PERGAMON

Archives of Oral Biology 47 (2002) 613–618

Archives of Oral Biology

www.elsevier.com/locate/archoralbio

Enhanced neutrophil emigration and *Porphyromonas gingivalis* reduction following PGG-glucan treatment of mice

Richard Niederman [a], Hans Kelderman [a,b], Sigmund Socransky [a,b], Gary Ostroff [c], Caroline Genco [d], Ralph Kent, Jr. [a,b], Philip Stashenko [a,b,*]

[a] *The Forsyth Institute, 140 Fenway, Boston, MA 02115, USA*
[b] *Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, MA 02115, USA*
[c] *Biopolymer Engineering Inc., 3388 Mike Collins Drive, Eagan, MN 55121, USA*
[d] *Boston University School of Medicine, 715 Albany St., Boston, MA 02118, USA*

Accepted 25 March 2002

Abstract

Periodontal disease is the consequence of a mixed Gram-negative infection in the gingival sulcus and has been associated with deficits in the neutrophil response. A novel, and heretofore untested, alternative approach to therapy is the use of biological-response modulators that enhance the neutrophil response. Poly-β1-6-glucotriosyl-β1-3-glucopyranose glucan (PGG-glucan) is an immunomodulator, derived from yeast, which specifically enhances neutrophil priming, phagocytosis and bacterial killing while failing to induce inflammatory cytokine expression. The hypothesis tested was that PGG-glucan could enhance host resistance to a Gram-negative periodontal pathogen, *Porphyromonas gingivalis*. Chambers were implanted subcutaneously in the dorsolumbar region of C57BL/6J mice and allowed to heal for 14 days. PGG-glucan was administered subcutaneously to one-half of the animals and saline to the other half. In the first set of experiments the chambers were inoculated with *P. gingivalis* (A7436) at 4×10^6, 4×10^7, and 4×10^8 colony-forming units (CFU). In the second set of experiments the chambers were inoculated with 5×10^8 CFU of either *P. gingivalis* or *Streptococcus sanguis*, a Gram-positive oral microbe that is not periodontopathic. Chambers were sampled over the following 2 weeks. The results demonstrated that: (1) bacterial CFU and neutrophils increased with increasing bacterial inoculum ($P < 0.02$); (2) bacterial CFU were lower in the PGG-glucan-treated animals than in the saline controls ($P < 0.02$); and (3) neutrophil counts were higher in the PGG-glucan-treated animals than in the saline controls ($P < 0.01$). These results indicate that PGG-glucan significantly enhances neutrophil emigration and bacterial killing, thus decreasing the bacterial infection in this model system.

Keywords: Neutrophils; Immunomodulation; Bacterial infection; *P. gingivalis*

1. Introduction

Oral infection with *Porphyromonas gingivalis* and *Bacteroides forsythus* has been associated with periodontal disease (Holt et al., 1988; Haffajee and Socransky, 1994; Socransky et al., 1998). Variations in bacterial virulence (Holt and Bramanti, 1991), the cellular host response (Schenkein and Van Dyke, 1994; Novak and Novak, 1996) and cytokine expression (Page and Kornman, 1997; Kornman et al., 1997) may account for variations in susceptibility to periodontal disease and lack of response to therapy. Treatment of these infections is mechanical, with or without antimicrobial therapy to reduce or eliminate the infection. The use of antimicrobials is, however, of concern in relation to accurate diagnosis, appropriate selection, microbial resistance and allergic reactions (Neu, 1992).

A potentially powerful, and novel, alternative approach to the problem of infection control is the use of biological-response modifiers that enhance the host phagocyte response without the expression of damaging inflammatory cytokines. A polysaccharide derived from yeast, PGG-glucan

Abbreviations: CFU, colony-forming units; PGG-glucan, poly-β1-6-glucotriosyl-β1-3-glucopyranose glucan

* Corresponding author. Fax: +1-617-456-7732.
E-mail address: pstashenko@forsyth.org (P. Stashenko).

