Endotoxin contamination in the dental surgery

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Dental waterlines contain large numbers of Gram-negative bacteria. Endotoxin, a component of such organisms, has significant health implications. Paired samples of dental unit water and the aerosols generated during dental procedures were collected, and assayed for bacteria and endotoxin levels, using heterotrophic plate counts and the *Limulus* amoebocyte lysate test. Consistent with published studies, the extent of bacterial contamination in the dental waters sampled for this investigation surpassed the levels associated with potable water, with counts in excess of 2.0×10^6 c.f.u. ml⁻¹ in some samples. Correspondingly high concentrations of endotoxin [up to 15 000 endotoxin units (EU) ml⁻¹] were present in the water. A statistically significant Spearman correlation coefficient of $\rho=0.94$ between endotoxin (EU ml⁻¹) and bacterial load (c.f.u. ml⁻¹) was demonstrated. All of the aerosol samples contained detectable endotoxin. Further studies of the consequences of dental endotoxin exposure, and evaluation of means to prevent exposure, are warranted.

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INTRODUCTION

Endotoxin, a heat-stable toxin associated with Gram-negative bacterial cells, is composed of LPS derived from the bacterial cell wall. Most of the pathogenic effects seen in Gram-negative bacterial infections are mediated by an endotoxin (Natanson *et al.*, 1994); the associated clinical syndrome may even occur in the absence of bacteraemia (Danner *et al.*, 1991; Graham & Brass, 1994). Endotoxin has been implicated in the pathogenesis of hepatotoxicity, hepatorenal failure, hepatic encephalopathy (Shibayama, 1992; Odeh, 1994), periodontitis (Trope *et al.*, 1995), mastitis (Tyler *et al.*, 1994), adult respiratory distress syndrome (Herbert *et al.*, 1992; Graham & Brass, 1994), disseminated intravascular coagulation (Graham & Brass, 1994), humidifier fever (Flaherty *et al.*, 1984; Mamolen *et al.*, 1993) and sick building syndrome (Teeuw *et al.*, 1994).

Dental unit waterlines are lined by a thriving biofilm composed predominantly of Gram-negative heterotrophic bacteria (Mayo *et al.*, 1990; Pankhurst & Philpott-Howard, 1993; Williams *et al.*, 1993). These organisms are released in high numbers into the dental unit coolant and irrigant water(s), and delivered through the distal outlet of the

dental instrument. With them is delivered the potential for endotoxin exposure.

In pharmaceutical products, endotoxin concentrations in fluids have to be carefully controlled and United States Pharmacopoeia (USP) standards for irrigation and parenteral fluids must be observed (USDHHS, 1987). The Centers for Disease Control and Protection (CDC) currently recommends that USP sterile water be used for all dental surgical procedures, and this stipulation requires the use of 'pyrogen-free' irrigant (USDHHS, 1987; CDC, 1993).

Despite the fact that there are numerous reports of Gram-negative bacteria in dental water, there is a paucity of published reports investigating dental water endotoxin (Bourassa *et al.*, 1995; Putnins *et al.*, 2001; Fulford *et al.*, 2004; Szymanska, 2005a, b).

Three questions were asked in the present study. (i) Are there significant levels of endotoxin in dental water? (ii) Is there any correlation between the number of bacteria and the level of endotoxin present in dental water? (iii) Is endotoxin aerosolized in detectable levels by routine dental procedures?

METHODS

Water and aerosol samples. Water samples were collected from the dental lines of operatories, as dental surgeries are called in the USA, in a large institutional setting and processed according to standard practices for water-quality evaluation using heterotrophic bacterial

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Abbreviations: CDC, Centers for Disease Control and Protection; EU, endotoxin unit; LAL, *Limulus* amoebocyte lysate.

plate counts (APHA, 1985). Samples (2–3 ml) were collected in sterile, pyrogen-free 12 × 75 polystyrene tubes, avoiding any contact between the instrument parts and the tube during collection. Samples were shipped overnight to the laboratory at 4°C.

