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Effect of *Porphyromonas gingivalis*-induced inflammation on the development of rheumatoid arthritis

P. Mark Bartold[1,2], Victor Marino[1], Melissa Cantley[3] and David R. Haynes[3]

[1]School of Dentistry, Faculty of Health Sciences; [2]Colgate Australian Clinical Dental Research Centre; [3]Discipline of Pathology, School of Medical Sciences, University of Adelaide, Adelaide, Australia

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Abstract

Background: Periodontitis is an extra-synovial chronic inflammatory condition, which has been proposed to be inter-related with rheumatoid arthritis.

Objective: We investigated the effect of an established extra-synovial chronic inflammatory lesion on the induction and severity of experimental arthritis.

Materials and Methods: Chronic inflammatory lesions were induced by the implantation of polyurethane sponges impregnated with heat-killed *Porphyromonas gingivalis* into the backs of DA rats. Thirty-five days later, adjuvant arthritis (AA) was induced in the rats by injecting a mycobacterium cell wall in complete Freund's adjuvant. The development of arthritis was then monitored for 2 weeks.

Results: Histological assessment of the implanted sponges confirmed that a chronic inflammatory lesion had been established after 21 days. Following induction of adjuvant arthritis, the severity of disease was scored and paw swelling was measured. Severe arthritis developed more rapidly in animals with a pre-existing *P. gingivalis*-induced inflammatory lesion elsewhere.

Conclusions: The results show that a pre-existing extra-synovial chronic inflammatory lesion induced by *P. gingivalis* promotes the development of arthritis in an animal model. These findings provide further evidence for a relationship between the presence of periodontal pathogen-associated inflammation and the development of rheumatoid arthritis.

Key words: chronic inflammation; extra-synovial; periodontitis; rheumatoid arthritis

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Close inspection of two of the most common chronic inflammatory diseases afflicting humans, periodontitis and rheumatoid arthritis, reveals remarkable similarities that warrant further investigation (Bartold et al. 2005, de Pablo et al. 2009). Indeed, a number of studies have been published reporting a significant association between these two diseases. For example, simple analyses of self-reported illnesses have indicated the likely inter-relationship between periodontitis and rheumatoid arthritis (Mercado et al. 2000, Lagervall et al. 2003, Georgiou et al. 2004). A number of case/control studies have also reported a significantly higher incidence of tooth loss and alveolar bone loss in patients with rheumatoid arthritis (Malmström & Calonius 1975, Albander 1990, Käßer et al. 1997, Mercado et al. 2000, 2001, Al-Shammri et al. 2005, Bozkurt et al. 2006). In addition, other studies have shown that rheumatoid arthritis and periodontitis share very similar pathological processes of chronic inflammation and associated tissue destruction (Bozkurt et al. 2000, 2006, Moen et al. 2003, Havemose-Poulsen et al. 2005, Ogrendik et al. 2005, Marotte et al. 2006). Of particular interest have been several studies reporting that periodontitis is a risk factor for arthritis development or it enhances the severity of rheumatoid arthritis (Ribeiro et al. 2005, Havemose-Poulsen et al. 2006). Furthermore, reports are emerging to suggest that reduction of extra-synovial chronic inflammation

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associated with periodontal treatment may have a beneficial effect on established rheumatoid arthritis (Ribeiro et al. 2005, Al-Katma et al. 2007, Ortiz et al. 2009).

One plausible explanation for the relationship between periodontitis and rheumatoid arthritis may be through a primed inflammatory response known as the ''two-hit'' model (Golub et al. 2006). This model suggests that a primary ''hit'' of chronic inflammation (e.g. chronic periodontitis or other extra-synovial chronic inflammation), followed by an arthritogenic hit to induce rheumatoid arthritis, could lead to an exacerbated response. It is also possible that the converse could occur and an initial hit of chronic inflammatory disease exacerbates the inflammatory response of developing periodontitis. If one were to take into account the total ulcerated periodontal tissue in an individual (28 teeth and periodontal pockets of around 5–6 mm), the total area of ulcerated tissue is about 75 cm^2 (Page 1998). Such a large amount of inflamed tissue is likely to have systemic effects and influence inflammatory reactions elsewhere in the body.

