Interferon-gamma Deficiency Attenuates Local \textit{P. gingivalis}-induced Inflammation

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What is This?
Interferon-gamma Deficiency Attenuates Local P. gingivalis-induced Inflammation

ABSTRACT
Infection with the periodontal pathogen Porphyromonas gingivalis causes a strong local inflammatory reaction. Using IFNγ-deficient mice, we tested the hypothesis that the absence of IFNγ would result in a reduction of the local pro-inflammatory response to P. gingivalis. Cytokine secretion by macrophages from IFNγ−/− animals was significantly attenuated. Addition of IFNγ restored cytokine secretion. In vivo injection of P. gingivalis into subcutaneous chambers increased the intra-chamber leukocyte counts and TNFα and IL-1β levels. This increase was significantly lower in the IFNγ−/− mice. Local reconstitution of IFNγ−/− mice at the site of inflammation with the IFNγ gene increased the levels of TNFα and decreased the IL-10 levels. This increase was significantly lower in IFNγ−/− mice. Anti-P. gingivalis IgG1 levels, a marker of Th2 response, were higher in immunized IFNγ−/− than in IFNγ+/+ mice. The results suggest that lack of IFNγ reduced the amplitude of the local pro-inflammatory response without decreasing the humoral protective response. The higher IgG1/IgG2a ratio observed supports the possibility of a Th2-dominant response in IFNγ-deficient animals.

KEY WORDS: IFNγ deficiency, Porphyromonas gingivalis, inflammation, cytokines, antibody.

INTRODUCTION
Porphyromonas gingivalis is a Gram-negative anaerobic bacterium that has been closely linked to the pathogenesis of periodontal disease (Socransky and Haffajee, 1992). Periodontitis is characterized by an inflammatory reaction localized to the periodontal tissues. The local inflammatory response is maintained and amplified by the in situ production of pro-inflammatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β (Van Dyke et al., 1993). This chronic inflammatory process results in periodontal tissue destruction and consequent tooth loss.

IFNγ is a characteristic cytokine of Th1 CD4+ T-cells (Mosmann and Coffman, 1989). It is secreted by Th1 cells and supports their growth. IFNγ has varied effects on the immune system, including the priming of macrophages toward the enhanced production of inflammatory cytokines and oxygen metabolites, as well as activation and growth enhancement of cytotoxic T-cells and Nk cells (Gemmell and Seymour, 1994). IFNγ also plays a major role in the control of immunoglobulin isotype switching by enhancing IgG2a production and suppressing other IgG isotypes and IgE (Snapper and Paul, 1987; Finkelman et al., 1988). In addition, IFNγ inhibits most of the activities induced by the Th2 cytokine IL-4.

Evidence derived from IFNγ knockout mice has suggested a central role for IFNγ in the pathogenesis of periodontitis (Baker et al., 1999). This study has shown that mice lacking the ability to produce IFNγ are resistant to P. gingivalis-induced experimental periodontitis. However, the effect of IFNγ deficiency on the molecular events at the site of P. gingivalis-induced inflammation is not fully understood.

To clarify this question further, we carried out experiments comparing the local inflammatory response to P. gingivalis in IFNγ-deficient (IFNγ−/−) with that in wild-type (IFNγ+/+) mice. We measured the inflammatory mediators secreted by mouse macrophages in vitro in the presence of LPS derived from P. gingivalis (Frolov et al., 1998), and the in vivo localized inflammatory response induced by P. gingivalis in the subcutaneous mouse model (Houri-Haddad et al., 2000).

MATERIALS & METHODS

Bacteria
P. gingivalis (ATCC 33277) was grown in anaerobic conditions and then heat-killed as previously described (Houri-Haddad et al., 2001). The bacterial concentration was standardized by optical density to 10⁶ CFU/mL (Baker et al., 1994), and stored at 4°C. Immediately before use, the bacteria were briefly sonicated, and 0.1 mL of the bacterial suspension was injected into each chamber.

Animals
Female IFNγ+/+ (control) and IFNγ−/− (knockout) Balb/c mice (6-7 wks old) (Jackson Laboratories, Bar Harbor, Maine, USA) were used in the present studies.
The chambers of IFN/H9253 challenge. Mice (n = 6, each group) were challenged with P. gingivalis. Levels of leukocytes between IFN/H9253. Results are expressed as mean ± standard error. Significant differences between IFN/H9253/H9253. Peritoneal macrophages of IFN/H9253/H9253 Board of the Hadassah-Hebrew University Medical Center. The animals were housed in specific-pathogen-free conditions. The experimental protocols were approved by the Internal Review Board of the Hadassah-Hebrew University Medical Center.