Aerosols were collected using AGI-30 glass impingers (Ace Glass) containing endotoxin-free sterile water, as described by Trudeau & Fernandez-Caldas (1994). Impingers were set up at a distance of 60 cm from the area of dental work in a patient's mouth and a total of 0.33 m³ of air was sampled for each of eight tested operatories: four from operatories using ultrasonic scaler lines and four from operatories using high-speed handpiece lines. Control collections were done in the early morning prior to the generation of any dental aerosols. Samples were shipped on ice via overnight courier and analyzed for endotoxin.

In view of the natural day-to-day fluctuations in bacterial contamination in dental water (Santiago *et al.*, 1994), both water and aerosol samples were obtained on multiple occasions, ultimately providing 47 separate water/aerosol pairs for our analyses.

Bacterial contamination. Bacterial contamination of the water samples was assessed by plating 100 µl aliquots of serial dilutions of each sample onto R2A plates and incubating as described previously (Williams *et al.*, 1993).

At each of the aerosol collection sites, additional collections were made using Anderson plate chambers. In this procedure, air is drawn over the surface of agar plates through steel plates, which deflect the particles onto the surface of the R2A agar for subsequent incubation and colony formation (Turner & Hill, 1975). In this way, the number of bacteria may be determined qualitatively, and the resultant colonies permit standard microbiological isolation and identification methods to be applied.

Endotoxin assay. The presence of endotoxin in the samples was assessed using the *Limulus* amoebocyte lysate (LAL) test (USDHHS, 1987), commercially available as a Pyrotell kit (Associates of Cape

Cod). The LAL assay is exquisitely sensitive to endotoxin, and mandates devoted adherence to the use of endotoxin-free glassware and plasticware (including tubes and pipette tips), diluent, etc. The assay was performed in accordance with the specified protocol. Results were obtained in endotoxin units (EU); 10 EU corresponds to 1 ng endotoxin. Commercially available endotoxin standards were used as controls.

Statistical analysis. The Spearman rank correlation coefficient for non-parametric data was employed to analyse the relationship between endotoxin and bacterial contamination levels.

RESULTS AND DISCUSSION

Contamination levels

Consistent with all studies to date, the extent of bacterial contamination in the dental waters sampled for this investigation far surpassed the levels associated with potable water, with counts in excess of 2.0×10^6 c.f.u. ml⁻¹ in some samples. Correspondingly high concentrations of endotoxin (up to 15 000 EU ml⁻¹) were present.

The relationship between bacterial concentrations and the results of the endotoxin assay are shown in Fig. 1. A Spearman rank correlation coefficient of $\rho=0.94$ between EU ml⁻¹ and c.f.u. ml⁻¹ was demonstrated, which was significant at $P<0.05$.

Aerosol sample cultures produced a varied mixture of bacterial organisms, which were rapidly overgrown by the abundant growth of a number of fungal varieties. This precluded quantification of bacterial numbers in aerosols. Nevertheless, a variety of bacteria was isolated, including Gram-positive cocci, Gram-positive coccobacilli, Gram-negative cocci and Gram-negative bacilli. Not all of the isolates were identifiable using standard microbiological and biochemical profiling techniques, but of those that could be definitively assigned to species, the following were found: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus haemolytica*, *Micrococcus* spp., *Micrococcus varians roseus*, *Pseudomonas vesicularis*, *Sphingomonas paucimobilis*, *Acinetobacter* spp. and *Flavomonas* spp.

All of the aerosol samples collected in impingers contained detectable endotoxin, although the concentration did not exceed 2.7 EU ml⁻¹ in any instance. No endotoxin was detectable in control air samples.

Water obtained from properly functioning distillation, reverse osmosis and ultrafiltration systems generally has undetectable levels of endotoxin. Clearly, the concentrations seen in the dental water samples far exceeded any acceptable standard for water intended for medical purposes. The generally acknowledged range of 0.06–0.5 EU ml⁻¹ for medical devices (including liquid devices), depending on application, applies to irrigation fluids and is regulated by the US federal government (USDHHS, 1993).