This study aimed to investigate whether an extra-synovial source of chronic inflammation, such as can be seen in chronic inflammatory periodontitis or other chronic inflammatory conditions, can influence the onset and severity of rheumatoid arthritis. To do this, we used a simple model of *Porphyromonas gingivalis*-induced peripheral inflammation and experimental arthritis in rats. Similar models have been used to study the exacerbation of experimental autoimmune encephalitis (Shapira et al. 2002) and T-cell function (Bronstein-Sitton et al. 2003). Therefore, the aim of this study was to investigate whether the onset and severity of experimental arthritis in a rodent model can be influenced by the presence of a pre-existing extra-synovial chronic inflammatory reaction to *P. gingivalis*.

Materials and Methods

Animal experimentation

This study was approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia. All experiments were carried out according to the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

Experimental groups

Healthy adult female DA rats in the weight range 140–150 g were acquired through the IMVS Animal Services Division. For this study, there were six experimental groups, each with six animals. Group 1: placement of sterile sponges soaked in sterile phosphate-buffered saline (PBS) for the duration of the experimental period; Group 2: placement of sponges impregnated with heat-killed *P. gingivalis* for the duration of the experimental period; Group 3a: placement of sterile sponges soaked in sterile PBS for 35 days, followed by induction of adjuvant arthritis (AA) (1/3 dose of the arthritogenic agent) and assessment over days 1–14 after arthritis induction; Group 3b: placement of sterile sponges soaked in sterile PBS for 35 days, followed by induction of adjuvant arthritis (1/5 dose of the arthritogenic agent) and assessment over days 1–14 after arthritis induction; Group 4a: placement of sponges impregnated with heat-killed *P. gingivalis* for 35 days, followed by induction of adjuvant arthritis (1/3 dose of the arthritogenic agent) and then assessment over days 1–14 after arthritis induction; Group 4b: placement of sponges impregnated with heat-killed *P. gingivalis* for 35 days, followed by induction of adjuvant arthritis (1/5 dose of the arthritogenic agent) and then assessment over days 1–14 after arthritis induction.

Preparation of heat-killed *P. gingivalis*

Cultures of *P. gingivalis* W50 (W83) were maintained on anaerobic blood agar (Oxoid, Adelaide, Australia) plates at 37°C in an atmosphere of N_2:CO_2:H_2 (90:5:5). Bacterial cell suspensions were prepared using sterile PBS (pH 7.2) in a volume of 2 ml directly from the plate. Suspended bacteria were then placed in a 10 ml sterile centrifuge tube. Cell density was estimated to be $>10^{11}$ organisms/ml [optical density (560 nm) >5.0]. The heat-killed *P. gingivalis* was prepared by incubating the suspension at 60°C for 5 min. The loss of *P. gingivalis* viability was confirmed by plating out an aliquot of the suspension following incubation.