**Elicitation and Culture of Mouse Macrophages**

Peritoneal macrophages of IFN/H9253 and IFN/H9253 mice were elicited and cultured as described previously (Frolov et al., 1998). The isolated macrophages were stimulated by LPS (100 ng/mL) extracted from P. gingivalis (strain A7436, kindly provided by T.E. Van Dyke, Boston, MA, USA) prior to LPS stimulation. 20 ng/mL recombinant mouse IFN (Pharmingen, San Diego, CA, USA) was injected into the chambers, followed, two days later, by the intra-chamber challenge of P. gingivalis. Chamber exudates were harvested after 4 hrs for analysis.

**Chamber Fluid Analysis**

Chamber exudates were centrifuged for 5 min at 4°C and 200 g. The supernatants were removed and stored at -20°C until analyzed. The pellets were immediately re-suspended in PBS (200 μL), and the total cell count in the exudates was calculated by means of a hemocytometer.

**Analysis of Cytokines**

The presence of TNF-α, IFNγ, IL-13, IL-12, and IL-10 in the chamber exudates or culture media was determined by two-site ELISA as previously described (Frolov et al., 1998). The assays were based on matched antibody-pairs matched for ELISA obtained from Pharmingen (San Diego, CA, USA). The optical density was read by means of a Vmax microplate reader (Molecular Devices, Palo Alto, CA, USA) against a standard curve based on known concentrations of the recombinant cytokine.

**Quantification of Anti-P. gingivalis Antibodies**

Levels of IgG1 and IgG2a antibodies against P. gingivalis in the serum were determined by a modification of an ELISA method described by Kojima et al. (1997; Houri-Haddad et al., 2001). The results were expressed as antibody titers by 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.

**Data Analysis**

Data analysis was performed with the use of a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). One-way repeated-measures analysis of variance (RM ANOVA) was used for testing the significance of the differences between the treated groups. When significance was established, the inter-group differences were tested for significance by Student's t test with the Bonferroni correction for multiple testing. The level of

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<td>IFN/H9253</td>
<td>815 ± 170</td>
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<td>IFN/H9253</td>
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* Significantly lower from IFN/H9253 mice, p < 0.05.

The In Vivo Localized Inflammation Model

Two chambers, constructed from coils of titanium wire, were implanted into the subcutaneous dorsolumbar region of IFN/H9253 and IFN/H9253 mice (n = 6, each group) (Houri-Haddad et al., 2000). After a healing period, the chambers were used as a compartment for confinement of an induced inflammatory response (Genco and Arko, 1994). At baseline, all chamber exudates were sampled followed by the intra-chamber challenge of P. gingivalis. From the two chambers in each animal, exudates were obtained from one chamber 2 hrs post-challenge and from the second chamber at 24 hrs.

As a test of the effect of prior immunization, IFN/H9253 and IFN/H9253 mice (n = 6, each group) received subcutaneous injections of P. gingivalis in alum adjuvant (Pierce, Rockford, IL, USA) (Houri-Haddad et al., 2001). The same injection was repeated 10 days later (booster). Eleven days after the booster injection, the immunized mice received the intra-chamber challenge of P. gingivalis as described above.

For verification of the role of IFNγ in the cytokine response to challenge, IFN/H9253 mice were reconstituted with IFNγ DNA (Porgador et al., 1993). The mouse IFNγ plasmid was constructed with use of the pLXSN vector with the IFNγ gene under the control of MuLV LTR promoter. The pLXSN vector alone was used as the negative control. The IFNγ gene or vector (50 μL, n = 6, each group) was injected into the chambers, followed, two days later, by the intra-chamber P. gingivalis challenge. Chamber exudates were harvested after 4 hrs for analysis.

**Figure 1.** Levels of leukocytes [A], TNF-α [B], IL-1β [C], and IL-10 [D] in the chambers of IFN/H9253 and IFN/H9253 mice following P. gingivalis challenge. Mice (n = 6, each group) were challenged with P. gingivalis, and chamber exudates were harvested for analysis after 2 and 24 hrs. Results are expressed as mean ± standard error. Significant differences between IFN/H9253 and IFN/H9253 groups at the same time point (p < 0.05) are indicated by an asterisk.

**Table.** Secretion of TNF-α and IL-12 from Macrophages Harvested from IFN/H9253 and IFN/H9253 Mice

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significance was determined at $p < 0.05$. All the results are presented as mean values ± the standard error of the mean.

RESULTS

Secretion of Cytokines from Macrophages Harvested from IFNγ+/+ and IFNγ−/− Mice

The levels of the pro-inflammatory cytokines TNF-α and IL-12 secreted by LPS-stimulated macrophages from IFNγ+/+ mice were significantly lower than levels secreted from IFNγ−/− macrophages (Table). The addition of IFNγ into the media enhanced the levels of these two cytokines in both IFNγ+/+ and IFNγ−/− mice, so that the significant differences between the cytokine levels in the 2 groups were eliminated.