We found levels of endotoxin in dental water several orders of magnitude below that reported in two published reports,

Fig. 1. Correlation between bacterial contamination and endotoxin levels in dental waterline samples. A positive correlation with a Spearman's rank correlation coefficient of $\rho=0.94$ between bacterial load and endotoxin level, significant at $P<0.05$, is evident. Note both axes are logarithmic.

both from the same investigator (Szymanska 2005a, b). Compared with others (Putnins *et al.*, 2001; Fulford *et al.*, 2004), our water endotoxin levels exhibited a broader range of values, encompassing their results but extending to both higher and lower levels. The reason for the discrepancy with the former (Szymanska 2005a, b) is not clear. Such a large difference in endotoxin level cannot be attributable solely to microbial content of the waterlines, as c.f.u. counts between those studies and ours were comparable. It is possible that significantly higher levels of endotoxin existed in the municipal water supplying the operatory and were showing up in the efflux. This would also explain the lack of correlation between dental water microbial levels and endotoxin found in the study. It is also conceivable that an unfortunate typographical error (mislabelling EU values as μg , a difference of several orders of magnitude) could have occurred, although this is deemed less likely as it would require the same error to persist through the peer-review process for two papers. Still, this is tempting to consider, as reading the endotoxin levels as EU rather than μg would bring these values into close agreement with that seen in this study, and those of Fulford *et al.* (2004) and Putnins *et al.* (2001). Aerosolized endotoxin levels documented in the present study were also exponentially lower than those found by Szymanska (2005a), who also found that disinfection of the dental unit waterlines, whilst decreasing the level of endotoxin detectable in the water, had no significant effect on the aerosolized endotoxin levels.

In light of the significant correlation demonstrated in Fig. 1, the proximate source of the dental water endotoxin contamination is likely to be the Gram-negative organisms resident in the dental waterline biofilms (Mayo *et al.*, 1990; Pankhurst & Philpott-Howard, 1993; Williams *et al.*, 1993). The correlation between bacterial and endotoxin contamination levels observed in this study is at odds with the findings of others (Bourassa *et al.*, 1995; Fulford *et al.*, 2004; Szymanska, 2005b). In those studies, a correlation between the levels was sought as a 'simple test for monitoring bacterial contamination'. They found no significant relationship between c.f.u. and endotoxin concentrations, even when the c.f.u. varied by up to three orders of magnitude. One group (Bourassa *et al.*, 1995) proposed that this lack of relationship may be due to bacterially produced inhibitors of the *Limulus* chromogenic test employed in their investigation (Roth *et al.*, 1990). The LAL assay may, in fact, be less prone than the chromogenic test to such inhibitors. However, another investigator failed to demonstrate a significant correlation using the same LAL techniques employed in our laboratory (Szymanska, 2005b), although the possibility of exogenous endotoxin being supplied in the municipal water could confound this, as noted above. The range of both bacterial and endotoxin levels found in our study was broader than that seen by Fulford *et al.* (2004), and could explain why a statistically significant correlation was not found in the latter. Other explanations could relate to sample size, variation in aliquot-handling technique or contamination

of diluents or laboratory-ware by exogenous endotoxins. Whatever the cause of the differences, whilst there is not an adequate basis on which to compute the number of bacteria ml^{-1} , the correlation between endotoxin and c.f.u. ml^{-1} is clear.

Implications

The presence of endotoxin in dental water has potential clinical significance, both medically and dentally. Endotoxin stimulates the production of numerous cytokines, which resulting in tissue injury (Graham & Brass, 1994). These may inhibit healing following dental or periodontal treatment. The significance of endotoxin in the pathogenesis of periodontitis is well documented (Trope *et al.*, 1995), and irrigation of highly vascular mucosal lesions with endotoxin-laden water during treatment for this condition is, at the very least, medically inappropriate. Irrigation of the site of any dental intervention with endotoxin-laden water has the potential for introduction of the endotoxin into the patient's bloodstream. Experimental injection of endotoxin into the systemic circulation of healthy volunteers elicits the signs and symptoms of endotoxaemia, including fever, elevated white blood cell count, elevated blood concentrations of stress hormones and decreased blood oxygenation (Suffredini *et al.*, 1989; van Deventer *et al.*, 1990; Herbert *et al.*, 1992). Endotoxaemia is typically associated with infection, but significant exposure via inhalation (Castellan *et al.*, 1987; Teeuw *et al.*, 1994) has been reported. Little is known about the significance of aspiration, ingestion, mucosal or dermal exposure.