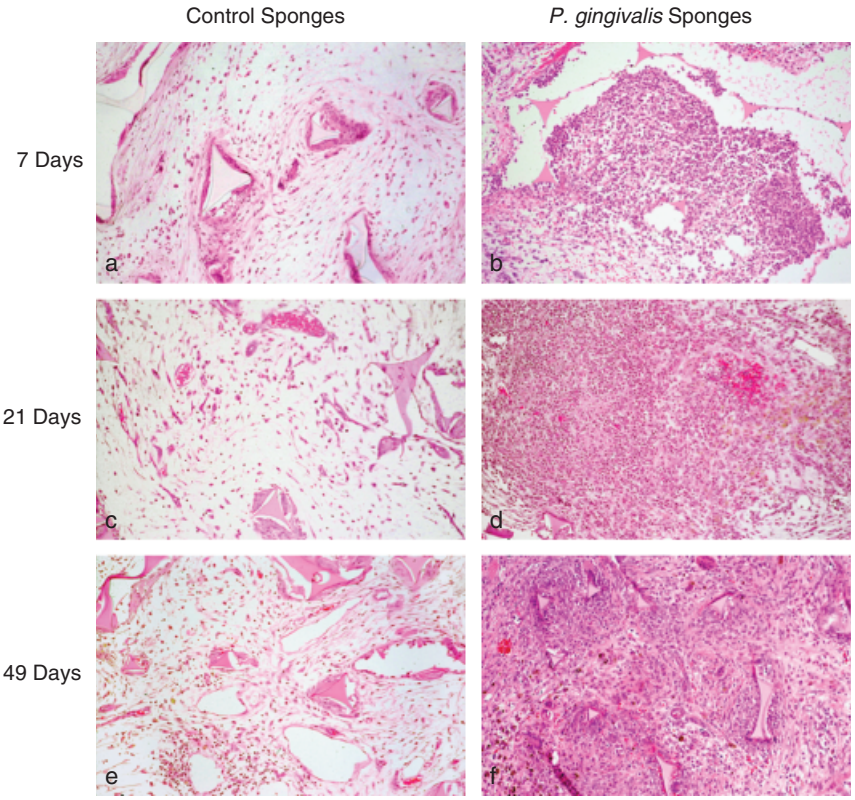

Fig. 1. Microscopic appearance of control sponges (a, c, e) and sponges impregnated with heat-killed *Porphyromonas gingivalis* (b, d, f) removed after 7 days (a, b), 21 days, (c, d) and 49 days (e, f). Magnification × 40.

Sponge implantation surgery

Sterilized dry polyurethane foam pieces ($7 \times 15 \times 15$ mm) were loaded with either sterile PBS (0.5 ml) or with a heat-killed *P. gingivalis* suspension (0.5 ml) approximately 24 h before implantation surgery. During this time, the sponges were kept separate in 12-well tissue culture plates and exposed to ultraviolet radiation 2 h before implantation.

The surgical implant procedure was performed on the rats following inhalation anaesthesia induced with 2% v/v isofluorane, with O_2 flow rates set at 2 l/min. A subcutaneous incision measuring approximately 20 mm was made along the ventral midline between the left and the right shoulders. Subcutaneous pouches below the right or the left shoulders were created for the placement of the sponges. The incision was closed using staples and swabbed with betadine. Post-operatively, the rats were administered 22.7 mg/ml enrofloxacin (Baytril®, Bayer AG, Leverkusen, Germany) orally for 1 week. The animals were monitored for a period of 35 days with clinical record sheets and their weights were recorded weekly until the induction of the adjuvant arthritis.

As determined from pilot studies, the sponges were left in situ 35 days to allow development of a chronic inflammatory lesion before induction of adjuvant arthritis.

At the completion of the study period, the sponges were removed, fixed in 10% neutral-buffered formalin solution and processed for routine histological assessment.

Sponges from animals in each test group were examined under light microscopy and scored semi-quantitatively for the number and type of inflammatory cells, the number of vessels and amount of inflammation. For these histological features, a numerical scale beginning at 0 for the lowest and 4 for the highest was used.

Adjuvant Arthritis induction

The animals were injected with 50 μl of the arthritis adjuvant near the tail base. The adjuvant was prepared from finely ground heat-killed delipidated human pathogenic strain *Mycobacterium tuberculosis* (Tuberculin Section, Ministry of Agriculture, Fisheries and Food, Weybridge, UK) dispersed in squalane (Fluka, Sigma-Aldrich, Castle Hill, NSW, Australia) at a stock concentration of 10 mg/ml. To moderate the level of adjuvant arthritis induced in the rats, different dosages (1/3 and 1/5) were made up from the stock preparation. The injected volume of adjuvant for each dose was the same (50 μl). This model of arthritis induction has been used routinely in our laboratory for the past 20 years (Haynes et al. 1988). Following the injection, the animals were assessed for development of arthritis over a period of 14 days. The parameters monitored were weight, front and rear paw inflammation and rear paw footpad thickness.

Arthritis assessment

A scale of 0–4 was used to assess the level of inflammation observed in the paws: (no sign of disease 0; erythema 1; erythema and malleolar induration 2; metatarsal and/or metatacarpal induration 3; and marked erythema and induration of the total paw including digits 4). The animals were scored independently by two observers with experience with the rat adjuvant arthritis model. The rear paw footpad thickness was measured using an electronic digital calliper (Sidchrome, Stanley Works Pty. Ltd., Somerton, Vic., Australia).

