 8 | | C. albicans (23000±6831) S. aureus (135000±1031) | C. albicans (44000±1424) S. aureus (156000±1987) | C. albicans (1100±1677) S. aureus (2900±2126) |
| 9 | | E. coli (1600±1566) | E. coli (1900±1545) | E. coli (900±683) Staph. spp. (600±766) |
| 10 | S. aureus (400±327) | S. aureus (2500±2891) | S. aureus (1000±766) C. albicans (800±1347) | S. aureus (1500±2220) |

The figures represent viable counts (± standard deviation) of the organisms isolated.

The effect of PDA on the viability of these potentially-pathogenic organisms can be seen in figure 3.7. Clinical strains of Gram-negative bacilli (*Ent. aerogenes, E. coli, K. pneumoniae* and *P. aeruginosa*) and *S. aureus* were shown to be equally susceptible to PDA (no statistically significant difference in susceptibility). Nearly 1 $\log_{10}$ reduction with 3.7 $J/cm^2$ light dose and 6 $\log_{10}$ reduction with 74.4 $J/cm^2$ light dose were obtained for all viable counts of the bacteria tested. A similar reduction in the viable counts of *C. albicans* was observed with 3.7 $J/cm^2$ light dose (no significant difference). However, with higher doses of light, the susceptibility of *C. albicans* was less compared to that of the Gram-negative bacilli and *S. aureus* (2 $\log_{10}$ reduction with 22.3 $J/cm^2$, $p<0.001$ and 3 $\log_{10}$ with 74.4 $J/cm^2$, $p=0.001$ of light dose).
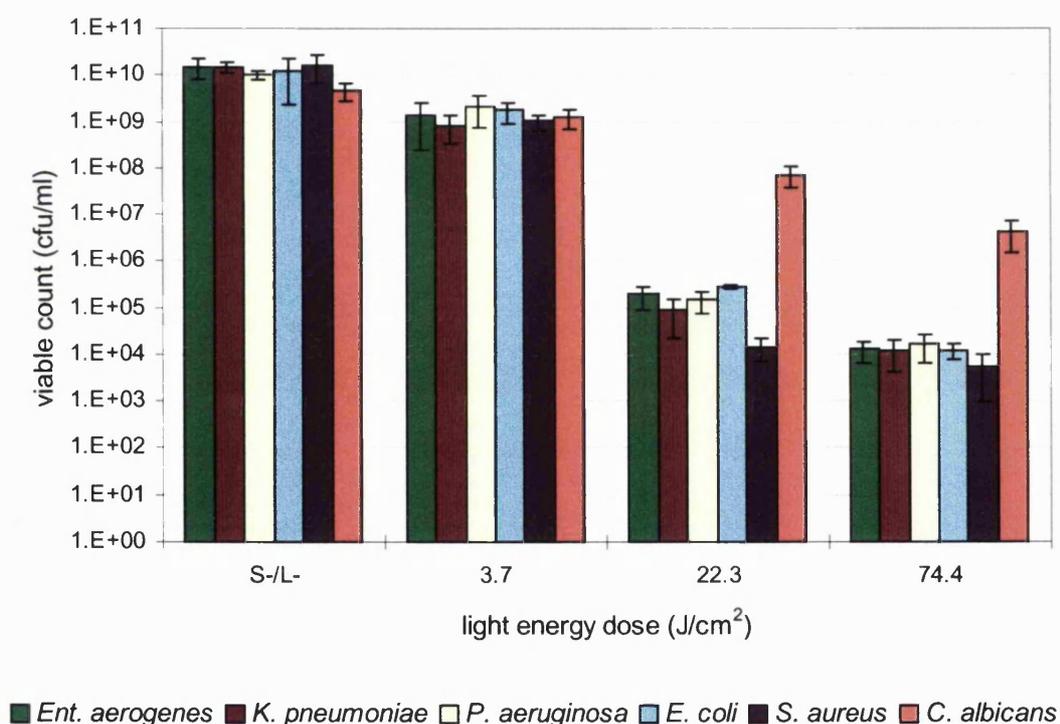


Figure 3.7    The susceptibility to PDA of clinical strains of potential pathogens. Suspensions of organisms were exposed to various light energy doses (3.7, 22.3 and 74.4 $J/cm^2$) in the presence of 12.5 µg/ml TBO. Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml). Bars represent the mean viable count (n=4) and error bars represent standard deviations.

## 3.4 DISCUSSION

This study examined the effect of TBO-mediated PDA on the viability of Gram-negative bacilli because of their close association with mucositis. In addition, the susceptibilities of other mucositis-associated micro-organisms, such as *S. aureus* and *C. albicans,* were of interest as well as those of oral commensal bacteria.

Substantial kills of all three target bacteria were achieved by TBO-mediated PDA, and the bactericidal effect achieved was found to be dependent on the light energy dose and the TBO concentration (but independent of PIT) with the doses studied. PDA was possible only when the photosensitiser and the light were present together, and the absence of either of these resulted in no significant reduction in bacterial viability.

The results of this study are encouraging as eradication of Gram-negative bacilli has been considered to be difficult due to the resistance of the bacteria to most other antibacterial strategies. In the case of photodynamic inactivation of micro-organisms, although a wide range of Gram-positive bacteria were demonstrated to be very susceptible with most of the photosensitisers studied, (Bertoloni *et al.,* 1984; Wilson *et al.,* 1993a) disappointing results were obtained for the killing of Gram-negative bacteria with certain photosensitisers, especially with porphyrin derivatives (Nitzan *et al.,* 1987b; Martinetto *et al.,* 1986; Bertoloni *et al.,* 1984). Although *Porphyromonas gingivalis* (anaerobic Gram-negative oral species), and *Helicobacter pylori* (gastritis-associated Gram-negative species) were reported to be sensitive to PDA with a range of photosensitisers (Wilson *et al.,* 1993b; Millson *et al.,* 1996a), differences in the surface components of these bacteria may contribute to their varying susceptibility to PDA. Some investigators have attributed the resistance of Gram-negative bacteria to PDA to the outer membrane of these organisms. Some outer membrane disrupting agents such as Tris-EDTA and polymyxin nonapeptide enabled photodynamic inactivation of Gram-negative bacteria with otherwise non-effective photosensitisers (phthalocyanines and deuteroporphyrin) (Bertoloni *et al.,* 1990; Nitzan *et al.,* 1992). In this study, it was found that Gram-negative bacilli could be killed without the need for any

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membrane-disorganising agent as a preparatory step to the photodynamic inactivation, when TBO was used as the photosensitising agent. Moreover, a higher degree of cell death was achieved when TBO-treated cells were irradiated for shorter periods compared to the kills achieved in both of the studies mentioned above (Bertoloni *et al.*, 1990; Nitzan *et al.*, 1992). One of the likely reasons for the effectiveness of TBO might be the direct effect of the TBO-activated PDA on the outer membrane, and subsequently, on the cytoplasmic membrane. In support of this suggestion, Ito (1980) reported that membrane damage was the major cause of cell death by TBO-mediated photodynamic inactivation.

The photosensitivity of bacteria may be related to the charge of the photosensitiser. Cationic compounds appear to be more effective sensitisers and TBO is a cationic and water soluble compound. Other cationic compounds have been reported to be effective photosensitisers. For example, cationic water-soluble zinc phthalocyanine was shown to be an active photosensitiser of both Gram-negative and Gram-positive bacteria while anionic and neutral phthalocyanines had no photosensitising effect (Minnock *et al.*, 1996). Similarly, meso-substituted cationic porphyrins were shown to efficiently photosensitise Gram-negative bacteria while negatively-charged meso-substituted porphyrins had no appreciable effect (Merchat *et al.*, 1996a). Although it is not clear why cationic compounds are more effective, the affinity of positively-charged compounds to highly anionic sites (e.g., bacterial outer membranes) may account for their effectiveness. Comparison of the results of the data presented in this study and those obtained with the above-mentioned cationic photosensitisers is extremely difficult due to differences between the studies in terms of the volume and density of the bacterial suspensions (thus the corresponding energy density for each bacterial cell), physiological status of the cells, the light source, its power, exposure times, PIT and photosensitiser concentration. However, greater kills could be achieved when TBO was used (as indicated in this study) with smaller energy doses compared to those obtained with the sensitisers used in the above-mentioned studies.

The data in this study indicate that photosensitisation is dependent on the concentration of TBO (from 3.1 to 12.5 µg/ml). However, there was no significant

difference in the kills obtained when the TBO concentration was increased from 12.5 to 50 μg/ml. There are two possible explanations for this. Firstly, in lower concentrations (up to 12.5 μg/ml), there is just sufficient light energy for each TBO molecule to interact while, at higher concentrations there will be free non-reacted TBO molecules with no more cytotoxic species production. The second explanation may be that with the higher TBO concentrations, the target sites on bacteria are saturated and although unbound TBO may absorb light and produce cytotoxic species, these species may be quenched before reaching the cells. Interestingly, Bhatti et al., in 1997, found a slight decrease in bacterial kills at higher concentrations (above 12.5 μg/ml). They proposed that the shielding effect of excess TBO was the reason for the impaired photodynamic effect on Por. gingivalis when used at higher concentrations.

The most effective minimum concentration of TBO when 22.3 J/cm$^2$ light energy was used was 12.5 μg/ml which corresponds to 0.001% (w/v). This concentration is 1000 times smaller than the concentration of TBO (1%) approved for use in the diagnosis of oral cancer (Silverman et al., 1984; Mashberg, 1981). However, before clinical use of TBO as a photosensitiser in the PDT of infections, its safety in the concentrations used in this study needs to be established in animal studies.

All three species of Gram-negative bacilli were equally susceptible to PDA when suspensions were incubated with TBO for 5 sec, 3 min and 15 min prior to irradiation, which implies that TBO sensitises the Gram-negative bacilli very rapidly. Although the PIT used in this experiment referred to the incubation of the photosensitiser and the bacteria prior to exposure, the period during the irradiation itself should also be taken into account as the continuous stirring (hence the interaction) of the bacteria-TBO suspension was taking place throughout the exposure period (3 min). The advantage of rapid sensitisation of Gram-negative bacilli by TBO from a clinical perspective is that it would substantially shorten the treatment time.

Depending on the photosensitiser and micro-organism used, different results were reported on the effect of PIT on the bacterial kill. Wilson and Pratten (1995)

showed that killing of *S. aureus* by TBO-mediated lethal photosensitisation was independent of PIT. In the presence of the same photosensitiser, greater kills of yeasts were achieved when the PIT was increased from 1 min to 3 min, though the kill remained the same after 3 min PIT (Wilson and Mia, 1993). In contrast, Ito (1980) observed a small but significant increase in the effectiveness of TBO when incubated for longer periods (30 min).

Rapid photosensitisation of bacteria with TBO may indicate either a rapid uptake of the sensitiser by bacteria or that the photosensitiser is effective from outside and no penetration into the cell is required. If the latter assumption is correct, then the outer membrane may be one of the sites of action for the photodynamic inactivation of cells.

It has been shown that bacteria can be killed by light from high power lasers such as the carbon dioxide laser (Dederich *et al.*, 1990) and the Nd:YAG laser (Schultz *et al.*, 1986). However, the effect is thermal and the power required to kill bacteria is too high for safe use on human tissue. In this study it was found that up to 111.6 $J/cm^2$ of laser light itself had no effect on the viability of bacteria. Several other studies have also demonstrated that red light at these energy doses has no effect on the viability of a range of bacteria (Burns *et al.*, 1993) and candida (Wilson and Mia, 1993). However, bacteria containing compounds similar to photosensitisers may be susceptible to killing by light energy. 3.5 J of red light was shown to reduce the viability of *S. aureus* and this was suggested to be due to the presence of endogenous sensitisers (Wilson and Pratten, 1994). *Propionibacterium acnes* has been shown to be susceptible to blue light with a wavelength of 415 nm and the endogenous photosensitisers responsible are thought to be porphyrins (Kjeldstad and Johnsson, 1986). In contrast to this, although *Por. gingivalis* contains protohaemin (a compound similar to the porphyrins), exposure of light from a HeNe laser at an energy dose of 2.2 $J/cm^2$ was reported to have no effect on bacterial viability (Bhatti *et al.*, 1997). This was explained by either the low absorption of light of this wavelength by porphyrins or by the insufficient concentration of porphyrins in the bacteria. The majority of oral

bacteria do not contain endogenous photosensitisers and therefore light alone will not produce any photosensitising effect.

Several photosensitisers, including haematoporphyrin and MB, have been reported to be toxic in the absence of light (Wilson *et al.*, 1993a). However, this study showed that TBO, at the concentrations used, exhibited no dark toxicity to Gram-negative bacilli.

The next stage of this investigation was to evaluate the susceptibility of a number of bacteria in mixed cultures. When the susceptibilities of the three target bacteria were tested separately, an accurate comparison of their relative susceptibilities was not possible due to the different numbers of cells present in each overnight culture prior to treatment with TBO and/or light. In early experiments *P. aeruginosa* appeared to be the least susceptible organism of the three. This is probably attributable to the higher density of the cell suspension used. Increasing the cell density may increase the competition of bacteria for free TBO molecules and the shielding effect on each other. Accordingly, when an approximately equal number of bacteria were present in the suspension, all three species were shown to be similarly sensitive to PDA.

Under the conditions employed in this study, the antimicrobial effect of photodynamic inactivation appeared to be non-specific, i.e., not targeted to a certain microbial group. All three Gram-negative bacilli appeared to be equally susceptible to PDA. In addition, Gram-negative and Gram-positive bacteria seem to have a similar degree of susceptibility. Moreover, similar results were obtained with commensal bacteria together with the non-commensal mucositis-associated bacteria except there was a reduction in the kill of yeasts. Although approximately equal numbers of each microbial species were mixed in the *in vitro* experiment, the number of candida is much smaller compared to the oral commensals in the oral cavity (hence the concentration of the candida per irradiation area) will be much lower than the bacterial population. However, selective photosensitisation could be achieved using TBO conjugated to antibodies against specific organisms. This approach, using the sensitiser Sn (IV) chlorin e6 linked to monoclonal

antibodies against *P. aeruginosa*, has been shown to be effective in selectively killing *P. aeruginosa* in the presence of *S. aureus* (Friedberg *et al.*, 1991). Similar results were found by Bhatti *et al.* (1998a) for the selective photosensitisation of *Por. gingivalis* in a mixed suspension with *Strep. sanguis*, using TBO-conjugated antibodies. Additionally, a new healthy flora may be maintained by the inoculation of the commensal oral flora immediately after the sterilisation of the mouth by PDT.

Although the aim of this part of the study was not to analyse the incidence of Gram-negative bacilli, candida or staphylococci in the oral cavity of cancer patients undergoing radiotherapy or the association between the degree of mucositis and the colonisation of these organisms, it was noted that all patients (except one) who developed mucositis were also colonised with Gram-negative bacilli, candida or staphylococci.

Strains of enteric Gram-negative bacilli, staphylococci and candida isolated from the mouths of patients were also shown to be as susceptible to PDA as the laboratory strains. Cancer patients receiving cytotoxic therapies are often on systemic prophylactic antibiotics, antiviral and antifungal agents. The antiviral effects of PDA have also been demonstrated and it has been used for sterilisation of blood prior to transfusion (Mohr *et al.*, 1997). Therefore, if proven to be an effective strategy for the elimination of pathogenic organisms from the oral cavity *in vivo*, use of PDT may eliminate the need to use separate antibacterial, antifungal and antiviral agents.

In summary,

1) When TBO-sensitised *E. coli, P. aeruginosa* and *K. pneumoniae* were exposed to HeNe laser light, significant reductions in viable counts were obtained. There was a positive correlation between the light energy dose and bacterial kill achieved, i.e., increasing the light energy dose resulted in higher kills. The kill was also increased by increasing the concentration of TBO up to 6 µg/ml. However, further increase in the concentration upto 25 µg/ml had no effect on the kills obtained. An increase in the PIT had no effect on the bacterial kills obtained. A

PIT of 5 sec was sufficient for PDA. When used alone, neither the laser light nor TBO were able to achieve any significant reductions in bacterial viability.

2) The three laboratory strains of *E. coli* tested were equally susceptible to PDA.

3) All three species were equally susceptible to PDA in mixed cultures.

4) Potentially pathogenic organisms (*E. coli*, *S. aureus* and *C. albicans*) were as susceptible as the commensal bacteria (*Strep. mutans*, *A. viscosus*, *V. dispar* and *N. subflava*) in mixed cultures.

5) Clinical isolates of mucositis-associated organisms (enteric Gram-negative bacilli, *S. aureus* and *C. albicans*) were similarly susceptible to the effects of PDA.

**Chapter 4**

**EFFECTS OF PHYSIOLOGICAL FACTORS ON PDA**

## 4.1  INTRODUCTION

The results discussed in the previous chapter have shown that all three of the target Gram-negative bacilli were susceptible to PDA in saline suspensions. Under *in vivo* conditions, however, there are several environmental factors that may influence the effectiveness of PDA on micro-organisms. In addition, bacteria may respond differently to antimicrobial strategies depending on their physiological status. Thus, it is essential to evaluate whether killing of target bacteria by this method is as effective under conditions that would be encountered in the oral cavity. In addition, understanding the effect of biological factors on PDA may create an opportunity to modulate its effect for optimal results.

Firstly, the effect of saliva on the degree of PDA was investigated. In the mouth, bacteria are mainly surrounded by saliva which contains many organic and inorganic components. The effect of serum on the PDA was also studied, as serum exudate from the ulcerated areas of mucositis lesions may contribute to the environment in which the bacteria are present. Serum and saliva may behave similarly and may impair the effect of photodynamic action physically and photochemically in a number of ways. Nevertheless, immune cells present in the saliva and serum may well augment the effect of PDT in the oral cavity.

Acidity of the saliva of cancer patients undergoing cytotoxic therapies has been reported to be in the lower pH range (around pH 5-7) (Ben-Aryeh *et al.*, 1975). Alteration of the acidity of saliva may have some effect on PDA by influencing the binding of sensitisers to target cells and also the stability of cytotoxic species. The effect of various pH values of the environment was, therefore, evaluated.

Finally, the effectiveness of PDA on bacteria when in various growth phases, was determined. Cells display an altered physiology and composition depending on their state of growth, and this may influence the susceptibility of cells to PDA. In the mouth, bacteria may be found in various physiological states, depending on several factors including available nutrients, competition with the other (both

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commensal and non-commensal) bacteria, and alterations in saliva, e.g., variations in pH values.