Of potentially greater significance than the occasional endotoxin exposure of patients undergoing dental care is the continuous exposure of those involved in the dental and dental-hygiene professions. Chronic endotoxin inhalation represents an occupational hazard to these groups. In other settings, Gram-negative organisms in the biofilms of plumbing and climate-control ductwork contribute medically significant quantities of endotoxin to their surroundings (Costerton *et al.*, 1987; Hugenholtz & Fuerst, 1992; Szymanska, 2005b). Inhaled endotoxin significantly lowers spirometric values in otherwise healthy subjects (Castellan *et al.*, 1987; Herbert *et al.*, 1992). Breathing endotoxin-tainted air has serious implications for exacerbation of chronic obstructive pulmonary disease and asthma in individuals with pre-existing conditions (Michel *et al.*, 1996). Studies linking airborne endotoxin to sick building syndrome (Teeuw *et al.*, 1994) suggest that even uncompromised individuals are at risk for the development of medical conditions due to inhaled endotoxin. Inhaled endotoxin has been suggested as the source of occupationally acquired asthma in dentists (Pankhurst *et al.*, 2005). Whilst there is no specific concentration of airborne endotoxin above which is defined as hazardous, experimental animals show respiratory dysfunction at $0.3 \mu\text{g m}^{-3}$. The use of face masks to prevent exposure is marginal at best, with inconsistent

effects that are heavily dependent on aerosol droplet size and a host of other variables (Lipp, 2003).

In addition to the clinical implications of dental water endotoxin, there are medico-legal ramifications. On top of the potential liability incurred by exposing patients to endotoxin, there is risk from an employee health perspective. In the USA, the Indoor Clean Air Act Amendments of 1990 mandate that, within buildings, exposure to airborne pollutants cannot exceed levels of exposure in the local outside air: dental office airborne endotoxin exposure may cross that threshold. The European Commission similarly regulates indoor air quality in the workplace (Stevens & Palmigianob, 1995). A World Health Organization working group has issued statements delineating the right to healthy indoor air, statements that have been successfully cited in lawsuits (Molhave & Krzyzanowski, 2003). Blaming an exacerbation of chronic obstructive pulmonary disease or asthma on workplace exposures could lead to both civil and criminal penalties.

The endotoxin literature contains an interesting report on the inhibition of *Legionella* growth within endotoxin-treated macrophages (Egawa *et al.*, 1992). In this study, macrophages from a murine strain permissive for *Legionella* growth became highly resistant to growth of the organism when pre-treated with endotoxin. This enhanced cytolytic activity occurs at some point subsequent to the initial bacteria-macrophage interaction. It is tempting to speculate that the high levels of endotoxin delivered in dental water might exert some sort of protective effect, contributing to the infrequency of severe legionellosis in those exposed (Fotos *et al.*, 1985; Reinthaler *et al.*, 1988) to the high levels of *Legionella* present in dental water (Atlas *et al.*, 1995).

Efforts to control the dental-line biofilm, the source of endotoxin in the dental operatory, have been largely disappointing. Flushing, long advocated as a solution, has been shown to be of no benefit (Santiago *et al.*, 1994), and biofilms are resistant to chemical disinfection (Mills & Karpay, 2002). Filtration is effective at removing the planktonic bacteria from the dental water (Mayo & Brown, 1999), but has no effect on the core biofilm and its endotoxin production.

In conclusion, this study has demonstrated that dental unit water contains high concentrations of endotoxin, and that there is a statistically significant positive correlation between endotoxin and the bacterial load present. This water is readily aerosolized during routine dental work. Exposure to either the endotoxin-laden water or the aerosolized endotoxin represents a potential health threat. Further epidemiological and clinical studies of the consequences of dental endotoxin exposure, and the means by which this exposure may be prevented, are warranted.

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