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The effect of immunization on the response to *P. gingivalis* infection in mice is adjuvant-dependent

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Abstract

Aim: Studies on vaccines against the periodontal pathogen *Porphyromonas gingivalis* have produced conflicting results, but no consideration has been given to the role of different adjuvants in these vaccines. We have previously shown that an intra-chamber challenge with heat-killed *P. gingivalis* was modified by immunization with different adjuvants. This study tested the hypothesis that different adjuvants in *P. gingivalis* vaccines would differentially modify the host response to a live *P. gingivalis* infection.

Results: Using *P. gingivalis*-infected subcutaneous chambers in mice, we show that vaccination with *P. gingivalis* in alum attenuated the pro-inflammatory cytokine levels at the site of infection, while the vaccine containing incomplete Freund's adjuvant did the opposite. Although both vaccines induced a similar humoral IgG response, *P. gingivalis*-induced abscesses were significantly smaller in the alum-adjuvant group.

Conclusions: The results suggest that the immune response and the resultant protection to a *P. gingivalis* infection, in *P. gingivalis*-vaccinated mice, are adjuvant-dependent.

Key words: cytokines; inflammation; *Porphyromonas gingivalis*/humoral response; *Porphyromonas gingivalis*; Th1/Th2; vaccine/adjuvant

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Studies on the development of a vaccine to prevent periodontal disease by immunization with antigens from *Porphyromonas gingivalis* have produced variable and conflicting results (Page 2000). Several studies using both mice and primates have demonstrated that immunization with *P. gingivalis* results in protection against experimentally induced periodontal destruction (Okuda et al. 1988, Persson et al. 1994, Genco et al. 1998, Gonzalez et al. 2003), while other animal studies have been unable to demonstrate protection (Ebersole et al. 1991, Moritz et al. 1998). In humans, numerous studies have demonstrated that patients with periodontitis mount a humoral immune response to plaque bacteria during the course of their disease (Tew et al. 1985, Mooney et al. 1997), and antibody titres to *P. gingivalis* have been shown to be inversely related to disease severity (Okuda et al. 1988, Mooney et al. 1997). On the other hand, it has also been demonstrated that patients with high antibody titres to *P. gingivalis* may experience bursts of disease progression and attachment loss (Okuda et al. 1994), suggesting that destruction of the periodontium can occur in spite of the establishment of a humoral response to the pathogen.

A protective immune response to a pathogen depends not only on serum and local antibody production, but also on the local T cells response, and the profile of cytokines they secrete (Carter & Dutton 1996). Th1 cells secrete pro-inflammatory cytokines such as interferon-γ (IFN-γ), Interleukin (IL)-2 and IL-12, which accelerate inflammatory-induced tissue destruction. On the other hand, Th2 cells secrete anti-inflammatory cytokines such as IL-4 and IL-10, which can diminish the inflammatory process (Mosmann & Coffman 1989, Romagnani 1996). These observations have led to the hypothesis that an immune response dominated by Th1 cells is associated with tissue destruction (Chen et al. 1990). Using the mouse subcutaneous chamber model, we have shown that a repeated intra-chamber challenge with *P. gingivalis* induces such a local pro-inflammatory Th1 response, as well as high anti-*P. gingivalis* titres (Houri-Haddad et al. 2000). A similar pro-inflammatory cytokine profile has been associated with destructive periodontitis (Stashenko et al. 1991). This type of response could explain how local tissue destruction may occur in spite of a successful humoral immune response. A similar but enhanced response was observed as a result of systemic immunization with a

P. gingivalis vaccine containing incomplete Freund's adjuvant (IFA) (Houri-Haddad et al. 2001).

We hypothesized that the nature of the adjuvant used in a *P. gingivalis* vaccine would modify the local inflammatory response and the clinical outcome to a live *P. gingivalis* infection, according to its effect on T-cell polarity. We have previously shown (Houri-Haddad et al. 2001) that a heat-killed *P. gingivalis* challenge results in the expression of pro-inflammatory cytokines at the local inflammatory site, and that this response was modulated by immunization according to the adjuvants used. The use of a heat-killed bacterial challenge does not necessarily reflect the response to a live bacterial challenge seen in a clinical infection. To test this hypothesis the present study was carried out using a live *P. gingivalis* induced abscess model to examine the protective effect of immunization with different vaccines on *P. gingivalis*-induced abscess formation and spreading in mice (Kesavalu et al. 1992). In addition, we examined the local intra-chamber inflammatory response to infection with live *P. gingivalis* in animals immunized with *P. gingivalis* in IFA, alum adjuvant and no-adjuvant. IFA and alum were chosen as adjuvants, which promote Th1 and Th2 responses, respectively, (Brewer et al. 1999, Houri-Haddad et al. 2001).