Therefore, the aim of this part of the study was to determine the effects on PDA of;

i) the presence of saliva

ii) the presence of serum

iii) pH variation

iv) the growth phase of the target bacteria

## 4.2 MATERIALS AND METHODS

### 4.2.1 Evaluation of the Effect of Saliva and Serum

2 ml of saliva was collected from five healthy volunteers, pooled and mixed vigorously to obtain a homogenous suspension. Overnight cultures of bacteria were centrifuged at 5000 $g$ for 15 min and the supernatants were removed. The bacteria were resuspended in 10 ml of saliva mixture, horse serum (Oxoid) or 0.85% NaCl (w/v). 100 $\mu$l of the resulting bacterial suspensions were sensitised with TBO to give a final concentration of 25 $\mu$g/ml, and after a 60 sec PIT, exposed to laser light (22.3 J/cm$^2$). Additional suspensions were treated in an identical manner using 37.2 J/cm$^2$ and 74.4 J/cm$^2$ of light. Control groups received 100 $\mu$l of 0.85% NaCl (w/v) instead of TBO, and were not irradiated. The remainder of the experiment was carried out as described in 2.1.1.5 except that bacteria were plated onto MacConkey agar, instead of Wilkins-Chalgren agar in order to suppress the growth of oral bacteria and to select for the enteric Gram-negative bacilli in order to determine the number of survivors.

### 4.2.2 Evaluation of the Effect of pH

Overnight cultures of bacteria were centrifuged at 5000 $g$ for 15 min and the supernatants were removed. The bacteria were resuspended in buffers at various pH values (pH 4.0-8.0) and in 0.85% NaCl (w/v) which acted as a control (pH 7.4). The citrate-phosphate buffers were prepared by the addition of 0.1 M citric acid solution and 0.2 M dibasic sodium phosphate solution in various proportions to obtain the desired pH values. TBO was dissolved in the appropriate buffers for each experimental condition. The bacterial suspensions (100 $\mu$l) at each pH value and 0.85% NaCl (w/v) were then either exposed to 22.3 J/cm$^2$ of light following the addition of 100 $\mu$l of 50 $\mu$g/ml TBO, or they received relevant buffer or 0.85% NaCl (w/v) with no laser irradiation. The experiments were completed as detailed in 2.1.1.5.

### 4.2.3    Evaluation of the Effect of the Growth Phase of the Bacteria

A few colonies of bacteria were inoculated in 10 ml Wilkins-Chalgren broth and incubated aerobically. The turbidity of the suspension was monitored on an hourly basis (up to 18 h) by reading the absorbance at 560 nm, and a growth curve was prepared. According to this growth curve, the lag, exponential and stationary phases of bacteria were identified. The susceptibility of bacteria to photosensitisation in each phase was then determined.

Suspensions at each phase were centrifuged at 5000 $g$ for 15 min and resuspended in 0.85% NaCl (w/v). Cultures in each phase had different numbers of bacteria i.e., lowest numbers in lag phase cultures and highest numbers in the stationary cultures. In order to make a valid comparison, approximately equal numbers of bacteria in each phase were obtained by diluting the high density suspensions to give similar optical densities. 100 µl aliquots of these suspensions received equal volumes of 50 µg/ml TBO and were exposed to 22.3 J/cm$^2$ of light. Control groups in each growth phase received 0.85% NaCl (w/v) but were not exposed to light. The assay was conducted as detailed in 2.1.1.5.

## 4.3  RESULTS

### 4.3.1  Effect of Saliva and Serum

The effect of saliva and serum on the PDA on bacteria can be seen in figure 4.1. In all cases, the kills achieved when organisms were suspended in saliva were lower than those achieved when bacteria were in saline but greater than those achieved in serum. In the presence of serum, a significant decrease in the kills was observed for all three bacteria (p at least 0.003). However, in the presence of saliva, the reduction in kill was not statistically significant for $K.$ $pneumoniae$, but was significant for $E.$ $coli$ (when 22.3 $J/cm^2$ light dose was used, p=0.003) and for $P.$ $aeruginosa$ (when 74.4 $J/cm^2$ light used, p<0.001).

Irradiation with 22.3 $J/cm^2$ of light resulted in a 3.9 $\log_{10}$ reduction in the viable counts of $E.$ $coli$ in saliva compared to a 6.1 $\log_{10}$ reduction in saline suspensions. Therefore, the presence of saliva impaired the effectiveness of PDA in saline. However, when light energy doses were increased to 37.2 and 74.4 $J/cm^2$, there were 4.8 $\log_{10}$ and 5.4 $\log_{10}$ reductions in cell viability in saliva compared to 6.5 $\log_{10}$ and 7.1 $\log_{10}$ reductions in saline. When bacteria were suspended in serum the bacterial kills were impaired further. 22.3 $J/cm^2$ had no effect on bacterial viability and a reduction of 0.7 $\log_{10}$ (85%) was found only after 74.4 $J/cm^2$ light irradiation.

In the presence of saliva, similar results were observed with the $K.$ $pneumoniae$ and $P.$ $aeruginosa$ suspensions. 22.3 $J/cm^2$ light led to a 3 $\log_{10}$ reduction and 74.4 $J/cm^2$ light resulted in reductions similar to those in saline suspensions (99.9999% kill). Similarly, 37.2 and 74.4 $J/cm^2$ light exposure achieved 2.7 $\log_{10}$ (98% kill) and 4.2 $\log_{10}$ (99.99% kill) reductions in the viability of $P.$ $aeruginosa$. Unlike $E.$ $coli$ suspensions, $K.$ $pneumoniae$ and $P.$ $aeruginosa$ were less affected by the presence of serum. 99% kills could be achieved by exposure of 37.2 $J/cm^2$ in $K.$ $pneumoniae$ and 74.4 $J/cm^2$ in $P.$ $aeruginosa$ suspensions.

a)



b)



c)
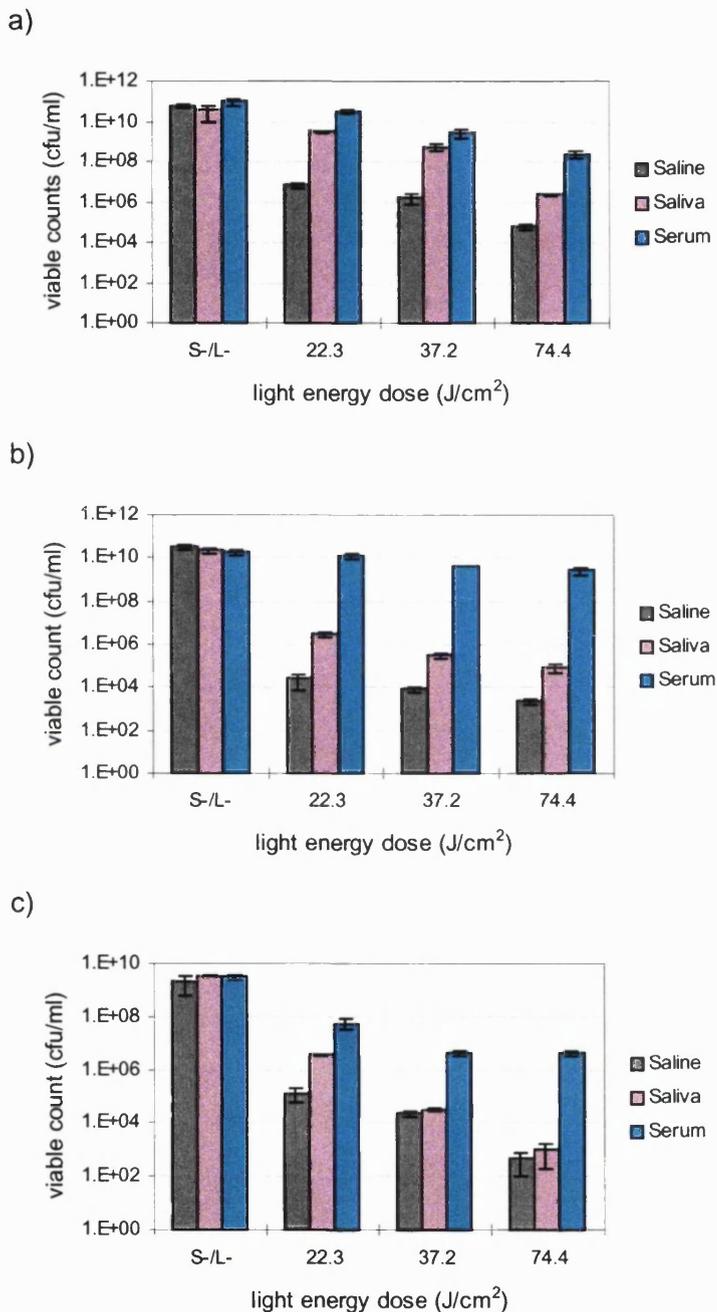


Figure 4.1    Effect of saliva and serum on the photodynamic inactivation of a) *P. aeruginosa,* b) *E. coli* and c) *K. pneumoniae.* Bacteria in each suspension either received 50 µg/ml TBO and exposed to various doses of light, following 60 sec PIT or received saline and not exposed to light (S-/L-).  Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).  Bars represent mean the viable count (n=4) and error bars represent standard deviations.

## 4.3.2 Effect of pH

The effects of various pH conditions are presented in figure 4.2. Of the 5 different pHs tested, highest kills were obtained at pH 8.0, giving a 5 $\log_{10}$ kill in all bacterial suspensions. When bacteria were irradiated at pH 8.0, there was 1 $\log_{10}$ increase in the kill compared to the saline control. (4 $\log_{10}$ kill obtained in saline group.) The viability of *P. aeruginosa* was adversely affected by acidic conditions (pH 4 and pH 5). Furthermore, these pH buffers resulted in the smallest PDA-induced reductions in viability in *E. coli* (1.5 $\log_{10}$) and *K. pneumoniae* (2.7 $\log_{10}$). At pH 4.0, the least kill was obtained for all the bacteria tested ($p<0.001$). All three species displayed a similar susceptibility in saline (pH 7.4) and in pH 7.0 buffer. Approximately 4,5 and 4 $\log_{10}$ reductions were obtained in *E. coli*, *K. pneumoniae* and *P. aeruginosa* suspensions respectively.

a)
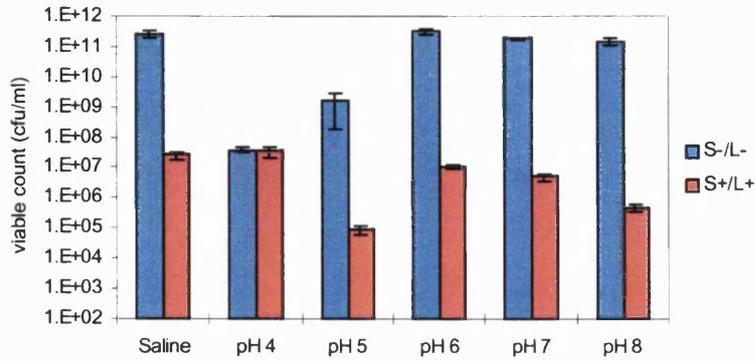


b)
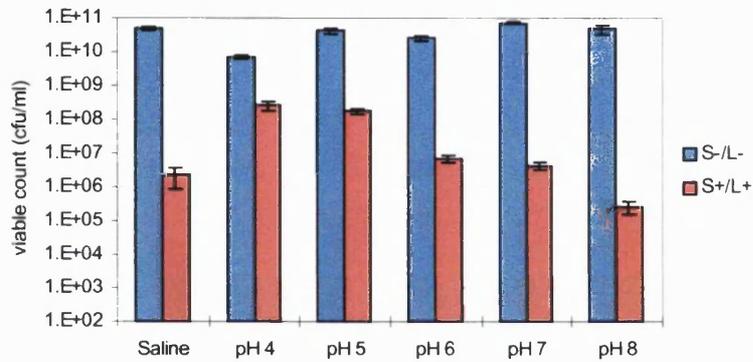


c)
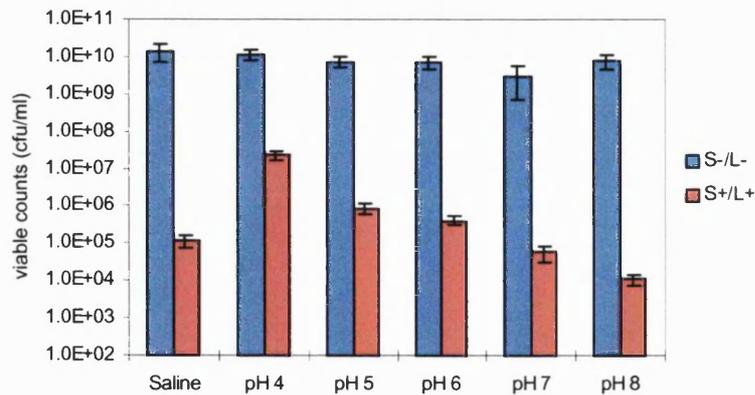


Figure 4.2   Effect of various pHs on the photodynamic inactivation of a) *P. aeruginosa,* b) *E. coli* and c) *K. pneumoniae.*   S-/L-: controls, S+/L+: 25 µg/ml TBO; 60 sec PIT; 22.3 J/cm$^2$.   Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).   Bars represent the mean viable count (n=4) and error bars represent standard deviations.

### 4.3.3  Effect of Growth Phase

The growth phases of each organism can be seen in figure 4.3.   All three organisms were in the lag phase in 2 hours. Mid-logarithmic phase was taken as 5 hours for *E. coli* and *K. pneumoniae* and 7 hours for *P. aeruginosa*. Bacteria in the stationary phase were photosensitised at 17 hours.

It can be seen from figure 4.4 that all three organisms in all growth phases had a similar susceptibility to PDA.  A 99.99% kill was achieved with *P. aeruginosa* in all states.  No statistical difference in the kills was found between any of the phases. In the case of *E. coli*, there were greater reductions in the lag phase than the stationary  phase  (p=0.007).    In  the  case  of  *K. pneumoniae,*  the  lag  phase appeared less susceptible than the other two phases (p=0.03 and p<0.001 for log and stationary phases respectively).
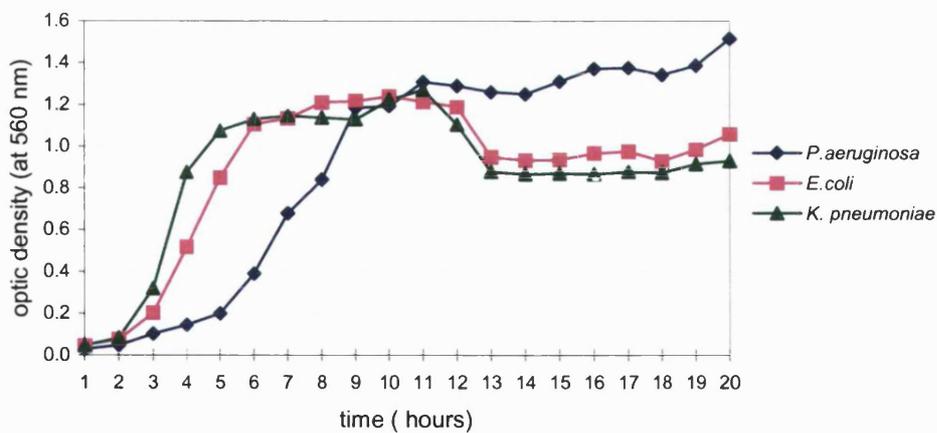


Figure 4.3   Growth curves of *E. coli, K. pneumoniae* and *P. aeruginosa*

a)
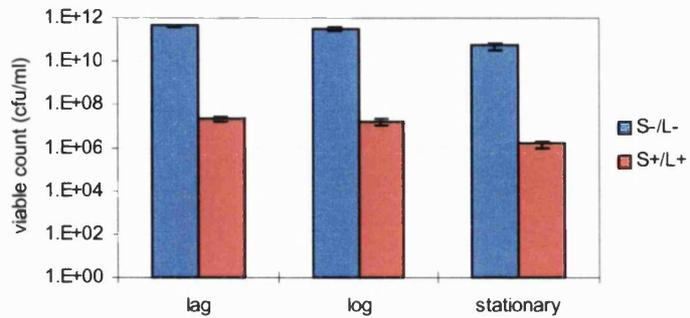


b)
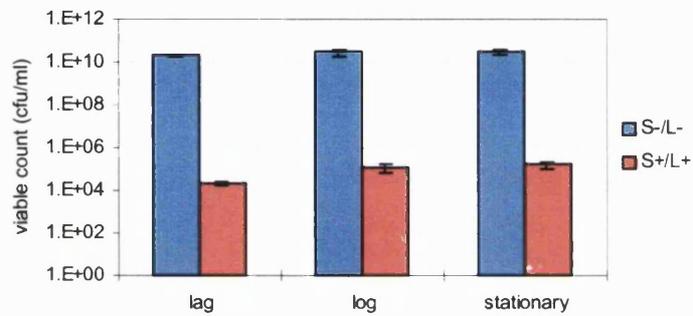


c)
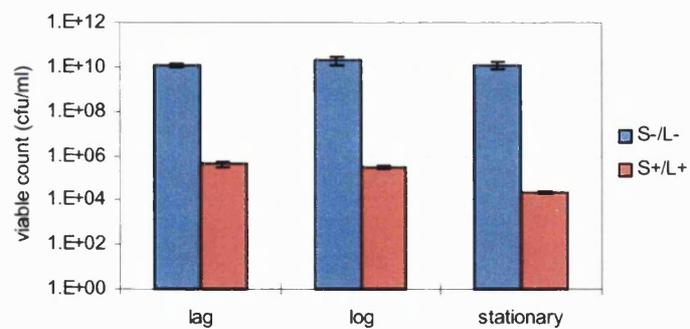


Figure 4. 4    The susceptibility to photodynamic inactivation of various growth phases of a) *P. aeruginosa*, b) *E. coli* and c) *K. pneumoniae*.  Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).  S-/L-: before and S+/L+: after PDA (TBO: 25 µg/ml, light: 22.3 J/cm$^2$ and PIT: 60 sec).  Bars represent the mean viable count (n=4) and error bars represent standard deviations.

## 4.4 DISCUSSION

In this part of the study, the effectiveness of the killing of bacteria by PDA was studied under conditions more likely to be found in the *in vivo* environment.