0003-9969/02/$ – see front matter
PII: S0003-9969(02)00042-0

(Kokoshis et al., 1978; Williams et al., 1978; Jamas et al., 1990), binds to specific β-glucan receptors on the phagocyte membrane (Czop and Austen, 1985). Systemic administration of PGG-glucan increases the production of polymorphonuclear leucocytes and primes phagocytic and bactericidal activity in vivo (Patchen and MacVittie, 1986; Onderdonk et al., 1992; Babineau et al., 1993; Stashenko et al., 1995). PGG-glucan reduces Gram-positive and Gram-negative sepsis in rodent models by enhancing leucocyte number, and both bacterial phagocytosis and killing (Onderdonk et al., 1992). In human clinical trials, PGG-glucan reduced the number and severity of postsurgical infections (Babineau et al., 1993).

In a previous study, we found that PGG-glucan reduced periapical and pulpal necrosis by 50%, and increased leucocyte phagocytosis two-fold, when compared to control animals (Stashenko et al., 1995). We have now examined the effect of PGG-glucan on a local *P. gingivalis* infection, using a chamber implanted subcutaneously in the rat.

2. Materials and methods

2.1. Animals

Two sets of experiments were carried out on 8–12-week-old female C57BL/6J mice ($n = 30$ and 52, respectively) (Charles River Laboratories, Wilmington, MA). Animals were kept in the Forsyth Institute animal facility under specific pathogen-free conditions. Their general health, and the size and consistency of primary and/or secondary lesions, were examined daily from the beginning of the experiment. The experiments were repeated on four other sets of mice with similar results.

2.2. Chamber implantation

Coil-shaped subcutaneous chambers were prepared from 0.028 in., stainless steel round wire (GAC International Inc., Central Islip, NY) and sterilized by autoclaving (Genco et al., 1991). Following anaesthesia by intramuscular injection of ketamine HCl (62.5 mg/kg) and xylazine (12.5 mg/kg) in sterile phosphate-buffered saline, an area of dorsolumbar skin was shaved, wiped with alcohol, incised and the sterile chambers implanted subcutaneously. The incision was closed with 4/0 silk sutures (Davis and Geck Inc., Manati, PR). Over the following 2 weeks the external incision healed completely in all mice.

2.3. PGG-glucan administration

Animals were randomly divided into two groups. One group was injected subcutaneously on the contralateral side of the body from the chamber with 0.5 mg/kg PGG-glucan (Biopolymer Engineering Inc., Minneapolis, MN) 24 h before, and on the day of bacterial challenge. This dose was maximally effective in previous studies (Patchen and MacVittie, 1986; Jamas et al., 1990; Onderdonk et al., 1992). The other group served as controls and received injections of sterile saline in the same location.

2.4. Bacterial strains

The periodontopathogen *P. gingivalis* A7436 was originally isolated from a patient with refractory periodontitis. *Streptococcus sanguis*, a Gram-positive strain associated with oral health, was obtained from the Forsyth Institute collection. Both strains were grown on anaerobic blood–agar plates in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. Two days before inoculation the bacteria were placed in supplemented Schaedler broth (Difco Laboratories, Detroit, MI). Following suspension culture, the bacteria were concentrated by centrifugation at 2500 × *g* for 10 min at room temperature, resuspended in 10-fold dilutions of the original volume in prereduced Schaedler broth, and their concentration estimated at OD $A_{660} = 1.2$, corresponding to approximately 10^9 CFU/ml. The actual bacterial inoculum was confirmed by serial dilution and plating.

2.5. Infection

In the first experiment, animals ($n = 30$) were divided into three groups of 10 each to determine the dose-response to three inocula of *P. gingivalis* (4×10^6, 4×10^7, 4×10^8 CFU) in the presence of PGG-glucan or saline ($n = 5$/group each). In the second experiment, animals ($n = 42$) were divided into three groups of 14 each to determine the response to two bacteria, *S. sanguis* (5×10^8 CFU) and *P. gingivalis* (5×10^8 CFU), and to Schaedler broth. Each group of 14 was also subdivided into two groups of seven each to compare the effects of PGG-glucan with saline.

2.6. Chamber fluid sampling

Fluid was aseptically aspirated from implanted chambers with a 25G hypodermic needle at days −1, 1, 4, 7 and 14 relative to bacterial infection. A 10 μl sample was obtained from each chamber and diluted 1:50 in Hank's balanced salt solution. Samples were used for bacteriological culture, total white blood cell and differential counts.