Materials and Methods

Bacteria

P. gingivalis strain ATCC 33277 was grown on blood agar plates in an anaerobic chamber (85% N_2, 5% H_2, 10% CO_2). After incubation at 37 °C for 2–3 days, the bacterial cells were inoculated into a peptone yeast extract for 1 week under the same conditions. The bacteria were washed three times with phosphate-buffered saline (PBS), and their final concentration was standardized using spectrophotometer to 10^{10} CFU/ml (Baker et al. 1994).

The *in vivo* local inflammation model

The experimental protocol was approved by the Internal Review Board of the Hadassah – Hebrew University Medical Center. Female Balb/c mice 5–6 weeks old (Jackson Laboratories, Bar Harbor, ME, USA), were used in this study. Two chambers constructed from coils of titanium wire were implanted into the subcutaneous dorsolumbar region of each mouse. After a 2-week healing period, the animals ($n = 24$) were divided into four groups. One group served as non-immunized control (saline-injected). The other three groups were immunized with two repeated subcutaneous injections (distant from the chambers) 7 days apart, with heat-killed *P. gingivalis* (0.1 ml of 10^{10} CFU/ml) in one of the following carriers: PBS (no adjuvant), alum adjuvant (ImjectAlum, Pierce, Rockford, IL, USA) or IFA (Sigma Chemicals Co., St. Louis, MO, USA). Seven days later the subcutaneous chambers in all four groups were infected by intra-chamber injection of live *P. gingivalis* (10^9 CFU in 100 µl PBS). Chamber exudates were collected at baseline (immediately before infection) and 2 and 24 h post-infection (each chamber was sampled only once). The exudates were centrifuged for 5 min., the supernatants removed and stored at − 20°C until analysed. The pellets were immediately re-suspended in PBS and the total leukocyte counts evaluated using a haemocytometer.

Analysis of cytokines

The presence of tumour necrosis factor-α (TNF-α), IFN-γ, IL-1β and IL-10 in the chamber exudates were determined by ELISA as previously described (Frolove et al. 1998). The assays were based on antibody-pairs matched for ELISA obtained from Pharmingen (San Diego, CA, USA).

Quantification of anti-*P. gingivalis* antibodies

Levels of IgG, antibodies to *P. gingivalis* were determined as previously described (Kojima et al. 1997, Houri-Haddad et al. 2001), using goat anti-mouse IgG antibodies (Jackson Immunoresearch, West Grove, PA, USA). The results were expressed as antibody titres with reference to serial dilutions of a serum pool prepared from immunized mice with high levels of the specific antibody. As a negative control we used serum from naïve mice.

The mouse abscess model

Five to six weeks old female Balb/c mice ($n = 6$ each group) were immunized with *P. gingivalis* in alum or IFA as described above. The control group received saline. Seven days after the last immunization, both groups were subcutaneously infected with live *P. gingivalis* (2×10^{11} CFU/100 µl) at the dorsolumbar area of the mice. Following infection, mice were evaluated daily during 7 days for signs of morbidity. Lesion diameters were measured using a calliper.

Data analysis

Data analysis was performed using a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). One-way repeated measures analysis of variance (RM ANOVA) was used to test the significance of the differences between the treated groups. When significance was established, the inter-group differences were tested for significance using the Student's *t*-test with the Bonferroni correction for multiple testing. The level of significance was determined at $p < 0.05$. All the results are presented as mean values ± the standard error of the mean.

Results

Vaccine in alum protected mice from abscess formation

Thirty percent of the non-immunized mice died by the second day following *P. gingivalis* infection. The surviving mice in this group showed local necrosis and abscess formation with a mean lesion diameter of 1.6 ± 0.16 cm. There was no mortality in the two immunized groups, but abscesses developed. In the IFA group, the abscesses were of greater