It was found that the medium in which the bacteria are suspended influences the effectiveness of PDA. The highest kills were obtained in the presence of saline whereas PDA was least effective in the presence of horse serum. Although the presence of saliva impaired the effectiveness of PDA, substantial kills could be achieved by using similar energy doses to those used in the saline experiments. The effects of various media on PDA have been examined in a number of other studies and generally similar results to those found in the present study were obtained. One exception was that photosensitisation of *C. albicans* appeared to be independent of the medium used (Wilson and Mia, 1994). Blood and nutrient broth were also found to reduce the susceptibility of bacteria to PDA (Wilson *et al.*, 1993c). Under conditions comparable to those used in this study, a significant decrease, although still substantial, in kills of *Por. gingivalis* was shown by Bhatti *et al.* (1997). Likewise, Wilson and Pratten (1995) demonstrated a reduction in kills of AlSPc-sensitised *S. aureus* in horse serum. What is more, Nitzan *et al.* (1987b) reported that even when diluted to 1:20, the presence of serum in the culture medium inhibited photo-inactivation of *S. aureus* by 90%.

Decreased photo-inactivation in the presence of both saliva and serum is most probably due to the protein content of these fluids. The acidity of both the serum and saline was in the range of pH 6.2-7.6 which is similar to that of saline (pH 7.4) The bacterial kills achieved in the presence of serum were not as great as in the presence of saliva. The reason for that could be the higher protein content (5-9.5 g/100ml) in horse serum compared to 0.15-0.25 g/100ml protein found in saliva (approximately 40 times less protein content in saliva) (Cole and Eastoe, 1988). Parallel to this, Nitzan *et al.* (1998) demonstrated that *Acinetobacter baumannii* could be photosensitised by a cationic porphyrin in low protein cultures (nutrient broth, containing 2 mg/ml protein) but in higher protein cultures (foetal calf serum, containing 15 mg/ml), the rate of photosensitisation was reduced. In the same

study, it was indicated that the type of protein as well as the concentration, influences photodynamic inactivation; various rates of bacterial killing were achieved in cultures containing equal amounts of serum and bovine serum albumin.

The presence of proteins in suspensions may decrease the effect of photo-inactivation by a number of mechanisms:

As suggested by Wilson and Pratten (1995), impaired photo-inactivation could be due to the shielding effect of protein molecules, causing decreased penetration of light through the suspension. Proteins may absorb the photons of light and therefore interfere with the interaction of light energy and photosensitiser molecules. Proteins may also compete with the bacteria to capture the available sensitiser in the medium thus leaving an insufficient amount of free sensitiser target for the cells. Bhatti et al. (1998b) reported that TBO binds to proteins and the uptake of TBO by bacteria was 5 times less in serum compared to that in saline. Singlet oxygen has an extremely short life time (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 the cytotoxic species produced in the supernatant. Culture medium has also been reported to quench singlet oxygen and free radicals (Nitzan et al., 1989).

This study has also demonstrated that the pH of the medium influences PDA. An alkaline environment (pH 8.0) promoted the effect of PDA as opposed to an acidic environment (pH 4.0 and 5.0), in which a decrease in bacterial kill (although still substantial) was observed. In accordance with this, oral bacteria (L. casei and A. naeslundii) were found to be less susceptible to lethal photosensitisation under acidic conditions (Burns, 1997). The reason for higher kills in an alkaline environment may be explained by increased binding of sensitisers to target cells at such pH values. Various enzymes and transport molecules of the cell are affected by pH. Therefore, a high pH may have some effect on the penetration of TBO and cytotoxic species into the cells. 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. The

pH can affect the absorbance properties and the quantum yields of TBO. Pottier *et al.* (1975) reported an increase in kills at higher pH values of TBO solutions and indicated that this was due to the increased efficiency of singlet oxygen molecules. Tuite and Kelly (1993) stated that the lifetime of the triplet state is increased at higher pH values due to the greater alkalinity of the excited state of the sensitiser compared to its ground state. Likewise, higher concentrations of protons in a lower pH environment may interfere with the generation of singlet oxygen or free radicals.

Nitzan *et al.* (1987b) and Ehrenberg *et al.*, (1985) demonstrated that deuteroporphyrin was most effective at killing *S. aureus* at pH 6.5 and less so at more acidic (pH 5.5), as well as more basic conditions (pH 8.0). The different results obtained in these studies may be related to the nature of the target organisms. Increased penetration of *S. aureus* by deuteroporphyrin due to changes in membrane fluidity was observed at pH 6.5. In addition, the properties and behaviour of different sensitisers in various pH conditions may also differ (Nitzan *et al.*, 1987b). In the same study, increased binding of porphyrin to the bacteria at pH 6.5 due to the higher monomeric or dimeric forms of the sensitiser was suggested. Cationic sensitisers, on the other hand, were reported to be more effective in neutral pH conditions (Nitzan *et al.*, 1987a). In parallel, inactivation of yeast cells (Ito, 1977) and *P. ginigivalis* (Bhatti *et al.*, 1997) was found to be increased in the neutral pH region. However, killing of *C. albicans* was demonstrated to be unaffected by changes in pH conditions (Wilson and Mia, 1994).

The inhibitory effects of serum, saliva and lower pH values on PDA may have an impact on the clinical effectiveness of PDT. As mentioned before, the saliva of patients who have cancer and are receiving radiotherapy has been shown to have increased viscosity because of a decrease in the flow rate, and have a tendency towards acidity. However, the detrimental effect of saliva and serum on the effectiveness of PDT could easily be overcome by increasing the light dose used. Alternatively, patients could rinse their mouth with saline before the application of

TBO and subsequent illumination. In fact, a pre-rinse with a mild alkaline solution may increase the bactericidal effect.

The results of this study showed that all three organisms were similarly susceptible to photo-inactivation in all physiological phases. However, this is not the case for other species. *Por. gingivalis* was reported to be less sensitive to photosensitisation with TBO in the stationary phase than in the lag or log phases although substantial kills were achieved in all growth phases (Bhatti *et al.*, 1997).. The susceptibility of *S. aureus* to lethal photosensitisation was found to be independent of growth phase when $AlS_2Pc$ was used (Wilson and Pratten, 1995), while Nitzan *et al.* (1987b) reported that this organism was most sensitive to the treatment in the mid-log phase, when deuteroporphyrin was used. However, Venezio *et al.* (1985) demonstrated that lag phase cells sensitised by HpD were most susceptible to PDA. In the stationary phase, due to diminished nutrients and accumulation of toxic product, organisms adjust their composition significantly. In this stage, cells are less permeable due to the thickened cell envelope. Therefore, stationary phase cells are usually affected less by environmental changes and antibacterial agents than are log phase cells. Surface associated material (SAM), which is found more abundantly on cells in the stationary phase, was suggested to be one of the reasons for less bacterial kill in this stage since it obstructs the cytotoxic species from reaching the critical sites, i.e., the cytoplasmic membrane (Bhatti *et al.*, 1997). The low membrane fluidity of the bacteria in the stationary phase was also suggested to be the reason for decreased kills of *S. aureus* (Nitzan *et al.*, 1987b).

In summary,

1) The presence of serum and, to a lesser extent, saliva decreased the extent of PDA on all three target organisms. Proteins present in serum and saliva are most probably responsible for this effect due to a) shielding effect from light, b) providing protection from cytotoxic species and, c) competition with organisms to bind available TBO.

2) In acidic conditions, the degree of PDA was reduced while the greatest kills were obtained at pH 8.0. This is probably due to the increased binding of TBO to bacteria and increased production and efficiency of cytotoxic species in alkaline conditions.

3) The growth phase of all three organisms had very little effect on their susceptibility to PDA.

**Chapter 5**

**MECHANISM OF ACTION**

## 5.1  INTRODUCTION

This chapter examines some aspects of the mechanism of PDA using one of the target bacteria (*E. coli*). Optimisation of PDA and further improvements to the therapy may be possible once the mechanism of cell damage has been established. Several factors may affect the mechanism of photo-damage in microbial cells. Bacteria, yeasts and viruses may respond differently to PDA. In the case of bacteria, the aerobic or anaerobic nature of the organism as well as its Gram staining characteristic, may play an important role in this mechanism. The type of photosensitiser and the environmental conditions could also be determinants of the mechanism of action.

Two types of process may be involved in photochemical reactions. A type II process involves singlet oxygen generation by energy transfer following the interaction of the triplet state sensitiser molecule with ground state oxygen. A type I process, on the other hand, involves free radical generation by electron and hydrogen transfer following the interaction of the triplet sensitiser molecule with molecules in its vicinity (Rosenthal and Ben-Hur, 1995). Many studies have shown that both type I and II mechanisms contribute to photo-damage, although the type II mechanism predominates (Valduga *et al.*, 1993; Dahl *et al.*, 1989; Burns *et al.*, 1996). The aim of this study was to determine which reactive cytotoxic species are involved in TBO-mediated *E. coli* cell damage. The effect of PDA on bacterial cell structure was also determined. The reactive species generated during the photodynamic interaction act on various constituents of cells leading to cell death and the cellular response to these cytotoxic species may also help to identify the target sites for PDA.

Determination of the uptake of TBO by the cells was an additional aim. The life-time of singlet oxygen is around 0.01-0.04 μsec and its diffusion distance in a cellular environment is estimated to be about 0.01-0.02 μm (Moan and Berg, 1991). Therefore, uptake and localisation of TBO molecules is of importance in determining the sites and route of photodynamic damage.

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In summary, the aims of this study were to determine:

i) the active species involved in killing of *E. coli* by PDA

ii) the morphological changes of photosensitised *E. coli*

iii) the characteristics of TBO uptake by *E. coli*

## 5.2 MATERIALS AND METHODS

### 5.2.1 Assessment of the Role of Reactive Species

The extent of bacterial killing in the presence of a singlet oxygen enhancer (deuterium oxide, $D_2O$), a singlet oxygen quencher (L-tryptophan) and a hydroxyl radical scavenger (n-propyl gallate) was examined. $D_2O$ enhances the life time of singlet oxygen (Rozenthal and Ben-Hur, 1995). Singlet oxygen and hydroxyl free radical scavengers, on the other hand, reduce the life-time of singlet oxygen and hydroxyl free radicals respectively (Nitzan *et al.*, 1989).

#### 5.2.1.1 Assessment of the effect of $D_2O$ on PDA

1 ml of an overnight culture of *E. coli* was aliquoted into four tubes. The pellets were harvested by centrifugation and were resuspended either in 0.85% NaCl (w/v) or in $D_2O$ (Sigma) containing 0.85% NaCl (w/v), and incubated for 3 h at 37°C aerobically. 100 μl of these suspensions were added to the wells of a 96-well microtitre plate and then either received the same volume of TBO (50 μg/ml) and were exposed to laser light, or received 0.85% NaCl (w/v) and were not exposed to light (controls) pursuing the steps described in 2.1.1.5, with the following exceptions: The TBO solution added to the bacterial suspensions in $NaCl/D_2O$ had been prepared by dissolving in $NaCl/D_2O$. A sub-optimal light dose of 3.7 $J/cm^2$ was used to compare more clearly any increased killing due to the presence of $D_2O$.

#### 5.2.1.2 Assessment of the effect of L-tryptophan and n-propyl gallate on PDA

The same procedure as described above was followed. However, cells were suspended in 0.001 M of L-tryptophan and in 0.001 M n-propyl gallate solutions (both obtained from Sigma). These scavengers, at certain concentrations, were toxic to the bacterial cells. The concentrations used in this study were determined by incubation of various dilutions of these reagents with organisms for 3 h, at

37°C, under aerobic conditions, and did not cause a reduction in viable counts of the bacteria. TBO solublised in each scavenger solution or in 0.85% NaCl (w/v); and a light dose of 22.3 J/cm$^2$ were used to compare the protective ability of scavengers against the photodynamic effect.

## 5.2.2 Evaluation of Morphological Changes of Photosensitised *E. coli*

An overnight suspension of *E. coli* was centrifuged and resuspended in 10 ml 0.85% NaCl (w/v). 2 ml of these aliquots were transferred into the wells of a 24 well microtitre plate. The experimental method used was identical to that described in 2.1.1.5. TBO concentration of 25 µg/ml; PIT of 60 sec and laser light of 22.3 J/cm$^2$ were used. After this, bacteria were harvested by centrifugation. Pellets were then resuspended in 2.5% gluteraldehyde for subsequent TEM. The technique used for TEM was as described in 2.1.2.

## 5.2.3 Uptake of TBO by *E. coli*

### 5.2.3.1 Removal of excess TBO before lethal photosensitisation

An overnight culture of *E. coli* was centrifuged and resuspended in 0.85% NaCl (w/v). Two aliquots (1 ml) of this suspension received equal volumes of 50 µg/ml of TBO and were incubated for periods of 1 min and 15 min respectively at 37°C on a roller, where continuous mixing took place. Control aliquots received 0.85% NaCl (w/v) instead of TBO and were incubated for the same periods of time. At the end of each incubation period, the excess TBO in the supernatant was removed by washing cells (by the addition of 0.85% NaCl (w/v) and centrifugation at 5000 $g$ for 15 min) several times until the supernatants were clear (showing an absorbance of around 0 at 633 nm). The washed cells were then resuspended in 0.85% NaCl (w/v) and 200 µl of each suspension was placed in the wells of a 96-well microtitre plate. Suspensions that were incubated with TBO were exposed to 22.3 J/cm$^2$ of light. Control suspensions in 0.85% NaCl (w/v) were not irradiated. Additional control suspensions were treated in the standard manner, i.e., they

were exposed to light straight after each incubation period with TBO, but to get comparable results, the same washing steps were followed before (but not after) the addition of TBO. Following the irradiation procedures, suspensions were plated as described in 2.1.1.5.

## 5.2.3.2 Uptake of Tritiated TBO ($^3$H-TBO) by cells

An overnight culture of *E. coli* was centrifuged and resuspended in 10 ml 0.85% NaCl (w/v). 1 ml of bacterial suspensions were allocated into eppendorfs. $^3$H-TBO (specific activity 278 Gbq/mmol, 7.5 Ci//mmol, Amersham Pharmacia Biotech Ltd., Little Chalfont, UK) was added to the suspensions to give a final concentration of 25 µg/ml. This was followed by incubation over periods of 5 sec, 1 min, 3 min, 5 min and 15 min. An additional suspension received the same amount of $^3$H-TBO but was washed a number of times after a 15 min incubation period. Controls were composed of suspensions with no $^3$H-TBO. At the end of each incubation period, the cells were harvested by centrifugation at 5000 *g* for 15 min. The supernatants were transferred into scintillation vials containing 5 ml of scintillation fluid. Pellets were further suspended in 1 ml of 0.85% NaCl (w/v) and were also transferred into another set of scintillation vials. To assess the total $^3$H-TBO reading, $^3$H-TBO was added to the scintillation fluid to give a final concentration of 25 µg/ml. Each condition was performed in triplicate. The amount of $^3$H-TBO present on the cells and in the supernatant was determined by scintillation spectrometry (WALLAC 1409 scintillation counter).

## 5.2.3.3 Detection of the uptake of $^3$H-TBO by cell by autoradiography

The method followed was as described in 5.2.2. However, in order to investigate the distribution of TBO molecules in the cells, $^3$H-TBO to give a final concentration of 25 µg/ml was added to the bacterial suspensions. Autoradiography was performed for the visualisation of TBO by TEM as described in 2.1.3.

## 5.3  RESULTS

### 5.3.1  Role of Reactive Species

Figure 5.1 shows the effect of $D_2O$ on the extent of PDA. When TBO-treated suspensions were exposed to 3.7 $J/cm^2$ in the presence of 0.85% NaCl (w/v), no statistically significant reduction in cell viability was observed. However, the same energy dose resulted in a 3.7 $\log_{10}$ reduction in the viability when organisms were suspended in $NaCl/D_2O$.
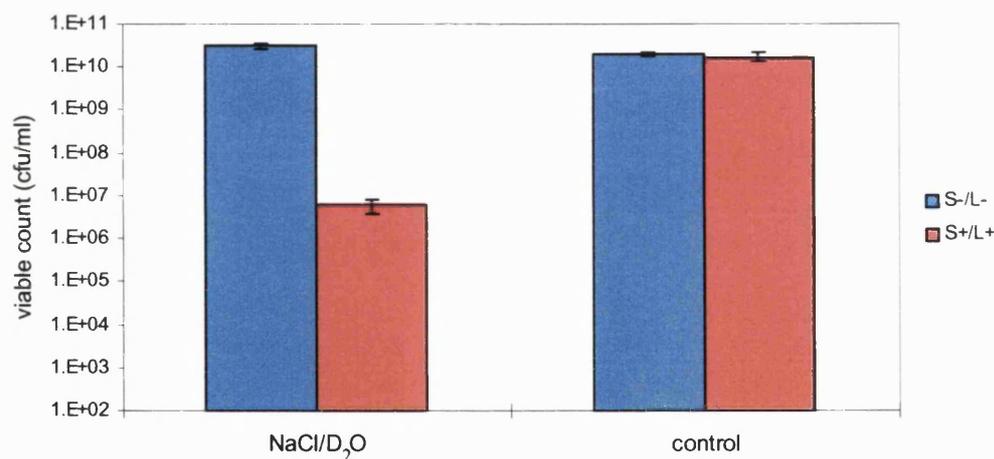


Figure 5.1   Viable counts of *E. coli* exposed to 3.7 $J/cm^2$ of light in the presence of 25 µg/ml TBO prepared in either $NaCl/D_2O$ or saline. Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml). S+/L+: sensitiser plus light, S-/L-: neither sensitiser nor light. Bars represent the mean viable count (n=4) and error bars represent standard deviations.