Statistical analysis

In order to compare the rear paw scores and rear paw thickness between the four treatment groups (HKPG+1/3 AA, HKPG+1/5 AA, PBS+1/3 AA, PBS+1/5 AA), a linear mixed-effects model was fitted to the data and the effects at the different time points were assessed using post hoc unpaired student *t*-tests, with significance levels at $p < 0.05$. All calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA).

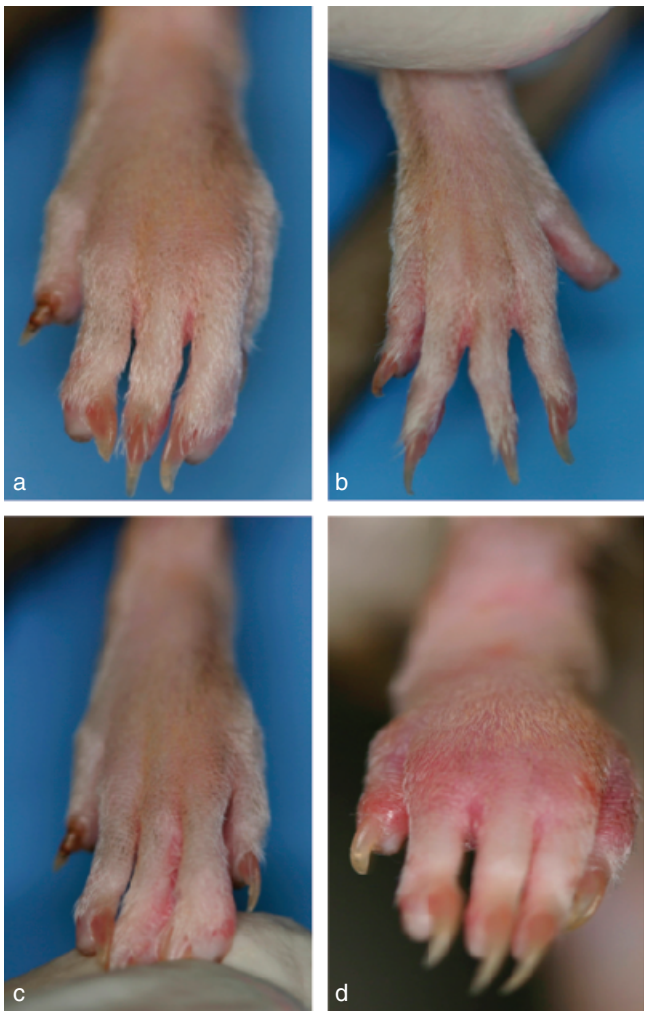

Fig. 2. Appearance of rear paws in (a) control phosphate-buffered saline sponge and no adjuvant arthritis, (b) control heat-killed *Porphyromonas gingivalis*-impregnated sponges and no adjuvant arthritis, (c) saline-impregnated sponges and adjuvant arthritis and (d) heat-killed *P. gingivalis*-impregnated sponges and adjuvant arthritis animals.

Results

The histological appearance of the tissue reaction within the *P. gingivalis*-impregnated sponges removed at various times is shown in Fig. 1. On day 7, dense aggregates of neutrophils and sparse fibrous tissue were noted. On day 21, a developing chronic inflammatory infiltrate consisting of lymphocytes and macrophages was observed. On day 49, a continuing presence of a chronic inflammatory response was observed. This chronic inflammatory lesion then persisted over the course of the experimental period. Throughout the course of the experiment, the control sponges showed very little inflammatory infiltrate and were slowly filled with fibrous tissue (Fig. 1a, c, e).

Figure 2 shows the macroscopic appearance of the rear paws of control animals (Groups 1 and 2) and those exposed to either PBS-impregnated sponges and adjuvant arthritis (Group 3) or heat-killed *P. gingivalis*-impregnated sponges and adjuvant arthritis animals (Group 4). The degree of inflammation was easily visualized, with the paws of the heat-killed *P. gingivalis*-impregnated sponges and adjuvant arthritis animals showing the greatest amount of paw swelling.