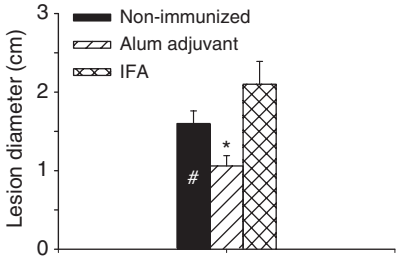

Fig. 1. Effect of immunization with *Porphyromonas gingivalis* on abscess formation 2 days following *P. gingivalis* infection. Mice ($n = 6$ in each group) were immunized with *P. gingivalis* using alum or incomplete Freund's adjuvant (IFA). Control (non-immunized) mice received saline. Results are expressed as mean ± standard error. # – two of the control mice died on the second day following *P. gingivalis* infection, therefore $n = 4$ for this group. Significant difference between the IFA and alum groups ($p < 0.05$) is indicated by an *.

diameter with larger areas of central necrosis than in the alum-adjuvant group (2.1 ± 0.29 and 1.06 ± 0.13 cm, respectively; $p < 0.05$; Fig. 1).

Adjuvants increased leukocytes recruitment into the chambers

At baseline, before infection (0 h), the total leukocyte counts in the chamber exudates were low (less than $10^4/\mu l$) in all the groups although the group immunized using IFA had higher cellular counts than the other three groups. This difference did not reach significance (Fig. 2). Two hours post-challenge, the leukocyte counts increased significantly in the IFA and alum-adjuvant groups only, with the increase being significantly greater in the IFA group (Fig. 2). The no-adjuvant and control groups had equally small, non-significant, increases from baseline. At 24 h, there was a further small increase in the leukocyte counts of the control and no-adjuvant groups and a significant decrease in the counts of IFA group. The leukocyte concentration in the alum group remained stable. These changes eliminated the significant differences seen between the treatment groups at the 2 h time point, but they were still significantly higher than baseline levels. It is interesting to note that almost half of the cells in the IFA group were ghost cells at this time point.

The relative ratios of polymorphonuclear leukocytes, monocytes and lymphocytes in the chamber exudates of the four treatment groups were similar at all time intervals (data not shown).

Vaccine adjuvants modified the inflammatory cytokine profile

IFN-γ, TNF-α and IL-1β were tested as representative of the pro-inflammatory group of cytokines. At baseline, only the IFA-treated group had levels of IFN-γ that were significantly higher than controls. Infection with *P. gingivalis* significantly increased the levels of IFN-γ in the control group 2 h post-challenge (Fig. 3), with a reduction of the levels at 24 h. In the IFA group, IFN-γ levels remained high following the local challenge during the 2 and 24 h time intervals. The levels of IFN-γ in the no-adjuvant and alum-immunized groups were significantly lower than the levels of the IFA and control groups post-infection.

Two hours post-infection, TNF-α levels increased dramatically (Fig. 3) and by 24 h the levels had returned to baseline in all four groups. However, the increase at 2 h was significantly attenuated in the alum-adjuvant group. IL-1β levels also increased dramatically 2 h post-infection in all four groups, with the increase in the IFA group being significantly higher. Twenty-four hours post-infection, the levels of IL-1β decreased but the decrease did not reach statistical significance. Because of the small amounts of chamber fluid obtained in the IFA group at the 24 h interval, we were unable to include a group for IL-1β analysis at this time interval.

IL-10 was used as a representative example of the anti-inflammatory cytokines. IL-10 was barely detectable in all

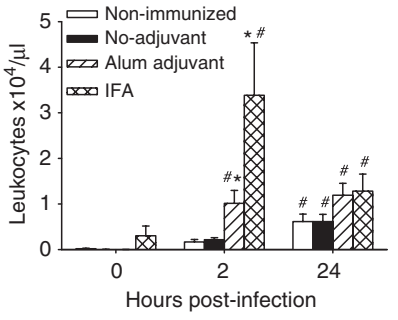

Fig. 2. Effect of vaccination with *Porphyromonas gingivalis* on leukocytes migration into subcutaneous chambers following *P. gingivalis* infection. Mice were immunized with *P. gingivalis* in alum or incomplete Freund's (IFA) adjuvants, or without adjuvant. Control non-immunized mice received saline. Seven days following the last immunization, mice ($n = 6$ in each group) received an intra-chamber challenge of *P. gingivalis*. Chamber exudates were obtained at baseline (immediately before infection), and 2 and 24 h post-infection. Results are expressed as mean ± standard error. Results that are significantly different from the other groups are indicated by an $^*p < 0.05$. Results that are significantly different from the other groups in the same time interval are indicated by $^*p < 0.05$. Results that are significantly different from baseline levels in the same group are indicated by $\#p < 0.05$.