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The effects of scavengers can be seen in figure 5.2. The viability of saline suspensions of *E. coli* was reduced by 5.5 $\log_{10}$ following exposure to TBO and 22.3 J/cm$^2$ light. When the saline suspensions included L-tryptophan (a singlet oxygen scavenger), the viability of the bacteria was reduced by only 0.5 $\log_{10}$ whereas the presence of propyl gallate (a hydroxyl free radical scavenger) in the suspension led to a 4.6 $\log_{10}$ reduction in the photosensitised cells. Therefore, L-tryptophan provided a 5 $\log_{10}$ (83%) protection ($p < 0.001$) against the photodynamic effect compared to a 0.9 $\log_{10}$ (9%) protection (no statistical significance) in the case of n-propyl gallate.
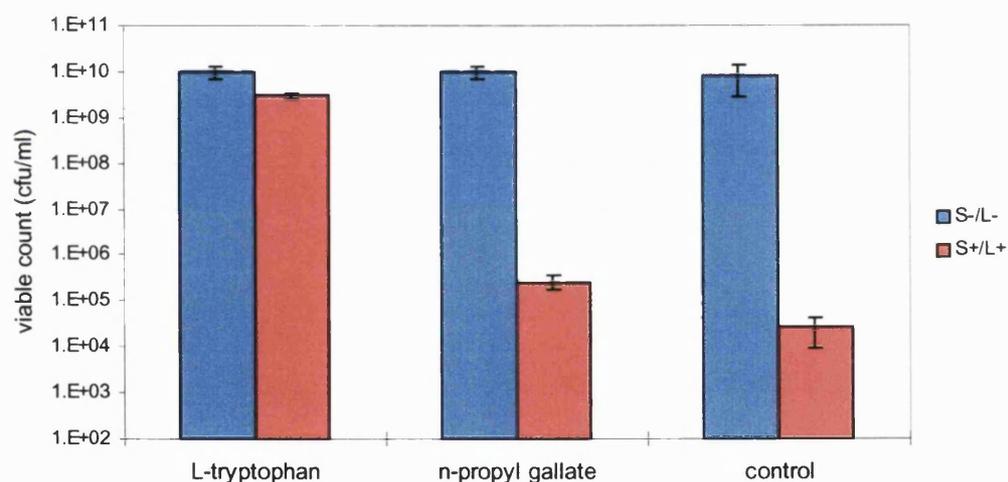


Figure 5.2    Viable counts of *E. coli* exposed to 22.3 J/cm$^2$ of light in the presence of 25 µg/ml TBO in 0.001 M L-tryptophan or n-propyl gallate. Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml). S+/L+: sensitiser plus light, S-/L-: neither sensitiser nor light. Bars represent the mean viable count (n=4) and error bars represent standard deviations.

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## 5.3.2 Ultrastructural Changes

Figures 5.3.a, b, c and d show the ultrastructure of the untreated (S-/L-), treated (S+/L+), TBO-treated (S+/L-) and light-treated (S-/L+) cells respectively.  There are obvious alterations in the various compartments of the photosensitised cells (S+/L+ group).  The most striking feature is that the outer membrane is displaced from the cytoplasmic membrane at regular intervals, which appear as wave-like exfoliations.  In addition, condensation of the cytoplasm with vacuole formation can be clearly seen.  In sensitiser-treated and light-treated cells, no alterations in cell structure were observed.

The appearance of vacuoles in treated cells should not be confused with those in the cytoplasm of control cells (blobs on one side of the cells) as the latter is a result of the technique used for the preparation of specimens for TEM investigation, and is regarded as an artefact.  The vacuoles in the treated cells are manifested as splits in the cytoplasm
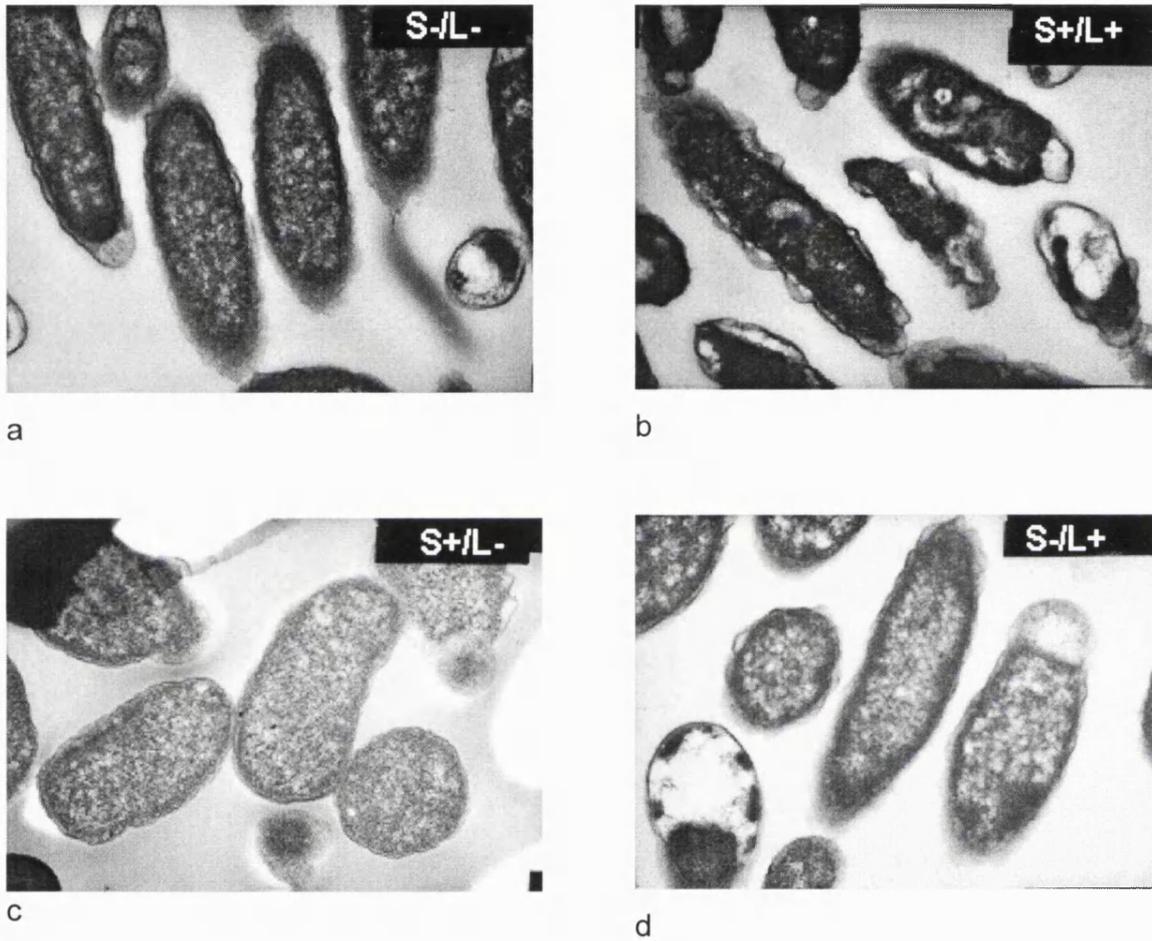
Figure 5.3     Transmission electron micrographs showing a: control (S-/L-); b: photosensitised (S+/L+); c: TBO-treated (S+/L-) and d: light-treated (S-/L+) *E. coli* cells. Magnification: x 16 000.

### 5.3.3  Uptake of TBO

When *E. coli* suspensions were washed to eliminate the excess (unbound) TBO in the supernatant, no kills were achieved on exposure to 22.3 J/cm$^2$ laser light. Increasing the incubation time of the bacteria with TBO from 1 min to 15 min had no effect on this result (figure 5.4).   In contrast, light exposure to unwashed suspensions in the presence of TBO, resulted in a 5 log$_{10}$ reduction.
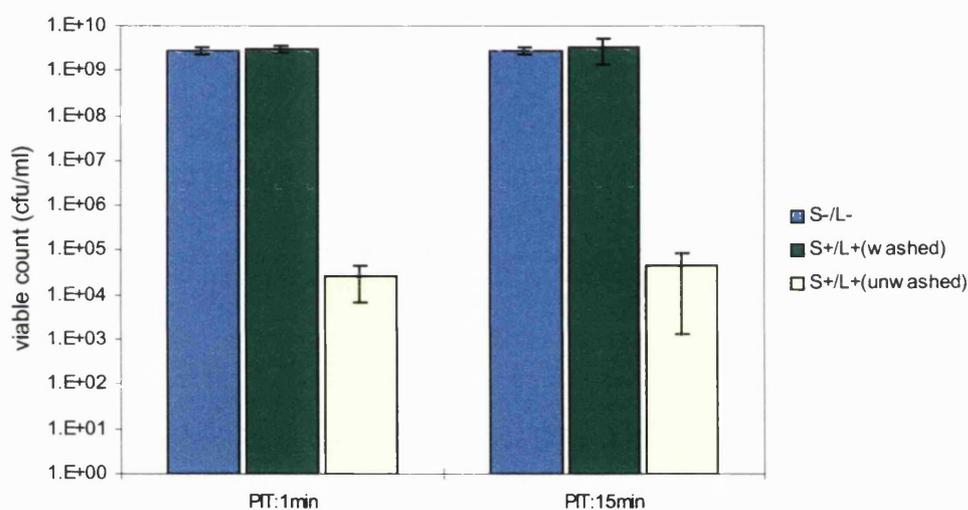


Figure 5.4    Viable counts of washed and unwashed *E. coli* cells after 1 and 15 min incubation with 25 µg/ml TBO, before (S-/L-) and after (S+/L+) light exposure of 22.3 J/cm$^2$.   Viable counts of bacteria are expressed as colony forming units per millilitre (cfu/ml).   Bars represent the mean viable count (n=4) and error bars represent standard deviations.

Figure 5.5 shows the amounts of $^3$H-TBO detected in the cells and in the supernatant. The measurements are expressed as counts per minute. $^3$H-TBO was measured in the supernatant and in the bacterial pellet. The count for the total $^3$H-TBO (25 μg/ml) used was 115857±25700 cpm. The count for the pellet was found to be 6185±1307 cpm compared to 106998±4518 cpm in the supernatant after 5 sec incubation time. Increasing the incubation time to 15 min had no effect on the uptake of $^3$H-TBO by the bacteria. When the bacterial suspension was washed following a 15 min incubation, even smaller amounts of H$^3$-TBO were detected in the pellet (46 ±20 cpm) and supernatant (153 ±46 cpm).
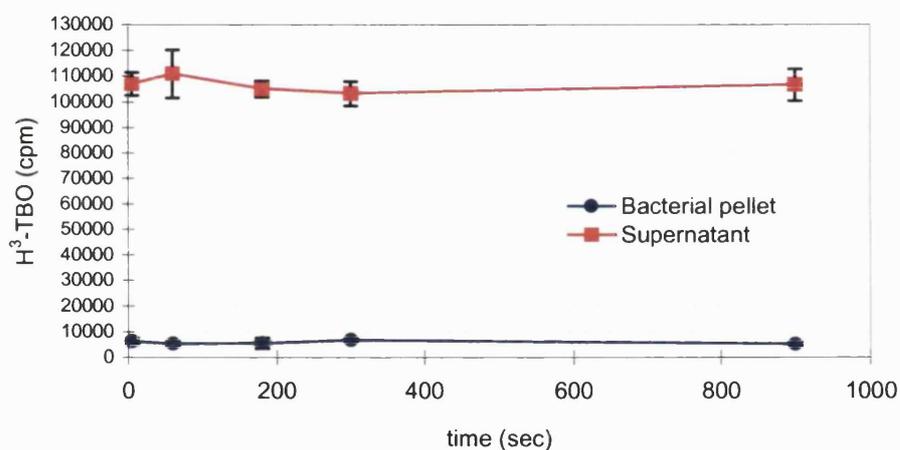


Figure 5.5   Uptake of 25 μg/ml $^3$H-TBO by *E. coli* after various incubation times (5 sec, 1 min, 3 min, 5 min and 15 min). $^3$H-TBO is expressed as counts per minute (cpm). Error bars represent standard deviations.

The distribution of the $^3$H-TBO molecules in the cells can be seen in figures 5.6.a (control cells), 5.6.b (photosensitised cells), 5.6.c ($^3$H-TBO-treated cells) and 5.6.d ($^3$H-TBO-treated + washed cells). The black particles are silver grains produced by radiation emitted by the $^3$H-TBO. Control cells do not show the presence of any black particles. (Very small particles on the cells are artefacts.) Particles can be seen both on the outer membrane as well as in the cytoplasm of those cells which had been exposed to $^3$H-TBO/light. Some particles can be observed on the cells that were incubated with $^3$H-TBO but were not exposed to light, but their number is smaller than those present in the photosensitised cells. When bacteria were washed after incubation with TBO, the number of particles present in the cells was reduced further.
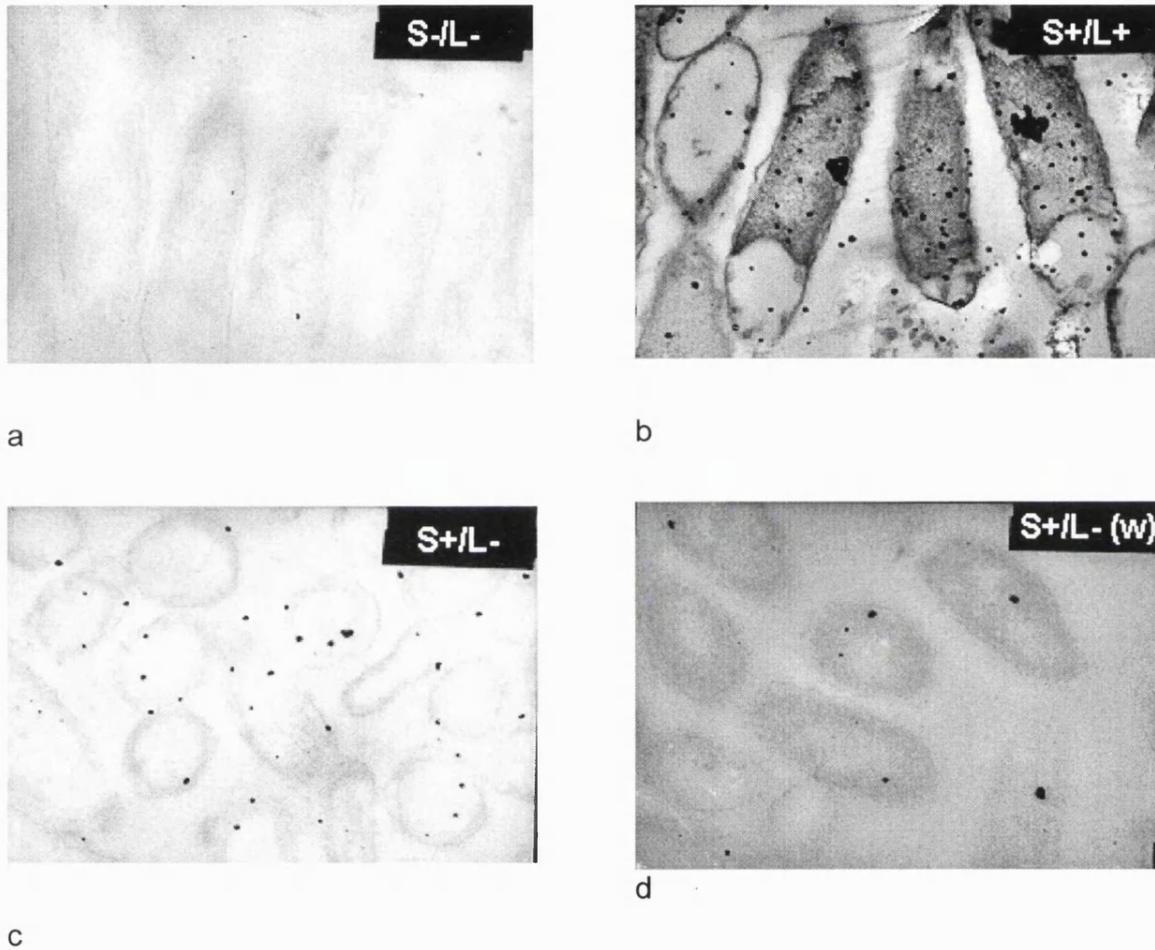
Figure 5.6    Transmission electron micrographs showing the distribution of $^3$H-TBO in a: control S-L-; b: photosensitised S+L-; c: TBO-treated S+L-; d: washed S+L-(w) *E. coli* cells.
Magnification: x16 000.

## 5.4 DISCUSSION

In this chapter, some aspects of the mechanism of photodynamic damage in relation to the production of cytotoxic species, the effect of lethal photosensitisation at a cellular level and the uptake of TBO by bacteria, were examined.

In the presence of $D_2O$, PDA on *E. coli* was enhanced. The presence of L-tryptophan (a singlet oxygen scavenger) and n-propyl gallate (a hydroxyl radical scavenger) were detrimental to PDA. This implies that photosensitisation of *E. coli* by TBO is mediated mainly by the generation of singlet oxygen. Hydroxyl free radicals appear to be involved in cell killing to only a small extent. Valduga *et al.* (1993) reported that singlet oxygen decreased the survival of Gram-negative bacteria only when the bacteria were pre-treated with Tris-EDTA. In addition, Gram-negative bacteria were shown to require a multi-hit killing while Gram-positive cells required single-hit kinetics to pure singlet oxygen generated outside the cells (Dahl *et al.*, 1989). TBO bound to sepharose beads has been shown to kill *Strep. sanguis* via the formation of singlet oxygen with no type I action taking place. Type I processes require the sensitiser to approach the substrate within sub-nanometre distances and are therefore only involved when TBO is in contact with a cell. This implies that singlet oxygen generation via a type II mechanism alone is able to inactivate Gram-positive cells. The results of this study suggest that Gram-negative bacteria are also killed predominantly by a type II process.

However, the use of scavengers and enhancers alone may be insufficient to draw conclusions about the precise proportions of reactive species involved in cell death. This is because $D_2O$ and the scavengers are not entirely specific enhancers or inhibitors (Rosenthal and Ben-Hur, 1995). In addition, Nitzan *et al.* (1989) reported that the inhibitory effect of several oxygen and hydroxyl free radical scavengers were found to provide various degrees of protection of *S. aureus* from porphyrin-mediated photodynamic inactivation.

Almost all photosensitisers, upon illumination, have been shown to form both singlet oxygen and free radicals, but PDA is strongly oxygen-dependent. The data presented here showed that TBO, too, generates both types of reactions. Nevertheless, the proportion of these intermediates may change depending on the conditions, such as the type of photosensitiser and the degree of oxygenation of the environment. Some dyes (e.g., flavin) generally act via the first pathway. Furthermore, phototoxicity may also occur under hypoxic conditions (e.g., when bacteriochlorophyll derivatives are used) (Fuchs and Thiele, 1998). Inactivation of Gram-positive and Gram-negative anaerobic bacteria was achieved under anaerobic conditions using different porphyrins and strong white light irradiation. It was suggested that under anaerobic conditions, the photodynamic damage is mediated only by the type I reaction (Nitzan *et al.*, 1994).

Since singlet oxygen plays a major role in PDA, it should be possible to increase the effectiveness of PDT by increasing the oxygen tension within the tissues to be treated. Oxygenated tissues have been shown to be more sensitive to PDT than hypoxic tissues. Theoretically, there are several approaches for improving the oxygenation of tissues which, in turn, would increase the yield of cytotoxic photo-intermediates. Administration of controlled hyperthermia, light fractionation and deuterated water were suggested as reasonable approaches to modulate oxygen tension and, therefore, to contribute to more effective PDT (Fuchs and Thiele, 1998).