Weight gain/loss was monitored throughout the study and is shown in Fig. 3. During the induction of chronic inflammation with the implantation of sponges containing heat-killed *P. gingivalis*, there was no discernable difference in weight gain between the treated and the test groups (Fig. 3a). However, it was noted that animals induced with a higher dose of arthritis adjuvant showed a higher percentage weight loss compared with the lower dose groups (Fig. 3b). There was no statistical difference in the weight loss recorded between the PBS and the heat-killed *P. gingivalis* sponge-loaded animals at the two administered doses of arthritis adjuvant ($p > 0.05$).

A visual assessment of the rear paw appearance (Fig. 4) and a direct measurement of rear paw thickness (Fig. 5) were used to assess the amount of inflammation and arthritis development of the rear paws. Animals in Groups 1 and 2 showed no evidence of arthritis development for the duration of the entire experimental period (data not shown). Animals induced with the lower dose (1/5 dose) of arthritis adjuvant and loaded with the heat-killed *P. gingivalis*

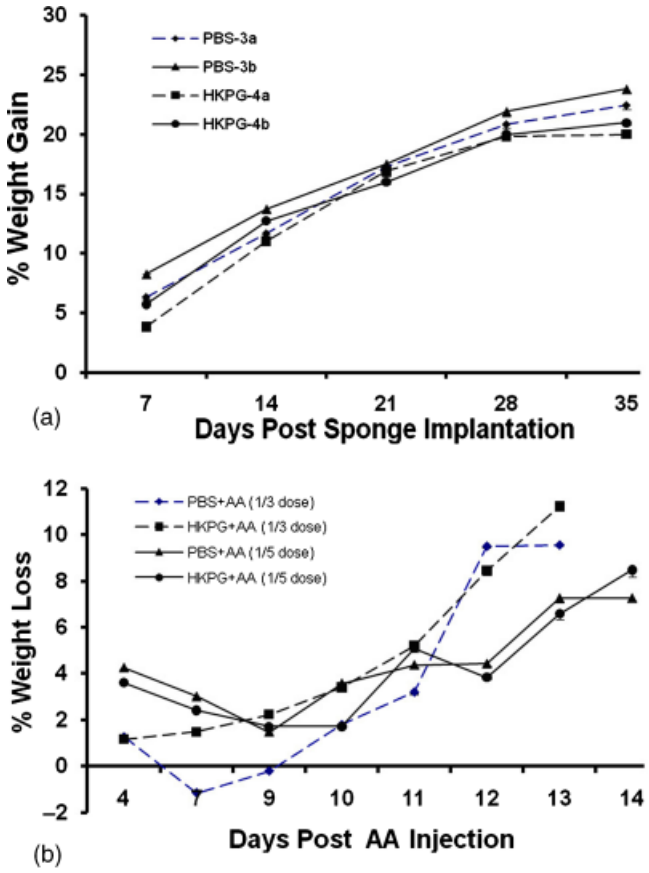

Fig. 3. (a) Monitoring of the percentage weight change in rats for 35 days following the implantation of sponges containing either PBS or heat-killed *Porphyromonas gingivalis* before induction of arthritis. Data represents the mean ± SEM, ($N = 6$) for each group at the different time points. Abbreviations: PBS, phosphate-buffered saline; AA, adjuvant arthritis; HKPG, heat-killed *P. gingivalis*. (b) Monitoring of the percentage weight change in rats after the initial sponge implantation (a) for 14 days following the arthritis adjuvant injection. Data represent the mean ± SEM, ($N = 6$) for each group at the different time points. Abbreviations: PBS, phosphate-buffered saline; AA, adjuvant arthritis; HKPG, heat-killed *P. gingivalis*.

sponges developed arthritis at the same rate as those animals that received the higher dose (1/3 dose). The rate of arthritis development in the PBS sponge-loaded animals with the lower dose (1/5 dose) of arthritis adjuvant was slower and less severe. For the higher dose of arthritis adjuvant (1/3 dose), there was no difference in the paw scores or paw thickness between animals loaded with the heat-killed *P. gingivalis* sponges and PBS controls. However, at the lower dose of adjuvant (1/5 dose), statistically significant differences in paw scores and paw thickness were noted between the animals pre-loaded with the heat-killed *P. gingivalis* sponges and the PBS controls at days 11, 13 and 14 ($p < 0.05$).