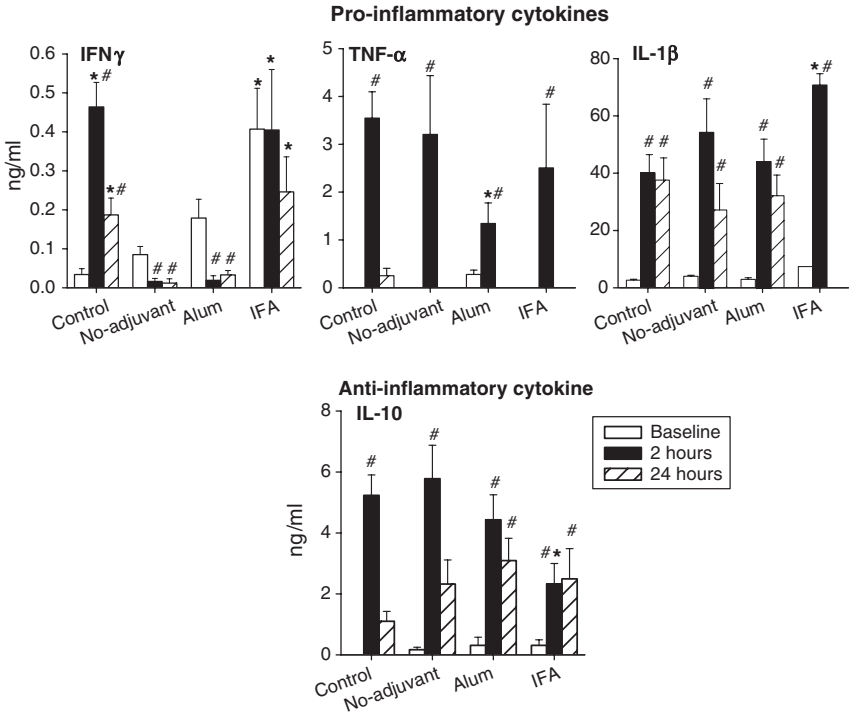

Fig. 3. Effect of immunization with *Porphyromonas gingivalis* on levels of IFN-γ, TNF-α, IL-1β and IL-10 in the subcutaneous chambers following *P. gingivalis* infection. Mice ($n = 6$ in each group) were immunized with *P. gingivalis* in Alum or incomplete Freund's (IFA) adjuvants, or without adjuvant (*P. gingivalis* only). Control non-immunized mice received saline. Mice received an intra-chamber challenge of *P. gingivalis*. Chamber exudates were obtained at baseline (immediately before infection), and 2 and 24 h post-challenge. The levels of each cytokine in the chamber exudates were determined by ELISA. Results are expressed as mean ± standard error. Results that are significantly different from those obtained in the other groups at the same time interval are indicated by $^*p < 0.05$. Results that are significantly different from baseline levels in the same group are indicated by $\#p < 0.05$.

four groups at baseline. Two hours following *P. gingivalis* infection, the levels of IL-10 increased significantly in all the groups, with the increase in the IFA group being significantly attenuated compared with the other groups (Fig. 3). Twenty-four hours post-challenge, the levels of IL-10 in the control and no-adjuvant groups decreased significantly, while the changes in alum-adjuvant and IFA groups between 2 and 24 h were not significant.

Adjuvants augmented IgG response to *P. gingivalis* vaccine

One day post-challenge antibodies to *P. gingivalis* were detected only in the three immunized groups. The titres were significantly lower in the no-adjuvant group compared with the alum and IFA groups (Fig. 4), with no significant difference between the latter two groups.

Discussion

The present study investigated the effect of two different adjuvants used for immunization on *P. gingivalis*-induced infection using two animal models. The results from both models suggest that immunization with *P. gingivalis* in alum attenuated the local inflammatory response as well as the clinical outcome of infection. In contrast, while immunization with IFA induced high antibody response, it failed to attenuate the local inflammatory response. These results correlated with the reduced ability of the *P. gingivalis* vaccine containing IFA to prevent *P. gingivalis*-induced tissue damage compared with the vaccine containing alum.