2.7. Microbial analysis

A portion (100 μl) of the diluted chamber fluid was streaked on to anaerobic blood–agar plates (Remel, Lenexa KA) and cultured in an anaerobic chamber. The CFU/ml was determined by direct colony counting.

2.8. White blood-cell analysis

A portion (200 μl) of the diluted sample was centrifuged in a Cytospin apparatus (Shandon Inc., Pittsburgh, PA)

for 3 min at 850 rpm/min. Following cytocentrifugation, slides were stained with Wright–Giemsa for differential cell counts, which were done by light microscopy at 40× magnification. All leucocytes in three random fields were counted using an ocular micrometer and the number of granulocytes determined.

2.9. Statistical analysis

Kruskal–Wallis ANOVA was used to compare responses to bacterial CFU and leucocyte emigration at day 1. Repeated-measures ANOVA was used to compare the effects of PGG-glucan and saline over the entire time course.

3. Results

3.1. Effect of PGG-glucan on bacterial infection

The initial experiments examined the ability of PGG-glucan-treated mice to lessen *P. gingivalis* infections from subcutaneously implanted stainless steel chambers. As shown in Fig. 1A–C, at day 1 the peak number of recoverable viable bacteria increased with increasing bacterial inoculum for all animals ($P = 0.02$ for PGG-glucan, $P = 0.004$ for saline; both Kruskal–Wallis ANOVA) and decreased thereafter. Injection with PGG-glucan also effected a reduction in the chamber bacterial CFU when compared to saline.