Discussion

In recent years, there has been renewed interest in the relationship between rheumatoid arthritis and periodontitis (Bartold et al. 2005). This interest stems mainly from the remarkable similarities in the pathology between these two common chronic inflammatory conditions. For example, both conditions are characterized by an exuberant inflammatory reaction, regulated by an infiltration of immune cells, enzymes and cytokines, characteristic of chronic inflammation, which results in both soft and hard tissue destruction. These similarities have led to proposals that both conditions may commonly co-exist in individuals due to similar dysregulated inflammatory responses (Bartold et al. 2005).

Increasing evidence suggests that chronic inflammation, such as that seen in chronic periodontitis, imparts a significant systemic inflammatory burden, which may affect other systemic conditions. For example, several animal studies have demonstrated that induction of periodontal inflammation exacerbates

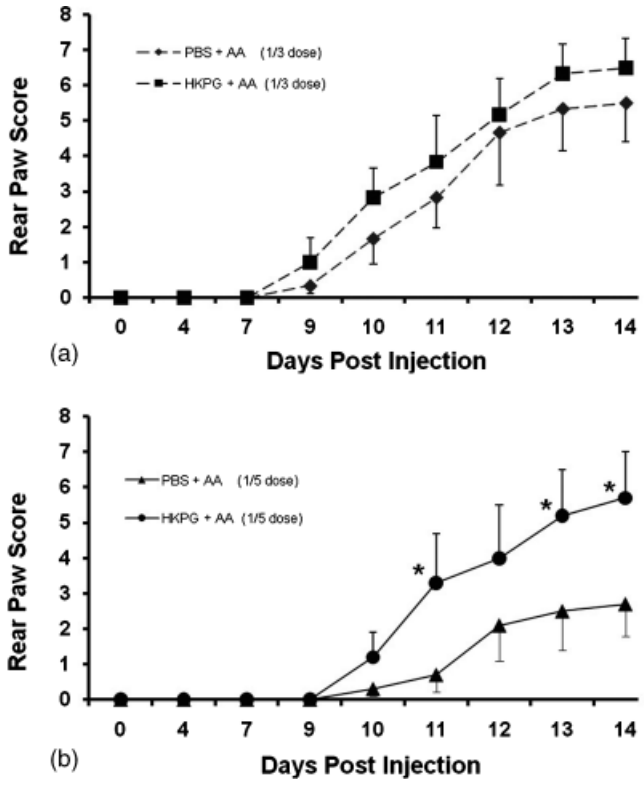

Fig. 4. Rear paw scores for control and heat-killed *Porphyromonas Gingivalis*-treated animals exposed to (a) high-dose (1/3 dose) and (b) low-dose (1/5 dose) arthritis adjuvant. Data represent the mean ± SEM, ($N = 6$) for each group at the different time points. Points marked with *indicate statistical difference at $p < 0.05$ at that time point.

the development of atherosclerotic lesions (Jain et al. 2003, Lalla et al. 2003, Serhan et al. 2003, Gibson et al. 2004, Ekuni et al. 2009). Because of the accruing evidence indicating that chronic periodontal inflammation and rheumatoid arthritis are significantly inter-related diseases, the present study aimed to determine whether an extra-synovial source of chronic infection impacts on the development of rheumatoid arthritis using an animal model. This was designed to be a ''proof-of-concept'' study using a simple model of chronic inflammation induced by heat-killed *P. gingivalis* rather than more complicated models of periodontitis that require infection with live bacteria. In this study, we have been able to show that extra-synovial chronic inflammation has a significant effect on the development of arthritis. This model is similar in concept to other studies that have investigated the effects of *P. gingivalis*-induced chronic inflammation on the exacerbation of experimental autoimmune encephalitis (Shapira et al. 2002) and T-cell function (Bronstein-Sitton et al. 2003).