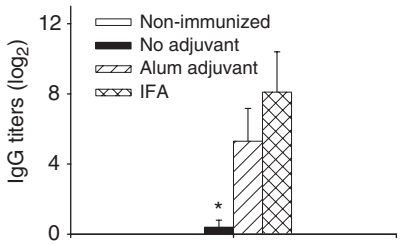

Fig. 4. Effect of immunization with *Porphyromonas gingivalis* on serum levels of anti-*P. gingivalis* IgG levels. Mice ($n = 6$ in each group) were pre-immunized with *P. gingivalis* using alum or incomplete Freund's adjuvant (IFA) as adjuvants or without adjuvant. Control non-immunized mice received saline. Serums were harvested 1 day following an intra-chamber infection with *P. gingivalis*, and anti-*P. gingivalis* IgG levels were analysed by ELISA. In the control (non-immunized) group, anti-*P. gingivalis* IgG levels were not detectable. Results are expressed as mean ± standard error. Results that are significantly different from those obtained in the other groups are indicated by an $^{*}p < 0.05$.

Many studies have examined the effect of immunization to *P. gingivalis* on experimental periodontitis, but the results have been inconsistent. For example, Persson et al. (1994) showed that vaccination with *P. gingivalis* provided protection against experimentally induced periodontitis in the monkey model. In contrast, Ebersole et al. (1991) could not demonstrate any protection against experimental periodontitis, using *P. gingivalis* vaccine in the same model. However, no study has raised the possibility, or tested the hypothesis, that these differences are because of the use of different adjuvants in the vaccine. Indeed, (Ebersole et al. 1991) used IFA, while Persson et al. (1994) used another commercially available adjuvant. The results using the abscess model show for the first time that the adjuvant in the vaccine may alter the clinical outcome of immunization with *P. gingivalis* infection. In addition, the present data suggest a possible mechanism for the different outcomes. The suppressed inflammatory response induced by alum compared with IFA may be responsible for the superior local protection achieved with alum as adjuvant.

In a previous study (Houri-Haddad et al. 2000), we have shown that the pro-inflammatory response to repeated, intra-chamber, challenges with heat-killed *P. gingivalis* is directly correlated to the number of challenges. In the present study, repeated exposure to a heat-killed *P. gingivalis* at a site distant from the chamber, resulted in marked suppression of IFN-γ levels within the chamber exudate that result from an intra-chamber challenge with live *P. gingivalis*. One of the possible explanations for this apparent contradiction is that the different sites of exposure result in different responses. Whereas repeated exposure at the site of the challenge result in a marked pro-inflammatory response, repeated exposure distant from the site of challenge result in a suppression of this pro-inflammatory response.

Immunization with *P. gingivalis* in alum adjuvant decreased the levels of the Th1 cytokine, IFN-γ, in the chambers to levels similar to those seen after immunization with *P. gingivalis* alone. In addition, it significantly decreased the levels of the pro-inflammatory cytokine, TNF-α, compared with the no-adjuvant group, further attenuating the inflammatory response. On the other hand, immunization with *P. gingivalis* in IFA attenuated the intra-chamber Th2 response and intensified the pro-inflammatory response. In contrast to the two other immunization protocols, immunizations with IFA resulted in a significant intra-chamber response at baseline prior to the *P. gingivalis* challenge. This response was expressed by high leukocyte counts and IFN-γ levels. The results suggest that a vaccine containing IFA is a more potent peripheral immune modulator (with Th1 characteristics) prior to an infectious challenge, than either the vaccine with no-adjuvant or the vaccine containing alum. A similar Th1 response was described by Gemmell et al. (1998) following immunization of mice with *P. gingivalis* antigens in IFA, resulting in the proliferation of predominantly IFN-γ positive T-cells in the mouse spleens. These cells may be the source of the high baseline levels of leukocytes and IFN-γ observed in the present study.

The titres of anti-*P. gingivalis* antibodies seen in the serum of the two groups immunized with *P. gingivalis* in either IFA or alum adjuvants were significantly higher than those seen in the group immunized with *P. gingivalis* alone, this suggests that immunization with *P. gingivalis* in either adjuvant is able to establish a superior protective immune response. Using the mouse abscess model, we tested the ability of vaccines containing different adjuvants to protect against abscess formation. The results showed that immunization with *P. gingivalis* in either adjuvant protected the immunized mice from death. However, the vaccine containing alum was significantly more effective at controlling the lesion size at the local site of infection than the vaccine containing IFA.

In conclusion, the results of the present study suggest that local modulation of the cytokine response at the site of *P. gingivalis* infection can be achieved by immunization using a suitable adjuvant. This modulation could be a key factor in determining the effectiveness of vaccines in protecting against *P. gingivalis*-induced inflammatory destruction such as seen in periodontal disease.

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