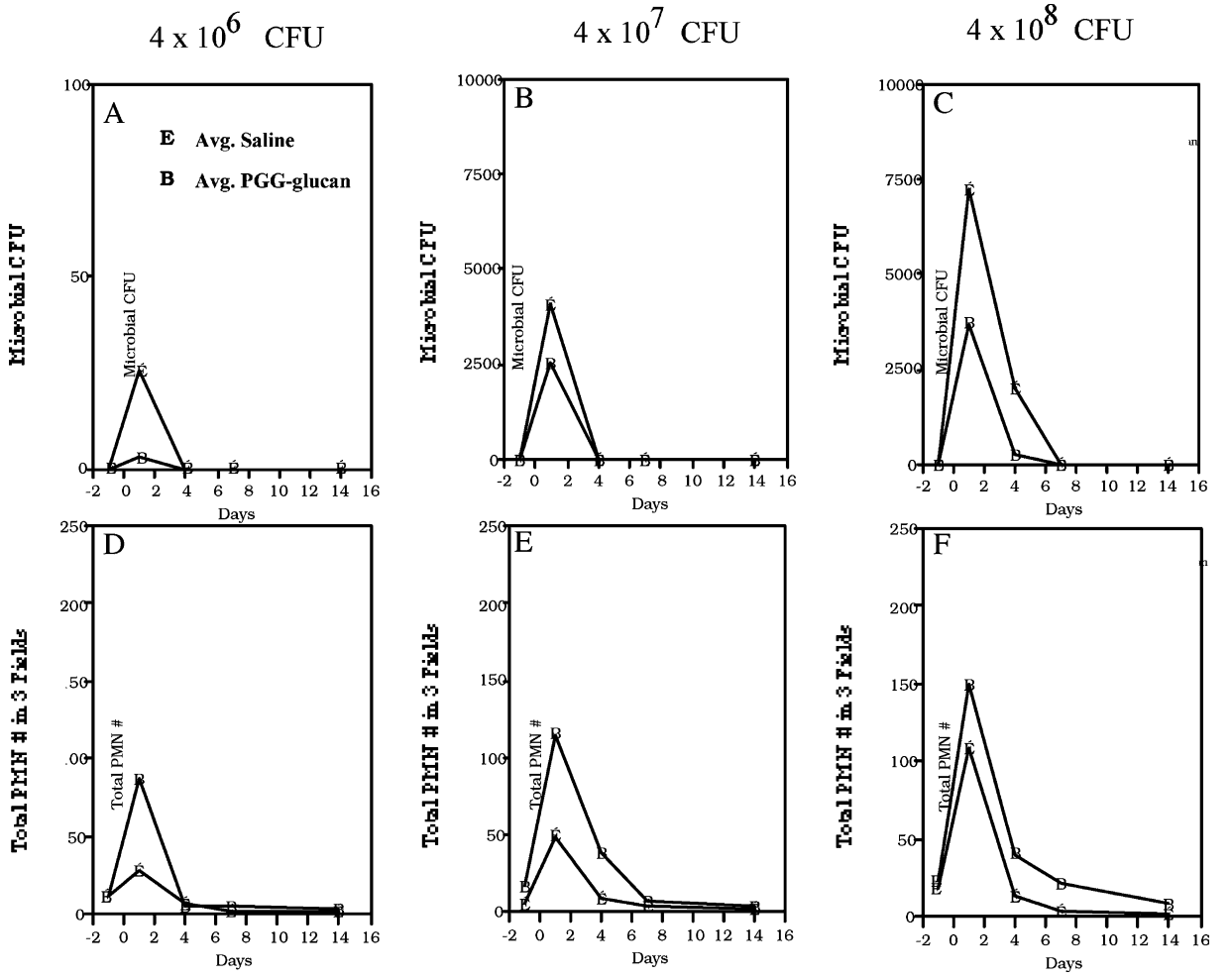

Fig. 1. Representative time courses and dose responses of PGG-glucan effects following chamber infection. Each data-point represents $n = 5$. Error bars omitted to increase visual clarity. (A–C) Clearance of bacteria from subcutaneous chambers following infection with *P. gingivalis*. Infection with (A) 4×10^6, (B) 4×10^7, and (C) 4×10^8 CFU. B: PGG-glucan; E: saline control, administered on contralateral side. Significant reduction in the 4×10^8 CFU-infected, PGG-glucan-treated group compared to saline ($P = 0.018$, repeated-measures ANOVA). (D–F) Polymorphonuclear leucocyte (PMN) emigration into subcutaneous chambers infected with *P. gingivalis*. Graphics are from the same animals presented in (A), (B) and (C), respectively. Significant PMN elevation in the 4×10^8 CFU-infected, PGG-glucan-treated group when compared to the saline control ($P = 0.007$, repeated-measures ANOVA).

Following infection with 4×10^8 CFU there was a significant reduction in bacteria in the PGG-glucan-treated group when compared to the saline-treated group ($P = 0.018$; repeated-measures ANOVA). Although there was a clear trend toward a reduction in PGG-glucan-treated animals inoculated with 4×10^6 and 4×10^7 CFU, the differences were not statistically significant. Similar reductions following PGG-glucan administration were seen in four additional experiments (data not shown).

3.2. Effect of PGG-glucan on leucocyte emigration

The ability of PGG-glucan to modulate the number of polymorphonuclear leucocytes emigrating into the chambers following infection with *P. gingivalis* was then examined. As shown in Fig. 1(D–F), the number of leucocytes increased with increasing bacterial infection in all animals ($P = 0.02$ for PGG-glucan, $P = 0.008$ for saline; both Kruskal–Wallis ANOVA). Emigration peaked at day 1 and declined thereafter. The number of leucocytes was higher and remained elevated longer in the animals treated with PGG-glucan than in those treated with saline. These differences were only statistically significant in the group inoculated with 4×10^8 CFU ($P = 0.007$, repeated-measures ANOVA). Similar qualitative, but not identical quantitative, results were obtained in four additional experiments (data not shown).

3.3. PGG-glucan effects following infection with a pathogenic or non-pathogenic oral species

In the second series of experiments, PGG-glucan- and saline-treated mice were infected with *P. gingivalis* or