In this model, heat-killed *P. gingivalis* was used as the ''priming'' inflammatory agent as a surrogate for periodontal inflammation induced by live bacteria. This model demonstrated that the presence of extra-synovial chronic inflammatory lesions, induced by heat-killed *P. gingivalis*, promoted the induction and severity of experimental arthritis.

Localized chronic inflammatory lesions, such as the one established in this study by heat-killed *P. gingivalis*, have the potential to result in the systemic dissemination of inflammatory cytokines and mediators, leading to an elevated systemic inflammatory condition (Bronstein-Sitton et al. 2003). Accordingly, it is conceivable that such a response has the potential to contribute to systemic inflammation and affect organs distant to the original site of inflammation. Indeed, such reactions have been purported to be, at least in part, responsible for periodontal inflammation and systemic interactions such as diabetes, cardiovascular disease and adverse pregnancy outcomes (Moutsopoulos & Madianos 2006, Chávarry et al. 2009). The precise mechanisms by which local inflammatory conditions influence systemic inflammation have not yet been fully elucidated. Nonetheless, the possibility that some individuals have a common predisposition to various inflammatory conditions has long been suspected. Conditions such as the ''hyperinflammatory phenotype'' have been proposed as one such mechanism (Beck et al. 1998).

The suggestion that local inflammation may influence the inflammatory response at distal sites is not new. Interestingly, chronic inflammation induced by the subcutaneous implantation of heat-killed *P. gingivalis* has been demonstrated to downregulate the expression of the T-cell antigen receptor ξ chain, leading to impaired T-cell function and a reduction of the hyperimmune response (Bronstein-Sitton et al. 2003). Such a response would be expected to impair the development of arthritis in our model. However, it has been suggested that in the presence of continuing chronic inflammation, persistence of ξ chain downregulation may prevent a full recovery by impairing immune responses. Thus, rather than resolving the condition, such a response may indeed contribute further to the pathological response (Bronstein-Sitton et al. 2003).

The host defence response to foreign material is generally considered to be a protective process whereby innate and acquired mechanisms are activated to dilute, destroy or negate damaging agents and initiate tissue repair. This response involves the co-ordinated activation of numerous biologic pathways of inflammation, resolution and repair, all of which may be observed in the chronically inflamed periodontium. If appropriately regulated, then tissue repair ensues; however, if not appropriately controlled, the inflammatory response becomes chronic and persistent, leading to further tissue destruction and progression of disease. It is this lack of control (dysregulation) that is thought to contribute to the pathogenesis of other chronic inflammatory diseases such as rheumatoid arthritis.

These findings are consistent with clinical observations that individuals with severe rheumatoid arthritis are more likely to suffer from advanced periodontitis and vice versa. However, the association seems to be in favour of rheumatoid arthritis impacting on periodontitis (Relative Risk 4.1) rather than periodontitis impacting rheumatoid arthritis (Relative Risk 1.5) (Mercado et al. 2001). In this context, it is interesting to