Ultrastructural examination of photosensitised *E. coli* shows disorganisation of the outer membrane with wave-like corrugations and separation from the cytoplasmic membrane. A condensation of the cytoplasm, with cleft-like cavities was also quite noticeable. A similar phenomenon was reported by Nitzan *et al.* (1992) when polymyxin nonapeptide-treated *E. coli* were exposed to deuteroporphyrin and light, but these bacteria also displayed elongation of the cells and the chromosomal region appeared more opaque.

These alterations suggest that the outer membrane is damaged by cytotoxic species first and, during the PDA process, extracellular TBO enters the cell via the damaged

membrane and interacts with the other cell components. The sequence of cell damage could also be in the reverse order. The appearance of cleft-like vacuoles together with the wavy disjunction of the outer membrane may also be a result of the shrinkage of the condensed cytoplasm. The results of autoradiograpy, however, support the former hypothesis. When bacteria were exposed to light following incubation with $^3$H-TBO, the amount of $^3$H-TBO in the cells was increased compared to unexposed cells, suggesting an influx of TBO into the cells following membrane damage by the cytotoxic intermediates. These results correlate well with the data presented by Paardekooper *et al.* (1995) who showed that the membrane permeability of yeast was increased during irradiation, and this was accompanied by accumulation of the sensitiser within the cell cytoplasm.

The leakage of TBO through the cell membrane and into the cytoplasmic region may then enable damage to the other structures by generating reactive species. This may partly explain the colour difference of the bacterial pellets of S+/L+ (dark blue) and S+/L- (white) groups. Damage to the outer membrane and subsequent leakage of TBO may be mechanical (photo-formation of pores) or electrical (depolarisation). As a result of the depolarisation of the cells, there will be free passage of ions and molecules through the cytoplasmic membrane. A total loss of cellular $K^+$ and $Na^+$ efflux was shown during haematoporphyrin-mediated photosensitisation of leukaemic cells, erythrocytes and *S. aureus* (Malik *et al.*, 1993) and damage to the membrane proteins were suggested to be responsible for the collapse of the transport systems (Ehrenberg *et al.*, 1993). Paardekooper *et al.*, (1992) demonstrated that photosensitisation of yeast cells by TBO caused a drastic change in plasma membrane permeability.

Different morphological alterations are seen with different sensitisers and organisms which implies that there is not just one type of cell damage. The formation of DNA fibres in the nuclear region, and mesosome-like multilamellar organelles in the cytoplasm of deuteroporphyrin-treated *S. aureus* has been reported. Some spore formation was observed in *Bacillus cereus* with no intracellular changes (Nitzan *et al.*, 1992). Membrane changes were also noted in zinc-sulphonated phthalocyanine

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sensitised *Strep. faecium*. A ragged membrane pattern was also found in yeast cells and the cells became less electron-dense (Bertoloni *et al.*, 1992).

Uptake studies showed that when excess TBO was removed from the supernatant, no kills could be achieved following exposure to 22.3 $J/cm^2$ of light compared to a 5 $log_{10}$ reduction in the viable counts of unwashed cells. A negligible amount of $^3H$-TBO could be detected on washed cells and this was considerably less than that in unwashed cells. These results were supported by the observation of a decreased amount of $^3H$-TBO on washed-cells compared to unwashed ones using autoradiography. The most probable explanation is that TBO molecules attach to the surface of the cells very weakly where they can cause cell damage when irradiated, but they can be displaced and removed easily by the washing steps. The remaining TBO molecules may be too few to produce sufficient reactive species and subsequent cell death. These results also support the hypothesis that the outer membrane is the first site to be damaged since the diffusion of singlet oxygen is in the range of 0.01-0.02 µm (Moan and Berg, 1991), and the thickness of the outer membrane of *E. coli* is approximately 0.03 µm. Therefore, singlet oxygen species are likely to reach the sensitive periplasmic targets when generated outside the cells. Nevertheless, TBO molecules (the ones in close proximity to the cells) which are abundant in the supernatant may also contribute to cell damage.

In general, penetration at a photosensitising molecule through the outer membrane depends on the nature of the bacteria, the photosensitising compound and the medium in which the organism is suspended. The outer membrane of Gram-negative bacteria is hydrophobic but has a number of outer membrane-associated proteins which enable the transport of hydrophilic substances (Nikaido, 1989). On the other hand, the negatively-charged outer membrane attracts positively-charged substances. TBO is a small, hydrophilic, cationic molecule. Therefore, some diffusion through, or at least a strong binding of TBO to, the outer membrane would have been expected. A capsule around the bacteria may be responsible for the weak attachment forming a barrier between the TBO molecules and the outer membrane. The binding of anionic, lipophilic substances

(porphyrins) to Gram-negative bacteria was shown to be possible only on the spheroplast forms (when the outer membrane, together with the capsule, was removed) (Nitzan *et al.*, 1992). This demonstrates that the main barrier to sensitiser uptake is the cell wall rather than the cytoplasmic membrane. Merchat *et al.* (1996b) reported that the driving force between the Gram-negative bacteria and the meso-substituted cationic porphyrins is electrostatic in nature. In contrast, Minnock *et al.* (1996) showed that both neutral and cationic forms of water-soluble phthalocyanines could bind to *E. coli* and *P. aeruginosa* but only the cationic one could kill the bacteria, and the strongly-bound fraction of the cationic dye accounted for its effectiveness.

Different photosensitisers may exert their action on different regions of the cell depending on their penetration and intracellular distribution. For non-penetrating dyes, it has been postulated that damage to the cell wall and the cytoplasmic membrane occurs either by the rupture of cells or loss of cytoplasm-associated biochemical functions (Ito and Kobayashi, 1977). In contrast, dyes penetrating inside the cells bind to the polynucleotides and damage the DNA (Bagchi and Sreeradha, 1989). Bhatti *et al.* (1998b) reported very low diffusion of TBO from the outer membrane to the interior of *Por. gingivalis*. 87% TBO was detected in the outer membrane as opposed to 5% in the plasma membrane, 8% in the cytoplasm and only 0.25% in the DNA. However, even though TBO was present in such small amounts, damage to the DNA was demonstrated.

No photosensitisation was achieved when cells were washed following incubation with TBO for up to 15 min. Furthermore, radiolabelling studies showed that increasing the incubation times of bacteria with $^3$H-TBO for up to 15 min did not result in any increased uptake of $^3$H-TBO. It is not surprising, therefore, that an increase in PIT did not lead to an increase in the kills achieved (see chapter 3). Ito (1980) reported that the action of TBO is effective only in close proximity to the cell surface since there is no detectable penetration into the cell interior. However, he also reported that TBO may penetrate into the membrane with increased incubation times (30 min) resulting in a slight, but significant, increase in kills.

Surprisingly, this study has shown that TBO is not taken up by *E. coli*, yet it has been shown to be a very active photosensitiser of Gram-negative bacteria. Hence, diffusion of the sensitiser to the plasma membrane does not seem to be a prerequisite for PDA to take place as suggested by Ehrenberg *et al.* (1985) and Nitzan *et al.* (1992). Certain properties of TBO such as its ability to generate both type I (minor) and type II reactions, its cationic nature and its water-solubility may contribute to the effectiveness of TBO at killing the Gram-negative bacteria (Rosenthal and Ben-Hur, 1995).

In summary,

1) A correlation was found between the life-time of singlet oxygen and the kills achieved by PDA as an increase in life-time enhanced while a decrease inhibited the bacterial kills. These results suggest that PDA on *E. coli* using TBO is most probably primarily mediated by singlet oxygen but, although not statistically significant, other reactive intermediates (hydroxyl radicals) are also likely to be involved.

2) PDA involves alterations to the outer membrane as well as to the cytoplasm. The outer membrane is most probably the primary site of photodynamic damage by reactive species. The damage to the plasma membrane and other sites is a result of TBO leakage through the damaged outer membrane.

3) No lethal photosensitisation could be achieved when cells were washed following incubation with TBO. A very small amount of $^3$H-TBO could be detected on the cells incubated with $^3$H-TBO for up to 15 min. TBO present on the surface of the cells, the neighbouring TBO in the supernatant (or a combination of both) are the most likely initiators of cell damage.

Chapter 6

**EFFECT OF PDA ON TWO BACTERIAL VIRULENCE FACTORS**

## 6.1 INTRODUCTION

Bacteria possess a number of virulence factors which are able to induce tissue destruction. Both cell-associated (e.g. lipopolysaccharide, LPS) and extracellular products (e.g. proteases) of Gram-negative bacteria may contribute to the inflammation, and therefore, to the severity of mucositis. Thus, ideally, treatment strategies should be targeted not only at destroying the bacteria but also at the inactivation of these virulence factors.

LPS is embedded in the outer membrane and exerts its toxic effects when released from multiplying cells in the soluble form, mainly when the bacteria are lysed. LPS stimulates a range of inflammatory cells, such as macrophages, as well as cells such as fibroblasts and keratinocytes, to induce the synthesis of many pro-inflammatory cytokines which are the mediators of inflammation. Pro-inflammatory cytokines may amplify the local immune response as well as tissue breakdown by promoting tissue inflammation (Wilson, 1995; Wilson et al., 1996). LPS also contributes to the outer membrane function of bacteria in providing a barrier to exogenous molecules thereby causing resistance to many antibacterial agents (Nikaido, 1989).

Once a bacterium has attached to host tissues, it may release extracellular enzymes such as proteases. A relation between P. aeruginosa proteases and tissue damage in a number of infections has been reported (Barletta et al., 1996; Cotter et al., 1996). Proteases are capable of cleaving proteins into low-molecular mass products which are taken up by the microbial cells and serve as nutrients (Birkedal-Hansen et al., 1988). The integrity of the surrounding tissue may be impaired by degradation of matrix proteins and this then promotes the spread of bacteria from the local site into the systemic circulation. Moreover, proteases have non-specific effects on nearly all protein structures of the host immune components and have been shown to reduce the phagocytic activity of leukocytes. IgA in the saliva may also be degraded by these enzymes (Frandsen et al., 1987).

The aim of this study was to investigate the effect of PDA on

i) the biological activity of *E. coli* LPS.

ii) the proteolytic enzyme activity of *P. aeruginosa*.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Assessment of the Photo-inactivation of LPS

#### 6.2.1.1 Photosensitisation of LPS

TBO was dissolved and diluted in PFW and aliquoted in sterile pyrogen-free falcon tubes (Sterilin). The LPS was diluted to the required concentration from a frozen stock solution containing 10 mg/ml of *E. coli* LPS.

100 μl of a 20 ng/ml LPS solution was transferred into each well of sterile pyrogen-free 96-well microtitre tissue culture plates (Sigma). 100 μl of various concentrations of TBO was added to the labelled wells to give final concentrations of 12.5, 25, 50, 100 μg/ml of TBO and 10 ng/ml LPS. The LPS+TBO solutions were then exposed to 74.4 J/cm$^2$ of light, following a 60 sec PIT. In order to determine the effect of TBO alone, additional suspensions received the same concentrations of TBO but were not exposed to light. Suspensions were then transferred into pyrogen-free eppendorfs. The experiment was repeated using various light energy doses. In this case, suspensions containing LPS and 25 μg/ml TBO were exposed to light doses of 22.3, 74.4, 111.6, 223.2 J/cm$^2$. Additional suspensions received PFW instead of TBO and were exposed to the same energy doses of light. Control groups received PFW and were not exposed to light. The experimental suspensions were then stored at -20°C until used for the LAL assay and for the assessment of cytokine release from PBMC.

#### 6.2.1.2 Semi-quantitative assessment of LPS

The effect of TBO/light on endotoxin activity was determined by LAL assay. The experimental method for the LAL assay was described in 2.1.4.

## 6.2.1.3  Assessment of pro-inflammatory cytokine release from PBMC

The effect of TBO/light on the ability of LPS to induce release of pro-inflammatory cytokines (IL-6 and IL-8) from PBMC was determined. PBMC were prepared, and incubated with test LPS solutions as described in 2.1.5. Release of these cytokines was assessed using ELISA as described in 2.1.6.

## 6.2.2  Assessment of the Photo-inactivation of Proteolytic Enzymes

An overnight culture of *P. aeruginosa* was centrifuged at 5000 *g* for 15 min. The supernatant was transferred to a universal container. 270 µl of the supernatant was transferred into the wells of a 24-well microtitre plate. An identical volume of TBO was added to give a final concentration of 12.5 µg/ml. The suspension was then exposed to light doses of 74.4 J/cm$^2$ after a 60 sec PIT. The experiment was repeated increasing the concentrations of TBO to 50, 100 and 200 µg/ml (final concentrations of 25, 50 and 100 µg/ml). Additional suspensions were prepared using the same concentrations of TBO but were not exposed to laser light. The effect of different light doses on the proteolytic activity was also studied using a constant TBO concentration of 50 µg/ml and light doses of 22.3, 74.4, 111.6, 223.2 J/cm$^2$. The effect of light alone was tested by the addition of 270 µl of 0.85% NaCl (w/v), instead of TBO, into supernatants, then exposure to the same light doses. The control group contained supernatant with 0.85% NaCl (w/v) and was not exposed to laser light. Samples of 500 µl were removed for the azocasein hydrolysis assay. Proteolytic activity was measured by the azocasein assay as described in 2.1.7.

## 6.3  RESULTS

### 6.3.1  Semi-quantitative Assessment of LPS

The LAL assay is a semi-quantitative assay, therefore a range of values is obtained rather than precise concentrations of LPS.

Table 6.1.a shows the effect of various concentrations of TBO alone, and in conjunction with light, on the LAL activity of LPS. Irradiation of LPS in the presence of TBO (with all the concentrations tested) reduced the LAL activity of LPS. The addition of TBO to the LPS suspension in the absence of light also reduced the LAL activity of LPS. However, light irradiation resulted in greater reductions. When LPS suspensions were exposed to 74.4 $J/cm^2$ light after the addition of 12.5 and 25 µg/ml TBO, the LAL activity of LPS was reduced to 0.15-0.3 ng/ml and 0.024-0.048 ng/ml respectively. Increasing the TBO concentrations to 50 µg/ml and 100 µg/ml resulted in increased reduction of LAL activity (0.003-0.006 ng/ml).

The effect of illumination alone, and in conjunction with TBO can be seen in table 6.1.b. Increasing the light doses resulted in a decreased LAL activity of LPS in the presence of 25 µg/ml TBO. Light doses of 22.3, 74.4, 111.6 and 223.2 $J/cm^2$ decreased the LAL activity to 0.3-0.6, 0.024-0.048, 0.012-0.024, 0.003-0.006 ng/ml respectively. The same doses of light alone had no effect on the LAL activity of LPS.

Table 6.1    The effect of TBO concentration (a) and light dose (b) on the photodynamic inactivation of the bio-activity of LPS

a)

|  | 0 | 12.5 µg/ml | 25 µg/ml | 50 µg/ml | 100 µg/ml |
|---|---|---|---|---|---|
| 0 | 2.4-4.8 | 0.3-0.6 | 0.15-0.3 | 0.024-0.048 | 0.006-0.012 |
| 74.4 J/cm$^2$ | | 0.15-0.3 | 0.024-0.048 | 0.003-0.006 | 0.003-0.006 |

b)

|  | 0 | 22.3 J/cm$^2$ | 74.4 J/cm$^2$ | 111.6 J/cm$^2$ | 223.2 J/cm$^2$ |
|---|---|---|---|---|---|
| 0 | 2.4-4.8 | 2.4-4.8 | 2.4-4.8 | 2.4-4.8 | 2.4-4.8 |
| 25µg/ml | | 0.3-0.6 | 0.024-0.048 | 0.012-0.024 | 0.003-0.006 |

## 6.3.2  Effect on Ability of LPS to Stimulate Cytokine Release from PBMC

When TBO was used together with light, the ability of LPS to stimulate IL-8 and IL-6 release from PBMC was reduced. This was dependent on the TBO concentration (p=0.009 and p=0.0047 for IL-8 and IL-6 release respectively) as well as on the light energy dose (p=0.008 for both cytokine release).

IL-8 release

Figures 6.1.a and b show the effect of different TBO concentrations and light doses respectively on the ability of LPS to stimulate IL-8 release from PBMC. All TBO doses tested resulted in statistically significant reductions in the ability of LPS to stimulate IL-8 release from monocytes (p<0.001). The total release of IL-8 from PBMC was found to be 2255 pg/ml in the control group which was reduced to nearly half (1298 pg/ml) by the addition of 200 µg/ml TBO plus irradiation of 74.4 J/cm$^2$ light (figure 6.1.a). While, 50, 25 and 12.5 µg/ml TBO caused 881, 811, 833 pg/ml reductions of IL-8 production respectively. 50 µg/ml and 100 µg/ml TBO in the absence of light exposure resulted in a reduced IL-8 production (p=0.004 and

p=0.003 respectively). However, when compared to the TBO+light group the IL-8 release was statistically significantly lower.

Increasing the energy doses of light, in the presence of TBO, resulted in a decrease in cytokine release and this was statistically significant (p=0.008). There were 608, 691, 790, 818 pg/ml reductions in IL-8 synthesis when 22.3, 74.4, 111.6, 223.2 J/cm$^2$ of light were used respectively. Light exposure alone had no significant effect on IL-8 synthesis by monocytes (figure 6.1.b).

IL-6 release

Figures 6.2.a and 6.2.b demonstrate the effect of TBO concentrations and light doses respectively on IL-6 release from PBMC. In the presence of light, TBO at concentrations of 12.5, 25 and 50 µg/ml resulted in 1032, 965 and 951 pg/ml of IL-6 synthesis compared to 1296 pg/ml in controls. When the concentration of TBO was increased to 100 µg/ml, IL-6 production was decreased to 350 pg/ml. TBO, on its own also caused a decrease in cytokine release (p<0.001).