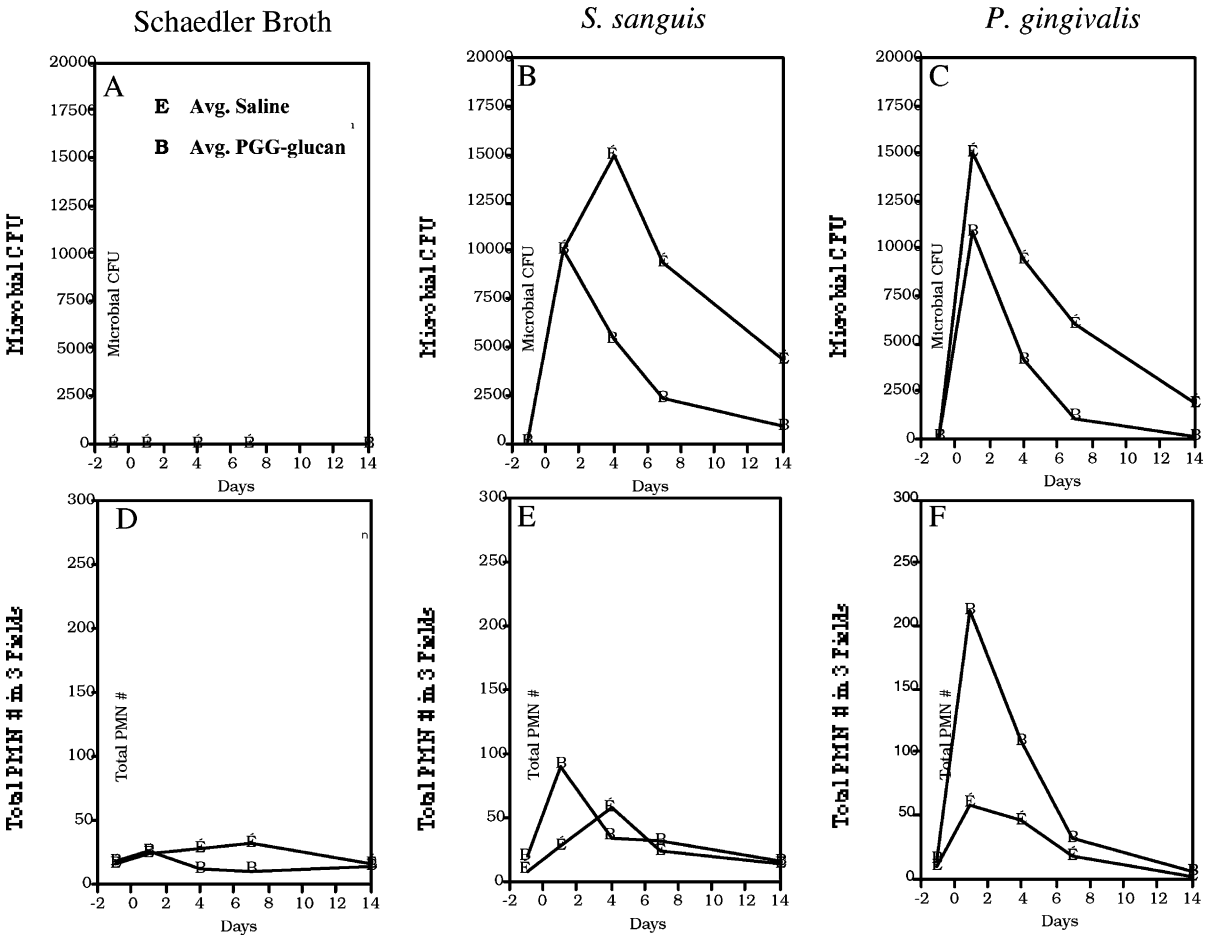

Fig. 2. Representative time courses of PGG-glucan effects following chamber infection. Each data-point represents $n = 5$. Error bars omitted to increase visual clarity. E: PGG-glucan; B: saline; both injected at days 1 and 0 at a site contralateral to the chamber. (A–C) Effect of PGG-glucan on clearance of bacteria from subcutaneous chambers following chamber infection with (A) Schaedler broth, (B) 5×10^8 *S. sanguis* (*Ss*), or (C) 5×10^8 *P. gingivalis*(*Pg*) at day 0. E: saline; B: PGG-glucan, injected into the contralateral side on day 1 and day 0. Each data-point represents $n = 7$. (E–G) Modulation of polymorphonuclear leucocyte (PMN) emigration into subcutaneous chambers following infection with (D) Schaedler broth, (E) *S. sanguis* (*Ss*) or (F) *P. gingivalis*. The graphics in (D–F) are from the same animals presented in (A–C), respectively.

with *S. sanguis*, a non-periodontopathic species. As shown in Fig. 2(A–C), the bacterial CFU for both *P. gingivalis*- and *S. sanguis*-infected, PGG-glucan-treated animals were significantly lower than for saline-treated control animals (both $P = 0.002$; repeated-measures ANOVA). PGG-glucan-treated animals were able to clear completely the *P. gingivalis* infection whereas the infection persisted in the saline-treated animals. The animals injected with Schaedler broth as a sterility control exhibited no bacterial growth in either the PGG-glucan- or saline-treated groups.