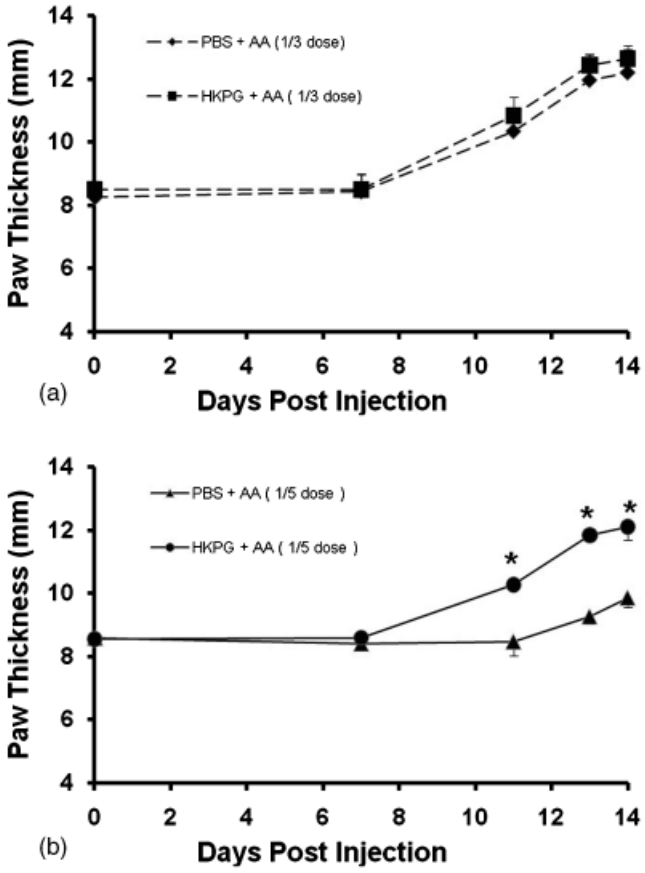

Fig. 5. Rear paw thickness for control and heat-killed *Porphyromonas Gingivalis*-treated animals exposed to a low-dose (1/5 dose) arthritis adjuvant. Data represent the mean ± SEM, ($N = 6$) for each group at the different time points. Points marked with *indicate statistical difference at $p < 0.05$ at that time point.

note the findings of one study that reported an increase in the amount of alveolar bone loss in rats with adjuvant arthritis (Ramamurthy et al. 2005). However, in the present study, we were unable to observe any evidence of increased alveolar bone loss in any of the animals with adjuvant-induced arthritis (results not shown). The reason for this discrepancy is not clear, although different species (Lewis *versus* DA) were used in both studies and so the possibility of genetic susceptibility in animals with regard to development of periodontitis cannot be discounted (Baker 2005). In an animal model, it has been demonstrated that HLA-B27 transgenic rats, which spontaneously develop arthritis, also develop advanced periodontitis (Tatakis et al. 2002, May & Tatakis 2004). However, this process took over 4 months to develop and was not directly comparable to our study as we could only follow arthritis development for 2 weeks due to the severity of the developing disease and ethical requirements. For similar reasons, we could not investigate whether adjuvant arthritis had any bearing on the development of experimental *P. gingivalis*-induced periodontitis in the long term.

In conclusion, using an animal model, we have demonstrated a potential mechanism whereby extra-synovial chronic inflammation (similar to that seen in chronic periodontitis) and rheumatoid arthritis might be inter-related. This model is based on the premise that a primary ''hit'' of *P. gingivalis*-induced chronic inflammation, followed by an arthritogenic hit to induce rheumatoid arthritis, can lead to an exacerbated response. Interestingly, these findings corroborate the results of studies investigating periodontal/cardiovascular inter-relationships in which local inflammation enhances the inflammatory responses at distal sites (Jain et al. 2003, Lalla et al. 2003, Serhan et al. 2003, Gibson et al. 2004, Ekuni et al. 2009). These findings of the present study, together with recent reports indicating that control of periodontal inflammation reduces the severity of active arthritis (Ribeiro et al. 2005, Al-Katma et al. 2007, Ortiz et al. 2009), lead us to believe that not only is there a significant pathological relationship between these two disease but removal or control of the chronic inflammatory burden of periodontitis has the potential to influence the clinical parameters of rheumatoid arthritis. Given the simplicity of the current model, further experiments are now required to test whether experimental periodontitis has a similar effect on experimental rheumatoid arthritis. Models are currently under development that will allow the development of both periodontitis and arthritis in the same animal.

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Address:
P. Mark Bartold
Colgate Australian Clinical Dental Research Centre
Dental School
University of Adelaide
Frome Road
Adelaide
South Australia 5005
Australia
E-mail: mark.bartold@adelaide.edu.au

Clinical Relevance

Scientific Rationale for Study: Accruing evidence indicates that periodontitis and rheumatoid arthritis are inter-related inflammatory diseases. *P. gingivalis* is one of many bacteria associated with the development of periodontal inflammation. It is possible that an extra-synovial source of chronic inflammation, as seen in periodontitis, may influence rheumatoid arthritis. Therefore, we studied the effect of an extra-synovial chronic inflammation, induced by *P. gingivalis*, on experimental arthritis.

Principal Findings: Pre-existing chronic inflammation induced by *P. gingivalis* exacerbated the onset and severity of experimental rheumatoid arthritis.

Practical Implications: Control of chronic inflammation at extra-synovial sites may reduce the severity of rheumatoid arthritis.