Similarly, when 22.3, 74.4, 111.6 and 223.2 J/cm$^2$ of light were used together with 25 µg/ml TBO, IL-6 release was decreased to 499, 388, 395 and 379 pg/ml respectively. Illumination in the absence of TBO did not have any significant effect on the ability of LPS to stimulate cytokine release from PBMC (figure 6.2.b).

a)
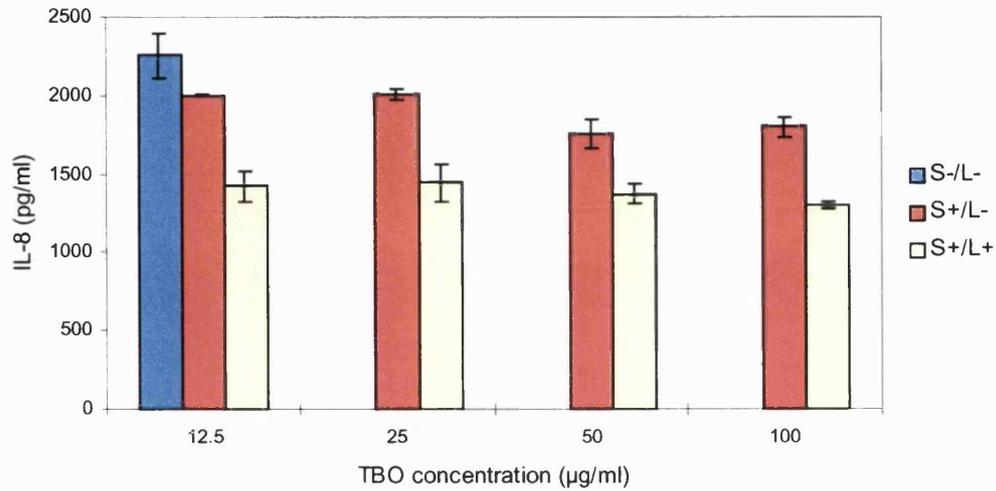


b)
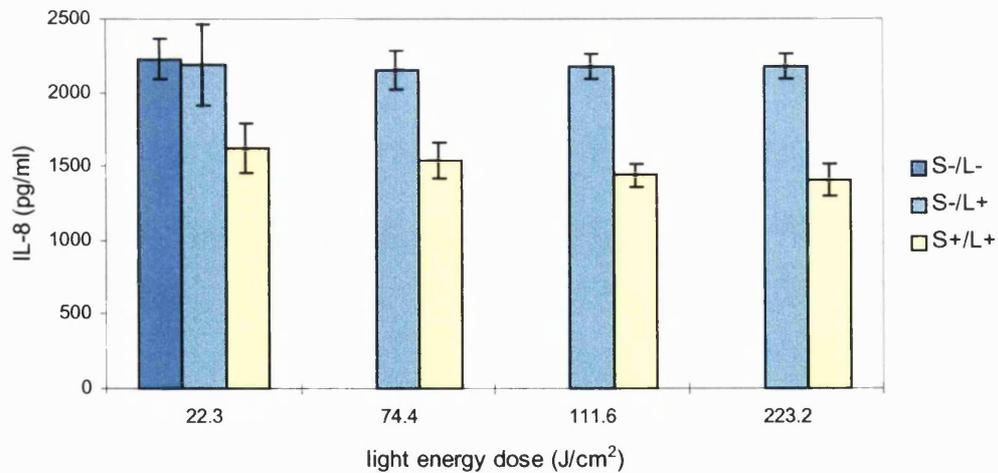


Figure 6.1   The effect of PDA on the ability of LPS to stimulate IL-8 release from human PBMC, a) using 74.4 J/cm$^2$ of light and various concentrations of TBO, b) using 25 µg/ml of TBO and various light doses.   S-/L-:exposure to neither sensitiser nor light, S+/L-: exposure to sensitiser but not light, S-/L+: exposure to light but not sensitiser, S+/L+: exposure to both sensitiser and light.   Bars represent mean values (n=3) and error bars represent standard deviations.

a)
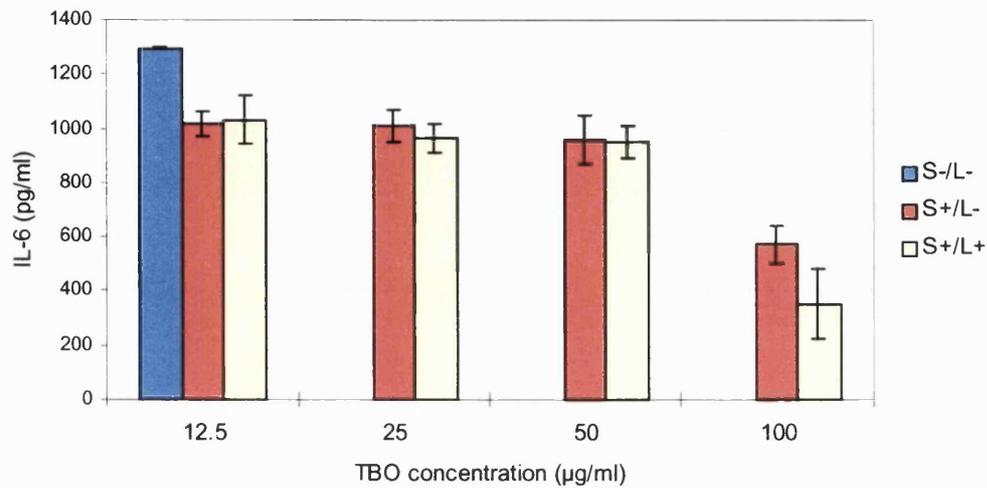


b)
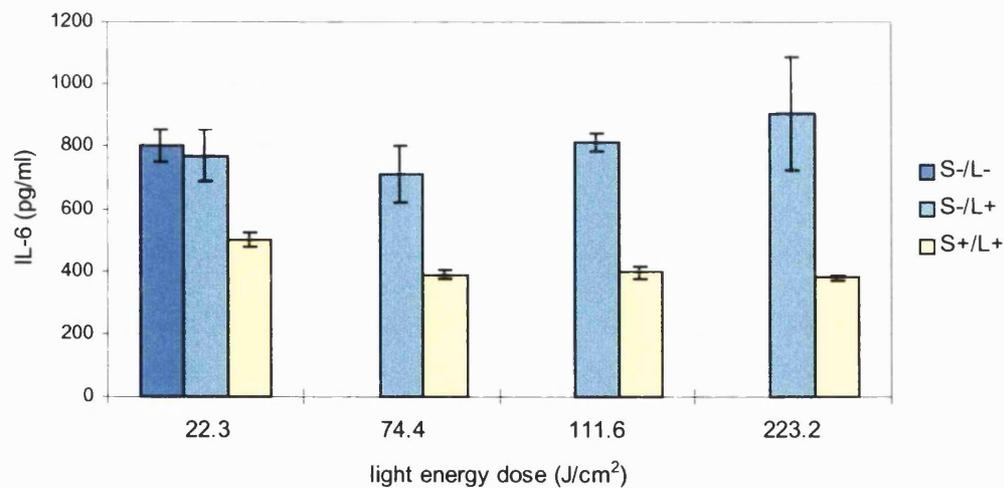


Figure 6.2   The effect of PDA on the ability of LPS to stimulate IL-6 release from human PBMC a) using 74.4 J/cm$^2$ of light and various concentrations of TBO, b) using 25 μ/ml of TBO and various light doses.   S-/L-:exposure to neither sensitiser nor light, S+/L-: exposure to sensitiser but not light, S-/L+: exposure to light but not sensitiser, S+/L+: exposure to both sensitiser and light.   Bars represent mean values (n=3) and error bars represent standard deviations.

### 6.3.3 Effect on Proteolytic Activity

Figures 6.3.a and b show the proteolytic activity of *P. aeruginosa* after treatment with various doses of TBO (a) and light (b). Neither TBO in the absence of light nor irradiation of the supernatants in the absence of TBO had any significant effect on the proteolytic activity. However, irradiation of the supernatants in the presence of TBO resulted in a substantial, light-dose dependent decrease in their proteolytic activity. A 22.3 $J/cm^2$ light dose did not significantly affect the proteolytic activity, which was reduced to 56% and 76% when 74.4 and 111.6 $J/cm^2$ light doses were used. A light dose of 223.2 $J/cm^2$, on the other hand, resulted in no detectable proteolytic activity ($p < 0.001$ for all three doses). A decrease in proteolytic activity was also dependent on the TBO concentration. When 12.5 µg/ml TBO was used, a 37% reduction in proteolytic activity was obtained. Increasing TBO concentrations (25-100 µg/ml) resulted in 58%, 60% and 55% reductions in proteolytic activity ($p < 0.001$ for all occasions).

a)
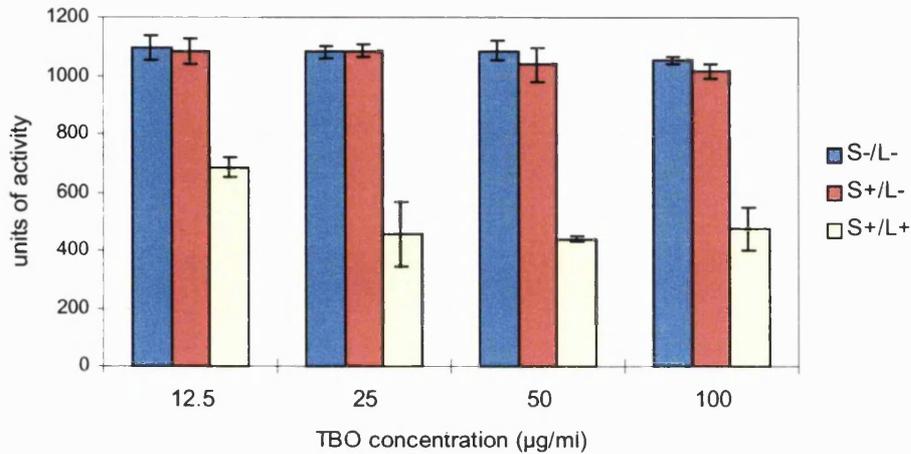


b)
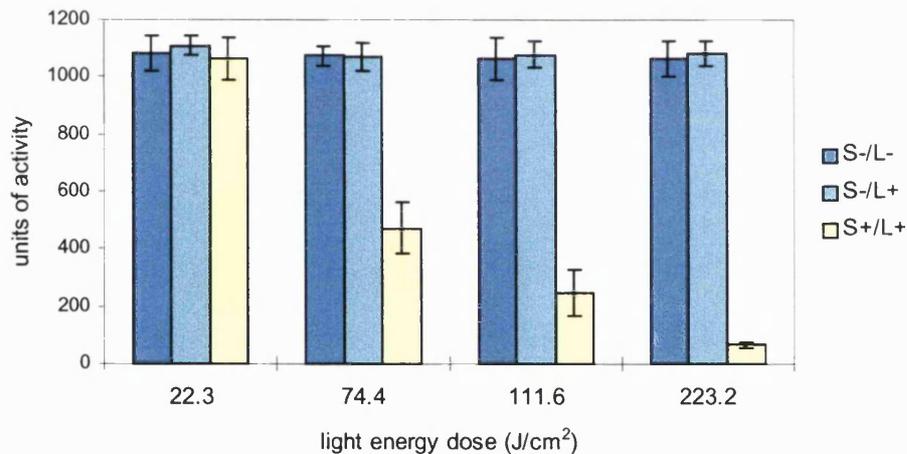


Figure 6.3　The effect of a) various light doses (in the presence of 25 µg/ml TBO) b) various TBO concentrations (on exposure to 74.4 $J/cm^2$ laser light) on the proteolytic activity of *P. aeruginosa*. S-/L-:exposure to neither sensitiser nor light, S+/L-: exposure to sensitiser but not light, S-/L+: exposure to light but not sensitiser, S+/L+: exposure to both sensitiser and light. Bars represent mean values (n=4) and error bars represent standard deviations.

## 6.4 DISCUSSION

In this chapter, the effect of TBO/light on the biological activities of LPS and proteases was studied, these being two important virulence factors in many Gram-negative bacteria.

TBO in conjunction with light was shown to be able to greatly reduce the LAL activity of LPS and also its ability to stimulate cytokine release from PBMC. TBO alone could also reduce both the LAL activity of LPS and its stimulatory effect on the monocytes, although to a lesser extent than TBO plus light.

Endotoxins released by Gram-negative bacteria are of great importance because of their potent inflammatory effects. Therefore, the total management of Gram-negative bacilli infection should be based not only on killing the bacteria but also on neutralising the endotoxins (van Saene and Martin, 1990). One of the problems associated with the chemotherapy of Gram-negative bacterial infections is that when bacteria are lysed by the antibacterial agents, the toxic effects on the tissues will accelerate due to the released endotoxin. Toxic effects of LPS occur via the stimulation of immune cells to synthesise pro-inflammatory cytokines. Pro-inflammatory cytokines including IL-1, IL-6, IL-8, TNF-$\alpha$ and TNF-$\beta$, when overabundant, can cause tissue damage e.g. due to collagenase induction in fibroblasts (van der Zee *et al.*, 1997) and by activation of osteocytes (Rams *et al.*, 1990). Although other components of both Gram-positive and Gram-negative bacteria can induce cytokine synthesis and subsequent inflammatory processes (Rams *et al.*, 1990), LPS is the main, and most commonly recognised, structure in the wall of Gram-negative bacilli to cause inflammation.

Leenstra *et al.* (1996) reported that the saliva of healthy volunteers contained a mean level of 1 mg/ml endotoxin, and postulated that the LPS was generated by the indigenous anaerobic Gram-negative bacteria such as *Bacteroides* spp., since no colonisation of facultative Gram-negative bacteria was found in the mouths of this study group. No study has been reported concerning the endotoxin level in the saliva of cancer patients. Agarwal *et al.* (1995) have demonstrated that the

extent of monocyte activation was dependent on the source of LPS, i.e., whether it was derived from anaerobic periodontal bacteria or from *E. coli*. The endotoxin potency of anaerobic Gram-negative bacteria has been reported to be 1000 times lower than that of aerobic Gram-negative bacteria (Leenstra *et al.*, 1996). Therefore, an increased carriage of aerobic and facultative Gram-negative bacteria in the mouth of cancer patients receiving radio/chemotherapy might cause, or at least contribute to, inflammation due to the increased potency of the endotoxins present.

The results of the azocasein assay indicate that proteolytic enzymes of *P. aeruginosa* are also affected by PDA. The protease activity was negatively correlated with light doses, i.e. decreased activity resulted from the use of higher light doses. With the parameters studied, no correlation was found between the TBO concentration and protease activity.

Paardekooper *et al.* (1995) reported that TBO, upon illumination, inactivated the intracellular enzymes involved in fermentation (alcohol dehydrogenase), glycolysis (glyceraldehyde-3-phosphate dehydrogenase and hexokinase) and oxidative phosphorylation (cytochrome c oxidase) in yeast cells. The damage to these enzymes was suggested to be subsequent to the initial damage to the cytoplasmic membrane. TBO alone did not affect the intracellular enzyme activity. In this study, TBO alone was also shown to be unable to inhibit the proteolytic activity.

The proteolytic activity of *P. aeruginosa* may play a role in the pathogenesis of mucositis by degrading the matrix proteins to provide nutrients for the bacteria and by cleaving host defence proteins. An increased proteolytic activity has been reported in the saliva of severely ill patients. A positive correlation between the colonisation of *P. aeruginosa* in the oral cavity and the level of proteolytic activity in saliva has also been found (Woods *et al.*, 1983). A number of studies have shown that protease inhibitors may have some beneficial effects in reducing the severity of tissue damage (Barletta *et al.*, 1996; Cotter *et al.*, 1996). A mixture of protease inhibitors decreased the circulating proteolytic activity in *K. pneumoniae*-challenged burned mice (Neely *et al.*, 1994). Antiulcer agents such as sucralfate

were reported to counteract the degradation of transforming growth factor (TGF) and platelet-derived growth factor (PDGF) by *H. pylori in vitro* (Slomiany *et al.*, 1996). Chlorhexidine was reported to inactivate the proteolytic activities of a number of dental plaque bacteria (Beighton *et al.*, 1991). These agents were reported to have some beneficial effects on mucositis (Franzen *et al.*, 1995; Rutkauskas and Davis, 1993). It may be postulated that inhibition of proteolytic activity may have contributed to their beneficial effects.

At present, aminoglycosides are one of the commonly used drugs of choice for Gram-negative bacterial infections. However, aminoglycosides, as well as disrupting protein synthesis in bacteria, were shown to destabilise the membrane of *P. aeruginosa* and increase the release of vesicles consisting of hydrolytic enzymes which may increase bacteria-related damage during treatment (Kadurugamuwa and Beveridge, 1997). In contrast to this, PDA on these bacteria by TBO reduces the activity of the LPS in the outer membrane, but does not increase the proteolytic activity.

The inactivation of proteases and LPS demonstrated in this part of the study suggests a multi-targeting effect of TBO-mediated PDA. LPS is composed of polysaccharide and lipid whereas proteases are proteinaceous. Most antibiotics exert their effect at a specific site or process (e.g. penicillin on cell wall synthesis). TBO mediated PDA may be considered to produce the effects of more than one antibiotic. Therefore, development of resistance to this strategy is highly unlikely.

As mentioned in chapter 5, the cytotoxic species can not diffuse well, and the damage exerted is at the immediate sites of production of these reactive molecules. Dyes penetrating inside the cells damage the internal structures, whereas dyes remaining outside will initially damage the outer membrane. Therefore, it may be anticipated that the effect of photo-damage is non-specific and the structures closest to the cytotoxic species will be damaged regardless of their type.

The hypothesised effect of TBO-mediated photo-damage on the cell wall structure of Gram-negative bacteria is depicted below:

E           E                                    E

Lipopolysaccharide

Phospholipid bilayer

Lipoprotein
Peptidoglycan
Periplasmic space

Cytoplasmic membrane

Cytoplasm
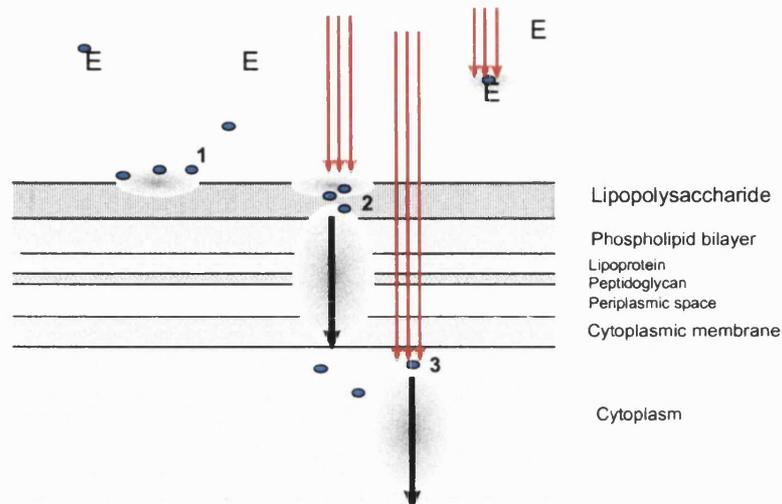
Figure 6.4    Proposed mechanism of photo-damage in Gram-negative bacteria.  The intact LPS forms a penetration barrier for the sensitiser. TBO, however, inactivates the LPS molecules [1] and further damage to LPS and other components of the outer membrane (unsaturated fatty acids and proteins) by the reactive species, occurs during illumination [2].  Damage to the outer membrane and cytoplasmic membrane will then permit the penetration of TBO molecules into cells.  TBO that enters the cell will then attack more sensitive targets when illuminated [3].  Red arrows represent laser light; black arrows represent singlet oxygen diffusion.  Blue dots symbolise TBO molecules. E stands for proteolytic enzymes.  The tissue damage is shown as grey ovals.