Fig. 2D–F shows how PGG-glucan affected the emigration of polymorphonuclear leucocytes. In the presence of PGG-glucan the leucocyte count increased significantly, compared to baseline, following infection with both *P. gingivalis* and *S. sanguis* (both $P < 0.001$; Kruskal–Wallis ANOVA). Counts were also significantly higher than in the saline-treated controls for *P. gingivalis* ($P = 0.001$; repeated-measures ANOVA) but not for *S. sanguis*. The leucocyte count in the PGG-glucan-treated, *P. gingivalis*-infected animals was significantly higher than in the PGG-glucan-treated, *S. sanguis*-infected animals ($P = 0.001$; repeated-measures ANOVA). Notably, the count did not increase after the chamber was injected with sterile Schaedler broth, indicating that the leucocyte immigration was not randomly stimulated by PGG-glucan in the absence of infection. Taken together, there results suggest that PGG-glucan increases the responses of polymorphonuclear leucocytes to both pathogenic and non-pathogenic species, and that the magnitude of this response is a function of the infecting microorganism.

4. Discussion

Our results support the hypothesis that an immunomodulator, PGG-glucan, can increase host resistance to pathogenic bacterial infections. This protective response appeared to be mediated by polymorphonuclear leucocytes, as an inverse relation between the number of infecting bacteria and the number of polymorphonuclear leucocytes was consistently observed (Fig. 2C and F). The response indicates that in addition to the previously noted effects of PGG-glucan on leucocyte production, priming, bacterial phagocytosis and killing (Onderdonk et al., 1992; Babineau et al., 1993; Stashenko et al., 1995), PGG-glucan also enhances leucocyte emigration to sites of infection.

Aggressive, early-onset or refractory periodontal disease has been consistently associated with a decrease in either the number or function of polymorphonuclear leucocytes (Schenkein and Van Dyke, 1994). Our findings with PGG-glucan, which enhances both leucocyte number and functional activity (Stashenko et al., 1995), demonstrate that this immunomodulator can increase resistance to a specific periodontal pathogen. The molecular mechanism(s) that underlie these effects are not well understood but are under investigation.

An interesting observation was that the PGG-glucan-stimulated leucocyte response, rather than being generically enhanced, appears to be specifically regulated by the host only to the extent necessary to combat the infection successfully. For example, following PGG-glucan administration, there was no leucocyte emigration into the sterile Schaedler broth-injected chambers (Fig. 2D), a modest but significant emigration into the *S. sanguis*-infected chambers (Fig. 2E) and a vigorous emigration into the *P. gingivalis*-infected chambers (Fig. 2F). Thus, PGG-glucan does not appear to stimulate random leucocyte emigration throughout the body, but rather enhances a response that is regulated by the host. Of interest, the magnitude of the leucocyte response might be dependent on the potential pathogenicity of the infection and be modulated to the extent necessary to bring the infections under similar control.

When comparing the first and second series of experiments (e.g. Fig. 1C and Fig. 2C), we found that in the first series the total recoverable bacterial CFU was lower, and the bacteria persisted in the chambers for a shorter time, than in the second series. This interexperiment variation was observed in four other subsequent replicates. The relative relation between saline and PGG-glucan animals was, however, always very similar, i.e. PGG-glucan-treated animals always had significantly lower bacterial CFU and higher leucocyte counts than the saline-treated animals, indicating the reproducibility of the effect.

Taken together, these data point to the possibility that PGG-glucan may provide an entirely new therapeutic approach to preventing and treating bacterial infections, including those of the periodontium. This host-oriented approach may be especially timely, given the increasing use of systemic antibiotics to treat infections and the concern about the rise in bacterial resistance to these agents (Neu, 1992). In the oral environment, this approach could prove particularly beneficial for patients who are susceptible to periodontal disease due to a number of predisposing factors. These include a reduced phagocyte response (Schenkein and Van Dyke, 1994; Novak and Novak, 1996), increased cytokine response (Stashenko et al., 1991; Page and Kornman, 1997; Kornman et al., 1997), the persistence of bacterial pathogens (Haffajee and Socransky, 1994; Socransky et al., 1998), the presence of antibiotic-resistant bacteria (Slots and Rams, 1990), autoimmune disorders that affect the host response, environmental factors such as smoking, patients who require premedication for dental procedures, or those who are at risk of systemic or local infections after undergoing oral surgery or chemotherapy.