In summary,

1) The *E. coli* LPS was inactivated by PDA, and there was an impaired release of pro-inflammatory cytokines from PBMC on stimulation by the photo-inactivated LPS.  The effect on LPS suggests a superior efficacy of TBO-mediated lethal photosensitisation over porphyrin-mediated photo-inactivation.

2) The activity of *P. aeruginosa* proteases was significantly reduced by PDA.

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3) Inactivation of virulence factors as well as the direct antimicrobial activity of TBO-mediated lethal photosensitisation, should make it a very effective treatment approach for mucositis.

Chapter 7

**THE EFFECT OF PDA OF TBO ON THE RAT BUCCAL MUCOSA**

## 7.1 INTRODUCTION

This chapter consists of 2 sections. Firstly, potential adverse effects of PDA on the rat oral mucosa were determined. Although a range of sensitisers and light doses have been tested on healthy tissues in animal models and human subjects, there has been no study of the TBO-induced photodynamic effect on the oral mucosa. PDT with systemic photosensitiser administration is not selective to cancerous tissues as healthy tissues are also damaged (Grant *et al.*, 1997). However, if PDT is to be used for the treatment of infections, it must not be detrimental to the host tissues while killing the target organisms. This is particularly important for mucositis where the integrity of the oral mucosa has already been compromised by the cytotoxic effects of radio/chemotherapy.

In the second section of this chapter, the biodistribution of topically-applied TBO on the rat buccal mucosa has been investigated. When patients rinse their mouth with TBO solution, a thin layer of photosensitiser on the surface or in the saliva would be sufficient to reach micro-organisms, which would then be subjected to a photodynamic effect upon light exposure. However, once micro-organisms have adhered to the tissues, invasion into the deeper layers of the epithelium is a possibility. Laser light of 633 nm wavelength has a penetration depth into tissues of around 1 cm. The cytotoxic effects due to PDA would correspond to the fluorescence distribution of the TBO as singlet oxygen diffuses through a distance less than 1 µm inside cells.

In summary, the aims of this study were to determine
i) the effect of PDA on the rat buccal mucosa
ii) the penetration of TBO (using fluorescence microscopy) into the buccal mucosa of rats

## 7.2  MATERIALS AND METHOD

### 7.2.1  Photosensitisation of Rat Buccal Mucosa

The effect of TBO (25, 50, 200 μg/ml) and/or light (111, 169, 338 J/cm$^2$) from a copper vapour pumped dye laser on the buccal mucosa of female Wistar rats was observed.  The experimental techniques used in these animal studies, and in the subsequent macroscopic and microscopic examination, were performed as described in 2.2.  Figure 7.1 shows the irradiation of the TBO-treated buccal mucosa of a rat via an optical fibre.
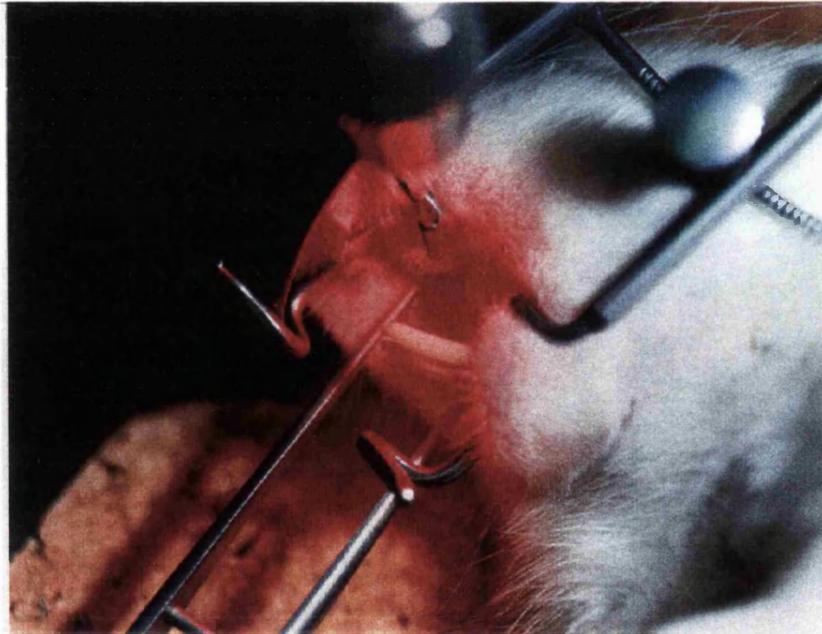


Figure 7.1   PDT of an experimental animal. The fibre has been positioned to expose the buccal mucosa pre-treated with topical TBO.

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## 7.2.2 Fluorescence Biodistribution of TBO

TBO (25 or 200 µg/ml) was applied to the mucosa in an identical manner to that used in studying the effect of TBO alone (see 2.2.3). After 1 min, the animals were sacrificed immediately by cervical dislocation and specimens were removed with care to avoid traumatising the surface of the mucosa. These specimens were frozen by submerging in a bath of isopentane placed in liquid nitrogen. 10 µm sections were prepared using a Cryocut E microtome (Reichert Ltd.) and stored at -70°C. The slides were thawed immediately before fluorescence microscopy and the fluorescence biodistribution of TBO in the tissues was detected as described in 2.2.5. Sections were prepared and imaged using a digital CCD camera with a minimum of light exposure to avoid any bleaching of the sensitiser. A minimum of 3 images were taken from each of the 3 sections of 3 rats. The autofluorescence of the mucosa from the unsensitised opposite side of the oral cavity was also measured (control sections).

The camera resolution of the images was 600-400 pixels. The images were recorded using a x10 objective and the scale of the micrographs was 880x550 microns. The mean fluorescence intensity was calculated by the computer after selection of three regions (e.g. 50x50 pixels) of each layer of mucosa.

## 7.4 RESULTS

### 7.4.1 The Effect of PDA on Rat Buccal Mucosa

During the 72 h observation period, no animal showed any distress. On macroscopic examination, none of the animals in any of the groups showed any visible changes to the mucosa. Figures 7.2.a and 7.2.b show the H&E stained histological sections of the control (right picture) and the treated mucosa with 200 µg/ml TBO and 338 J/cm$^2$ light (left picture). All muscle fibres, connective tissue and epithelia were intact and appeared unchanged (on the basis of microscopic examination) in all groups. No damage to blood vessels was detected and no necrotic or inflammatory changes were observed. Some cellular infiltration was noted with some eosinophilia, but this was considered to be within normal limits.

The laser light and sensitiser control groups produced clusters of abscess formation around the line of mucosal and dermal junction. This was consistent in both the treated and untreated sides of the buccal mucosa. Laser light and sensitiser alone experiments were then repeated on 3 animals from each group. The second experiment resulted in a normal mucosal picture with no signs of abscess formation. The formation of abscesses in the initial experiment was thought to be due to the mechanical trauma of the retractor applied for retraction of the cheek.

a                                                              b
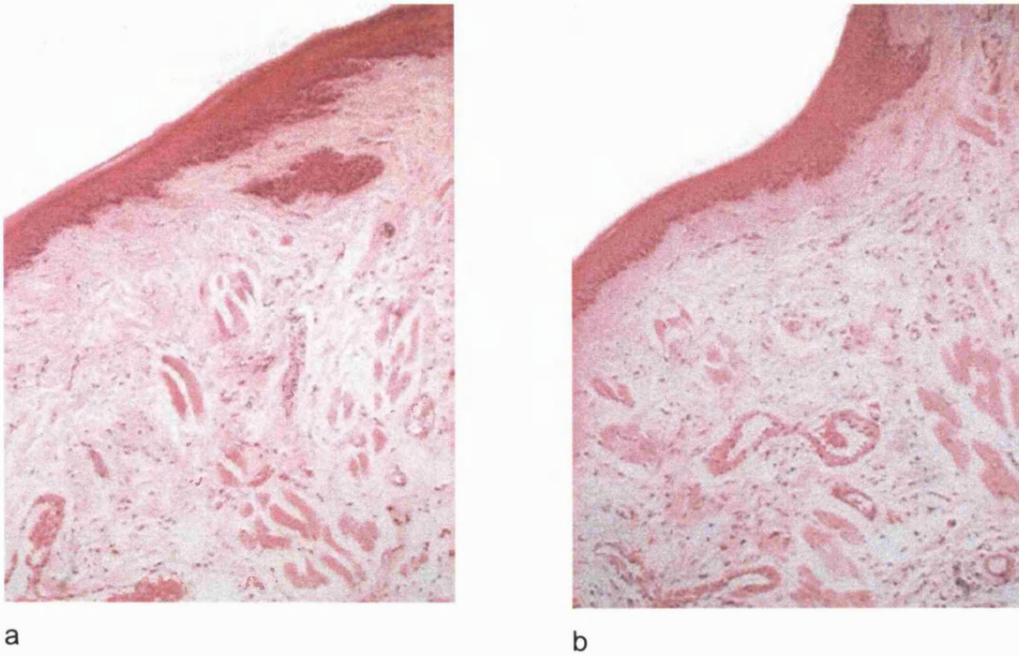
Figure 7.2   Histologic section of a) PDT-treated mucosa (200 µg/ml TBO; 338 J/cm$^2$), b) untreated mucosa. (Magnification: x100)

## 7.4.2  The Fluorescence Biodistribution of TBO in Rat Buccal Mucosa

False colour-coded CCD fluorescence images of the rat buccal mucosa are shown in figures 7.3.a and 7.3.b. The arbitrary units of the scale of fluorescence can be seen at the top of the image showing black at 75 and white at 303 counts per pixel with blue peaking at around 200. The white regions represent the highest intensity of fluorescence and black the lowest. H&E stained images of the same sections were depicted in figure 7.3.c and 7.3.d (lower photographs).
The images shown here were representative of all the sections photographed.

The photomicrograph shows that the fluorescence of TBO is very strong where it is absorbed and that this was confined only to the epithelial layer. In the connective tissue, the fluorescence signals were very similar to those from controls and this may be the result of tissue autofluorescence e.g. low levels of endogenous porphyrins. Furthermore, upon re-scaling, it was evident that the keratinised layer contained the highest level of fluorescence penetrating throughout the epithelium with decreasing intensity (picture is not shown).
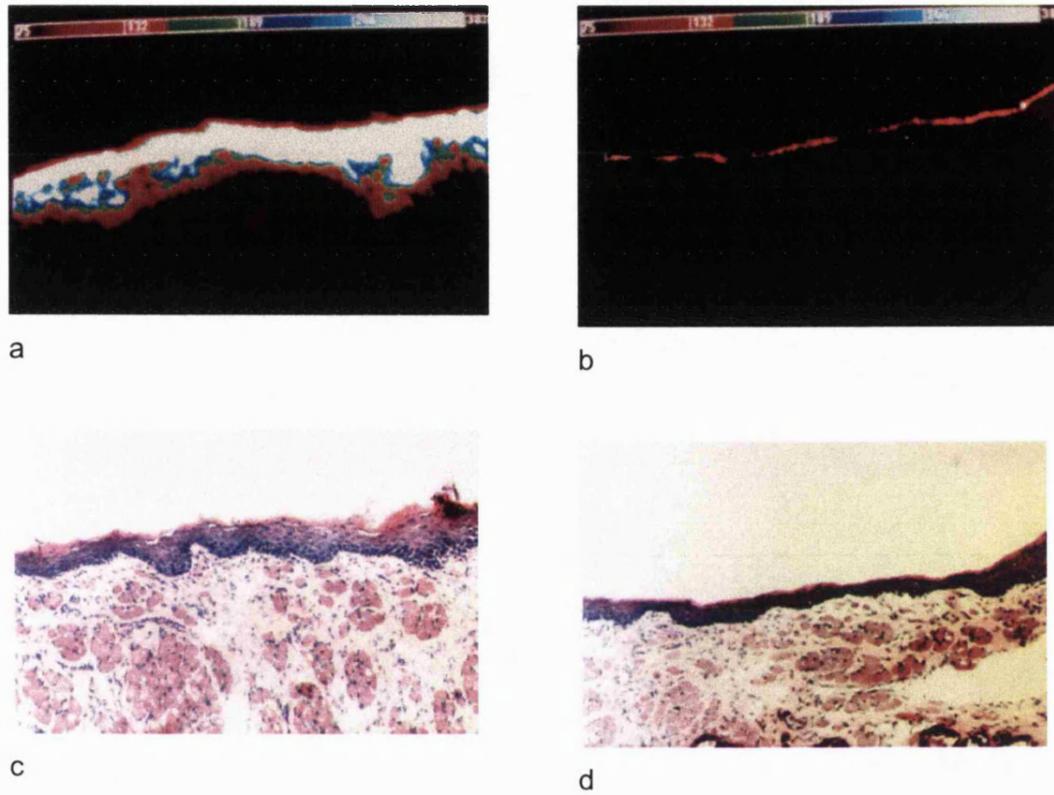
Figure 7.3    Fluorescence CCD micrograph (a) and corresponding H&E stained section on the same scale (c) of rat buccal mucosa 60 sec after sensitisation with 200 µg/ml TBO.    Figures b and d are the control sections.    The scale on the top shows the fluorescence level of the mucosa.  The CCD image scale was 880-550 microns.

Not surprisingly, the magnitude of the peak fluorescence intensity in the buccal epithelium was found to be dose-dependent with around a 7 fold greater intensity in the epithelium using 200 µg/ml TBO relative to 25 µg/ml. Figure 7.4 shows that a much higher intensity of fluorescence (expressed in arbitrary units) was observed at 200 µg/ml TBO (480±44) compared to 25 µg/ml (68±13), where the autofluorescence of the tissues was 35±10.
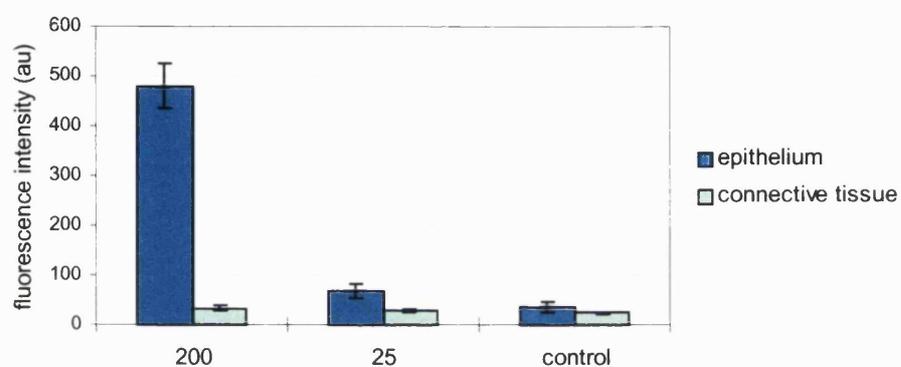


Figure 7.4    Comparison of fluorescence intensity in the buccal mucosa following application of 200 µg/ml and 25 µg/ml TBO. Each value is the mean from 50-50 areas in 3 rats. The control group shows the unsensitised side (autofluorescence).

## 7.4 DISCUSSION

In this chapter the rat buccal mucosa was examined for the distribution of TBO and for any potential damage induced by the PDA.

It was found that when up to 338 $J/cm^2$ of light and 200 µg/ml of TBO was used, either alone or in combination, no damaging effects on the rat buccal mucosa were detectable.

The response of normal tissues to TBO-induced PDT determines the feasibility of this treatment as a possible antimicrobial regime. The results presented in this part of the study are encouraging since the doses tested were much higher than the optimum parameters required to kill bacteria and yeasts *in vitro*. (In chapter 3, it was shown that nearly all micro-organisms were killed using 25 µg/ml TBO and 111 $J/cm^2$ light.) This implies that whilst it is possible to kill the causative micro-organisms by PDT, the normal adjacent tissues will most probably remain intact and unaffected. This is important, especially in the case of mucositis, where the integrity of the oral tissues is disturbed due to the adverse effects of radiotherapy and/or chemotherapy.

The power of laser light capable of damaging the tissues (thermally) is of the order of a few watts compared to the miliwatt range used in PDA studies (which is a non-thermal effect). Similarly, the concentration of TBO required to kill organisms upon illumination is at least 500 times less than that used for the detection of cancerous changes in the oral cavity (10 mg/ml) (Mashberg, 1983; Warnakulasuriya and Johnson, 1996). There has been no report of any harmful effect on the mucosa of TBO in such concentrations following clinical use. The data presented in this chapter confirm the safety of up to 200 µg/ml TBO when used in conjunction with light on oral mucosa in the animal model used.

A variety of sensitisers in conjunction with various energy doses of light on healthy animal tissues have been shown to elicit different tissue responses. Meyer *et al.*, (1991) reported that administration of 5 mg/kg $AlS_2Pc$ and subsequent 100 J of

laser light exposure in rabbits induced muscle necrosis and necrotising sialometaplasia in salivary glands while gingival ulcerations occurred with relatively lower light doses (50 J). Nevertheless, the healing was uneventful in all cases. In another study, collagen and elastin were shown to be unaffected by the same regimen in the rabbit carotid artery. The vessel walls showed full thickness cell death but the mechanical properties stayed intact with no inflammatory changes (Grant et al., 1995).

Michael (1993) indicated that the degree of tissue response in the mouse tongue was parameter-dependent when HpD was used. 20 mg/kg HpD in conjunction with both 90 $J/cm^2$ and 180 $J/cm^2$ light produced the most severe response. Oedema and cellular infiltration, despite a reduction in the number of vessels, were observed. Vesicle formation, disturbing the stratification of the epithelium, was also noted. However, the muscle fibres remained intact. Healing was complete in all cases in 5 days post-PDT, with regeneration of normal tissues.

The route of application of the photosensitiser may also be related to the degree of tissue reaction. Topically-applied HpD (50 $\mu g/ml$) with 10 $J/cm^2$ light elicited no histologic abnormalities in swine gastric epithelium. Minor transient changes like oedema and rare focal mononuclear cell infiltration were noted only at day 1 and 2 with higher doses of HpD (500 or 5000 $\mu g/ml$) and light (100 $J/cm^2$). In the present study, the effect of intravenous or oral administration of TBO was not investigated because of the superficial nature of mucositis. TBO was used in the form of an aqueous solution with a maximum concentration of 200 $\mu g/ml$ chosen because of the effectiveness of lower concentrations in killing the micro-organisms.

In vitro experiments also elicited different responses for the specific sensitisers. HpD with HeNe light irradiation resulted in significant toxicity to keratinocytes, which was dependent on both the doses of dye and the light. Massive damage to the outer membrane of the cells and lysozyme digestion with activation of the lysosomes was observed (Artuc et al., 1989). Soukos et al. (1994) reported cytotoxic effects of $AlS_2Pc$ on normal human gingival keratinocytes and fibroblasts

*in vitro*. Following 24 hours administration of 25 µg/ml AlS$_2$Pc, exposure to 0.6 J/cm$^2$ of light, resulted in 100% kill of keratinocytes and fibroblasts. However, in the absence of light, the dye was also toxic even with lower concentrations (2 and 5 µg/ml respectively). On the other hand, there was no reduction in cell viability of human keratinocytes and fibroblast using 2-5 µg/ml TBO and 0.9 J/cm$^2$. In contrast, 0.6 J/cm$^2$ of light in the presence of 2.5 µg/ml TBO was enough to kill *Strep. sanguis in vitro* (Soukos *et al.*, 1996).

TBO has also been tested for its photosensitising effects on various cancerous cell lines. Canete *et al.* (1993) reported that MB and TBO administration followed by red light irradiation induced photo-inactivation of HeLa cells. The effect was dependent on the incubation time and the light dose when 10$^{-5}$ M solutions of thiazine dyes were used. At equimolar concentrations, TBO was found to have the most potent photosensitising effect. TBO, among other dyes, was reported to preferentially photosensitise carcinoma or other tumour cells and to have less effects on normal cells. A 90% reduction of the colonogenicity of human epidermoid carcinoma was achieved with 0.67 µM TBO and 1 J/cm$^2$ of white light, whereas normal hamster ovary cells were highly resistant to the photosensitising effects (Darzynkiewicz and Carter, 1988). Although, using the parameters investigated in this part of the study, TBO was shown to cause no effect on the healthy oral tissues in an animal model, with concentrations higher than 200 µg/ml, its potential use as a photosensitising agent of cancerous tissues is still open to further investigation.

Millson *et al.* (1996b) reported the photosensitising activity of TBO on *Helicobacter mustelae* implanted on the ferret gastric mucosa when illuminated with light from a copper vapour dye laser. A 90% reduction in counts of bacteria sensitised with 0.75 mg/kg TBO were seen after irradiation with 200 J/cm$^2$. However, from the experiments on rats, the energy required to kill the sensitised bacteria was insufficient to damage the underlying mucosa. In contrast to TBO, several photosensitisers were shown to be phototoxic to the mammalian cells with no effect on Gram-negative bacteria. A possible explanation would be that TBO-mediated photo-damage is targeted to specific and highly susceptible sites on the

procaryotic cell surface which mammalian cells lack. The results presented in chapter 5 suggest that the damage to the outer membrane causes cell death. In addition, it was shown that LPS which is situated on the outer membrane is inactivated by PDA (see chapter 6).

The digital CCD fluorescence images obtained here clearly demonstrate that TBO accumulates on the keratinised epithelium with high levels throughout all layers of the epithelium. However, no detectable penetration occurs into deeper layers of the mucosa. TBO, if its photosensitising ability is proven on cancerous cells *in vivo*, may be of use in the PDT of superficial epithelial neoplasms such as basal cell carcinoma. However, alternative use of TBO in the PDT of neoplasms is beyond the scope of this discussion.

In the case of topical infections, its penetration through the epithelium, into which the invasion of micro-organisms may extend is most probably sufficient to eradicate the causative organisms. The assumption that the localisation and intensity of microscopic fluorescence correlates reasonably well with PDT-induced damage is generally accepted. The efficacy of the sensitiser is strongly dependent on its location because singlet oxygen has a short life time and short diffusion range (Moan and Berg, 1991). In the mouth, facultative Gram-negative bacteria and yeasts generally adhere to the surface of the epithelium and are mainly suspended in saliva. However, microbial penetration through the epithelium may be encountered, e.g., candida may penetrate through the epithelium following mycelia formation. Therefore, light application to those areas after topical application of TBO should result in the successful killing of microbes.

The process of preparation for the CCD fluorescence images involves the samples being snap-frozen as soon as possible after sacrifice of the animals and excision of the specimens. Therefore, this technique may allow some useful interpretation of the intensity of TBO in saliva. The thin band on the surface of the epithelium showing a high level of fluorescence may correspond to TBO in the saliva layer, which may in turn indicate a high uptake of TBO by salivary components.

The distribution of the topically-administered sensitiser may vary from that given intravenously or orally. The high fluorescence of TBO in the epithelium is most probably a consequence of its topical application. However, even when used systemically in ferrets, the surface of the gastric epithelium was found to be the most bright under fluorescence (Millson et al., 1996b). Following oral administration of ALA in patients, maximum fluorescence was found in the oral epithelium (in both normal and abnormal areas). The ratio between the fluorescence in the epithelium and underlying subcutaneous tissue was approximately 2-3:1. The photodynamic effect, following subsequent illumination, was limited to the epithelium (Fan et al., 1996). On the other hand, intravenous application of mTHPC resulted in slightly higher levels in the connective tissue (Fan et al., 1997).

It is difficult to make a direct comparison of the TBO penetration depth of healthy mucosa with that of impaired mucosa. Whether the penetration of TBO through the epithelium is related to the structure of the tissues or depends solely on the diffusion rate of TBO, penetration through intact tissues may be expected to be lower than that through the erosive mucosa. Therefore, it may be presumed that increased levels of absorption may take place through regions of the mucositis lesions where the integrity of the oral epithelium is disturbed.

In summary;

1) Safety studies showed that when using up to 200 µg/ml TBO on exposure to 338 J/cm$^2$ laser light there was no histological nor visible damage to the healthy rat buccal mucosa, which suggests that PDT can be safely used.

2) The fluorescence biodistribution of TBO was demonstrated to be highest in the epithelium. This suggests that topically-applied TBO may be ideal for the management of localised, topical infections. However, the optimum concentration of the photosensitiser and the light dose remain to be investigated in clinical trials.

Chapter 8

FINAL DISCUSSION

## 8.1  SUMMARY OF RESULTS

In chapter 3, the susceptibility of Gram-negative bacilli to TBO-mediated PDA was evaluated.   In addition, other mucositis-associated organisms, as well as commensal oral bacteria, were tested for their susceptibilities to lethal photosensitisation.

Gram-negative bacteria were found to be very susceptible to killing by PDT.   With the parameters studied, the effectiveness of PDA was dependent on the doses of both TBO and light. However, increasing the PIT up to 15 min had no effect on the kills achieved, which saves patients from the inconvenience of keeping TBO in their mouth for a long period and shortens the clinical time involved in the application of this treatment.

PDA was found to be non-specific as all organisms tested, including both mucositis-associated organisms and commensal bacteria, were susceptible to this technique to a similar extent.   This may mean a total sterilisation of the oral cavity could be achieved with PDA.   However, the use of antibody-conjugated sensitisers may provide better selectivity for target organisms.   In addition, implanting "friendly bacteria" may also help to reconstitute the normal oral flora.

The susceptibility of isolates from patients with mucositis to PDT was also evaluated.   Clinical isolates of all organisms tested were shown to be as susceptible to PDA as their laboratory counterparts.

In chapter 4, the effects on PDA of the physiological factors most likely to be encountered in the oral environment were elucidated.

It was possible to kill the bacteria under all physiological conditions tested.   In the presence of saliva, the extent of bacterial kills was not as high as in the presence of saline.   The numbers of bacteria killed in the presence of serum were further reduced.  When the experiments were carried out at various pH values (pH 4.0-8.0), the highest kills were obtained at pH 8.   Increasing the acidity caused a

reduction in the numbers of bacteria killed. The pH of saliva in patients undergoing cytotoxic therapies was reported to be in the acidic region (at around pH 5.0). The efficiency of PDT could be increased by asking patients to rinse their mouth with slightly basic solutions such as bicarbonate just before the application of TBO. This would also cleanse the mouth and reduce the effect of serum from the lesions and saliva. The growth phases of bacteria had no effect on their susceptibility, which indicates that bacteria from all stages of growth can be killed at a similar rate.

In chapter 5, some aspects of the mechanism of PDA on *E. coli* (uptake of TBO, type of photochemical process and cellular targets) were studied.

Results of the experiments concerning the photochemical mechanism suggest that singlet oxygen (type II process) is mainly responsible for cell death via TBO-mediated PDA. However, although minor, the type I process (generation of free radicals by the interaction of biomolecules and excited sensitiser) also appeared to be involved. The uptake of TBO by bacteria was found to be very minor and/or weak. Therefore, the effect does not seem to rely on the penetration of the sensitiser into the cell. This was supported by the finding that when TBO in solution was eliminated, no kills were obtained. The TEM studies showed that the outer membrane was one of the sites , and most probably the initial site, damaged by PDA, which then allows TBO to gain access into the cell interior. Condensation and shrinkage of the cytoplasm also imply damage to internal structures such as cytoplasmic proteins and DNA.

The results of mechanistic studies may have some clinical implications. Increasing the oxygenation of the area to be treated may increase the effects of PDA and reduce the treatment time, as singlet oxygen is the main cytotoxic species inducing cell death. A $D_2O$ rinse may also be helpful as it may increase the life-time of singlet oxygen. Alternatively, the use of fractionated irradiation may allow recovery from oxygen depletion in the area. As TBO does not need to enter the cells to exert a photodynamic effect, and the outer membrane is one of the main structures damaged, specific bacterial kills could be achieved by sensitiser

conjugated to structures which have an affinity for bacterial surface receptors e.g., antibodies.

In chapter 6, the effects of PDA on two of the virulence factors of Gram-negative bacilli were investigated.

It was found that PDA reduced the ability of *E. coli* LPS to stimulate cytokine synthesis by PBMC. The activity of *P. aeruginosa* proteolytic enzymes was also shown to be reduced.

Inhibition of these virulence factors by PDA is potentially a great advantage as they are thought to be involved in damaging host tissues. LPS is capable of initiating and perpetuating the inflammatory process by stimulation of cytokine release and thus may increase the severity of mucositis. A reduction in proteolytic activity by PDA may help reduce the degradation of the structures of the mucosa as well as the effector components of immune cells.

In chapter 7, any possible harmful effect of PDA together with the fluorescence biodistribution of TBO on mucosal tissues was elucidated.

No detectable macroscopic or microscopic effect could be observed when up to 200 μg/ml TBO and 338 J/cm$^2$ were used either alone or in combination. TBO, when applied topically, can penetrate throughout the epithelium with no detectable levels in the connective tissue layer.

The favourable results obtained in the *in vitro* studies and safety studies using an animal model suggest the feasibility of PDT as a possible antimicrobial strategy for the treatment of topical infections.

## 8.2 CURRENT STATUS AND FUTURE DIRECTIONS OF PDT FOR INFECTION

PDT could be considered as constituting one regime for all types of infection regardless of their aetiology since PDA combines the effects of all antimicrobial agents, i.e. antibacterials, antifungals and antiviral agents. Bacteria, yeasts and viruses have all been shown to be photosensitised to light with various dyes. Although TBO has the properties of a desired photosensitiser, and could be the compound of choice, different sensitisers could be used according to the nature of the infection. Once the efficacy of PDT for infections is approved clinically, it could eliminate the need for selecting a particular agent for each type of infection. TBO-mediated PDT would have many advantages over conventional antimicrobial regimes:

PDT is localised. The photochemical reactions will take place only when the photosensitising agent is exposed to light. Therefore, if the photosensitiser has accumulated in irrelevant areas, the treatment region can be controlled by directing the light to the desired area. Topical application of TBO was confined only to the epithelial layer in the animal model, therefore the effect of PDT would be at the local and superficial level.

It is a repeatable technique. Only low concentrations of TBO are needed for the antimicrobial effect and there is no cumulative toxicity. Therefore, the treatment could be employed as often as necessary.

The development of complete resistance seems very unlikely. TBO was shown to have no effect on the viability of cells unless it is used in combination with light. It was also demonstrated that for TBO to be effective, it does not have to enter the cell interior. Therefore, there is no direct interaction of un-excited (ground state) TBO with viable cells and so resistance to the photosensitiser is less likely to occur.

Antibiotics or antifungal drugs target a specific component of the cells such as the inhibition of cell wall or protein synthesis, whereas cytotoxic species, produced by

the interaction of photosensitiser and light, exert their effects at the molecular level via oxidative damage which affects a number of cellular targets. Modification of all of these targets to provide resistant strains would be very unlikely. However, the likelihood of resistance can not be completely overlooked, since some compounds in the cells, such as carotenoids, may act as singlet oxygen or hydroxyl radical scavengers which may inhibit the effect of this action by quenching the singlet oxygen before it produces any cytotoxic effects.

The simultaneous use of different photosensitisers may increase the effectiveness of PDA. Depending on the binding site or location, each photosensitising compound, upon illumination, has been shown to act mainly on a range of structures. For exogenous sensitisers like TBO, the cell wall appears to be the main target in the bacteria. On the other hand, MB-induced PDA was reported to mainly damage DNA. Therefore, if a resistance mechanism evolves at one target site, damage to the other sites should still be sufficient to cause cell death. Similarly, simultaneous use of two sensitisers, one mediating predominantly type I and the other type II processes, may overcome any quenching mechanism developed by the organisms (Kochevar et al., 1996).

TBO may display a dual-function. 1% solutions of TBO have been demonstrated to be of value for the detection of early malignant changes in the mouth (Mashberg, 1983). When TBO is rinsed prior to light illumination, a quick examination for any excessive uptake of TBO by dysplastic areas may prove to be useful in detecting any possible recurrences or primary lesions.

PDT for infections would not be without its draw-backs:

Photodynamic inactivation is non-specific as the results of this project demonstrated, all the organisms tested were susceptible to this treatment. This may be a disadvantage, especially in the oral cavity which is naturally inhabited by a wide range of bacteria. Killing the indigenous bacteria as well as the pathogenic exogenous organisms may result in the establishment of an inappropriate oral microflora.

This may be overcome by the implantation of members of the normal oral microflora after PDT, which would then re-establish a normal microflora in the oral cavity.

Conjugation of antibodies to the dye molecules may be an alternative to improve the specificity of a sensitiser. Tin (IV) chlorin e6 bound to anti-*P. aeruginosa* monoclonal antibody has been shown to efficiently kill *P. aeruginosa* in pure cultures and selectively in mixed cultures with *S. aureus* (Friedberg *et al.*, 1991). Gross *et al.* (1997) reported that bacteriochlorophyllide conjugated to IgG bound with high specificity to protein A on the cell surface of *S. aureus* and was 30 times more effective than the control (non-targeted) bacteriochlorophyllide-serine conjugate. Selective killing of *Por. gingivalis* (periodontopathogenic, anaerobic Gram-negative bacilli) was achieved in mixed culture with very little effect on *Strep. sanguis* when TBO was conjugated to the antibody (Bhatti *et al.* 1998a).

Selective targeting to bacteria by conjugates with no effect on mammalian cells can also be achieved. Chlorin e6 conjugated to poly-L-lysine was shown to produce 99% killing of *Por. gingivalis* and more than 99.99% killing of *A. viscosus* while oral epithelial cells remained intact *in vitro* (Soukos *et al.*, 1998).

In addition to promoting selective PDA, the advantages of such immunoconjugates might include an increased clearance of the bacteria, and a more specific treatment might reduce the energy dose and sensitiser concentration required to kill the bacteria.

Several other systems such as the use of liposomes and plasma lipoproteins have also been reported to improve the selective distribution of the photosensitiser in cancerous cells and these may be adaptable to the targeting of bacteria.

Application of PDT requires clinical settings and trained staff. Unlike antibiotics, the patients cannot self-administer this regime as application of PDT needs careful monitoring. However, patients receiving cytotoxic therapies need to visit the clinic on a daily basis. Oral examinations also need to be performed regularly

on these patient groups. The application of this therapy should not take more than a few minutes so it could be performed after a regular check up of the oral cavity.

The light sources used in this project were the HeNe gas laser (for *in vitro* studies) and the copper vapour dye laser (for animal studies). Dye lasers have mainly been used for PDT application in clinics. These lasers are bulky and require a physicist to adjust the machine prior to use. Recently, more portable laser machines (diode lasers) have been developed and the small size of the laser light sources may enable the use of this therapy more easily in standard clinical settings. These lasers are also user-friendly and cheaper than the dye lasers. In addition, non-laser light sources such as the LED appear to be very promising. Development of more efficient light sources and light delivery systems will also allow a wider range of tissues to be treated. Treatment of the whole oral cavity may be possible with total illumination of the complete mucosal surfaces in a few minutes.

This project has demonstrated that sensitised Gram-negative bacilli can be killed by light under conditions which would not cause any unacceptable damage to the underlying mucosa in an animal model. However, the parameters for PDT should be adjusted very carefully during the treatment since the production of cytotoxic species is of key importance. Since oxygen is consumed during PDT, monitoring the oxygen status during illumination may facilitate control of PDT dosimetry. According to oxygen depletion or excess oxygen in the area, treatment parameters could be adjusted which would produce an optimum outcome and avoid any damage to the host tissues. Treatment could also be controlled by the use of low or high fluence or the use of continuous or fractionated light delivery (McIlroy *et al.*, 1998).

The results of the experiments reported in this thesis showed that TBO is an effective sensitiser. As the physio-chemical properties of TBO seem to play an important part in its efficacy. It would be of interest to investigate whether derivatives of TBO are more effective sensitisers.

At present, treatment of infections with PDT appears best for localised and superficial infections. Apart from the oropharyngeal cavity, infection of the other cavities, especially maxillary sinuses and female genitalia, may be other possible areas for PDT. In addition, PDT would also be used for infections associated with burns and wounds. However, with improvements in the properties of both sensitisers and light, treatment of deeply-seated infections with this method may be possible. For the deep-seated infections other routes of application of the sensitiser (i.v. and oral) and light (interstitial) would need to be evaluated.

The PDT effect on normal tissue has been evaluated in animals, but more information is needed on its effect on normal human tissues. More specifically, the effect of PDT on the already-damaged tissues should be determined.

Although there are several aspects requiring further evaluation, the results of this project suggest that PDT could be used for the treatment of infections and might, in future, offer a credible alternative to conventional antibiotic therapy. More studies are required to check the optimal doses and the efficiency of the therapy by well-designed and well-controlled clinical trials.

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