Acknowledgements

This work was supported by Grants DE-11664 and DE-13747 from the N.I.D.C.R. We thank Tamoki Uematsu for technical assistance and Megan Mack for assistance with the manuscript.

References

Babineau, T., Marcello, P., Swails, W., Kenler, A., Wakshull, E., Oldman, F., 1993. Randomized phase I/II trial of a macrophage-specific immunomodulator (PGG-glucan) in high-risk surgical patients. Surg. Forum 44, 51–53.

Czop, J.K., Austen, K.F., 1985. Properties of glycans that activate the human alternative complement pathway and interact with the human monocyte β-glucan receptor. J. Immunol. 135, 3388–3393.

Genco, C.A., Cutler, C.W., Kapczynski, D., Maloney, K., Arnold, R.R., 1991. A novel mouse model to study the virulence of and host response to *Porphyromonas* (Bacteroides) *gingivalis*. Infect. Immun. 59, 1255–1263.

Haffajee, A.D., Socransky, S.S., 1994. Microbial etiological agents of destructive periodontal diseases. Periodontology 5, 78–111.

Holt, S.C., Bramanti, T.E., 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit. Rev. Oral Biol. Med. 2, 177–281.

Holt, S.C., Ebersole, J., Felton, J., Brunsvold, M., Kornman, K.S., 1988. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. Science 239, 55–57.

Jamas, S., Easson, D.D.J., Ostroff, G.R., 1990. PGG—a novel class of macrophage activating immunomodulators. In: Proceedings of the International Congress on Infectious Diseases. Medizin Verlag, New York, p. 252.

Kokoshis, P.L., Williams, D.L., Cook, J.A., DiLuxio, N.R., 1978. Increased resistance to *Staphylococcus aureus* infection and enhancement in serum lysozyme activity by glucan. Science 199, 1 340–342.

Kornman, K.S., Page, R.C., Tonetti, M.S., 1997. The host response to the microbial challenge in periodontitis: assembling the players. Periodontology 2000 14, 33–53.

Neu, H.C., 1992. The crisis in antibiotic resistance. Science 257, 1064–1073.

Novak, M.J., Novak, K.F., 1996. Early-onset periodontitis. Curr. Opin. Periodontology 3, 45–58.

Onderdonk, A.B., Cisneros, R.L., Hinkson, P., Ostroff, G., 1992. Anti-infective effect of poly-β1-6-glucotriosyl β1-3-glucopyranose glucan in vivo. Infect. Immun. 60, 1642–1647.

Page, R.C., Kornman, K.S., 1997. The pathogenesis of human periodontitis: an introduction. Periodontology 2000 14, 9–11.

Patchen, M.L., MacVittie, T.J., 1986. Hemopoietic effects of intravenous soluble glucan administration. J. Immunopharmacol. 8, 407–425.

Schenkein, H.A., Van Dyke, T.E., 1994. Early-onset periodontitis: systemic aspects of etiology and pathogenesis. Periodontology 6, 7–25.

Slots, J., Rams, T.E., 1990. Antibiotics in periodontal therapy: advantages and disadvantages. J. Clin. Periodontol. 17, 479–493.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C., Kent Jr., R.L., 1998. Microbial complexes in subgingival plaque. J. Clin. Periodontology 25, 134–144.

Stashenko, P., Jandinski, J.J., Fujiyoshi, P., Rynar, J., Socransky, S.S., 1991. Tissue levels of bone resorptive cytokines in periodontal disease. J. Periodontology 62, 504–509.

Stashenko, P., Wang, C.Y., Riley, E., Wu, Y., Ostroff, G., Niederman, R., 1995. Reduction of infection- stimulated periapical bone resorption by the biological response modifier PGG-glucan. J. Dent. Res. 74, 323–330.

Williams, D.L., Cook, J.A., Hoffmann, E.O., DiLuzio, N.R., 1978. Protective effect of glucan in experimentally induced candidiasis. J. Reticuloendothel. Soc. 23, 479–490.