Recruitment of Leukocytes into the Chambers Following P. gingivalis Challenge

The total leukocyte count in the chamber fluid was very low prior to the baseline challenge with P. gingivalis (day 0) in both the IFNγ+/+ and IFNγ−/− animals ($< 10^6/\mu L$). The leukocyte count increased markedly over the 24-hour study period, with the increase being significantly greater in the IFNγ+/+ group (Fig. 1a). Concentrations of the leukocytes 2 hrs following the intra-chamber challenge were significantly higher than baseline levels in the chambers of the IFNγ+/+ mice, but not in the IFNγ−/− mice ($p < 0.05$). At 24 hrs, both groups showed levels significantly higher than baseline, but the IFNγ+/+ group had significantly higher levels than the IFNγ−/− mice.

Levels of Cytokines in the Chamber Following P. gingivalis Challenge

P. gingivalis challenge increased the levels of all the cytokines tested in both the IFNγ+/+ and IFNγ−/− animals (Figs. 1b-1d). As expected, the IFNγ+/+ group showed increased levels of IFNγ during the 24-hour experiment, while IFNγ was not detected in the chamber exudates of the IFNγ−/− animals (data not shown).

At baseline, the levels of the two pro-inflammatory cytokines TNF-α and IL-1β were very low to undetectable. TNF-α levels peaked 2 hrs post-challenge in both experimental groups, and then decreased to very low levels in the IFNγ+/+ and to undetectable levels in the IFNγ−/− animals. At 2 hrs post-challenge, TNF-α levels were significantly lower in the IFNγ−/− group than in the IFNγ+/+ mice (Fig. 1b). IL-1β levels showed a continuous increase in the IFNγ+/+ group over the study period, whereas in the IFNγ−/− group, the levels peaked at 2 hrs and then leveled off. At the 24-hour time period, IL-1β levels were significantly lower in the IFNγ−/− group than in the IFNγ+/+ mice (Fig. 1c).

The anti-inflammatory cytokine IL-10 was detectable in both experimental groups at baseline, with the levels in the IFNγ−/− group significantly higher. Following P. gingivalis challenge, IL-10 levels increased, but there were no differences between the 2 groups (Fig. 1d). The levels of the other anti-inflammatory cytokine, IL-13, peaked at 2 hrs and then decreased, with no differences being evident between the 2 groups throughout the study (data not shown).

Levels of Cytokines in the Chambers of IFNγ−/− Mice Following Local IFNγ Gene Transfer

The introduction of the IFNγ gene into chambers implanted into IFNγ−/− mice induced detectable levels of IFNγ in the chamber fluid (Fig. 2). In addition, levels of TNF-α were significantly higher in the IFNγ−/− group than in the vector-injected group, while the levels of IL-10 were higher in the vector-injected control group.

DISCUSSION

The present study demonstrated an in vitro reduction in the pro-inflammatory response to LPS of macrophages from IFNγ-deficient mice. Furthermore, in vivo, the IFNγ−/− animals exhibited a reduction in the pro-inflammatory parameters at the local inflammatory site (reduced levels of leukocytes, IL-1β and TNF-α), as well as higher baseline levels of the anti-inflammatory cytokine IL-10. These results were confirmed by the addition of IFNγ to the macrophage cultures and by reconstitution of the IFNγ gene at the local inflammatory site, shifting the response of the knockout mice to one similar to that seen in control, IFNγ+/+, mice. The present results support the concept that IFNγ is a central and important inflammatory mediator, participating in the regulation of inflammatory processes resulting from local infection.

To test the hypothesis that the differences between the
IFNγ−/− and IFNγ+/− mice were actually due to the lack of IFNγ, we injected expression plasmid with the mouse IFNγ gene into the chambers of IFNγ−/− mice, in an attempt to create a local source of IFNγ. This model of gene delivery to the inflammatory site provides a more extended and stable source of the cytokine compared with the introduction of the protein itself, which has a relatively short half-life. The results proved that this delivery system of the IFNγ gene restored the production of IFNγ at the local site, suggesting the incorporation of the IFNγ gene and its expression into chamber cells. We did not attempt to use hybridization or immunochemistry to demonstrate that cells in the chamber expressed IFNγ, and the presence of the detectable levels of IFNγ following gene delivery was satisfactory for testing the hypothesis. Indeed, we found that the local production of IFNγ reversed the changes induced by IFNγ deficiency (i.e., elevation of the levels of TNF-α and reduction in the levels of IL-10 compared with the mock-treated IFNγ−/− mice), supporting the central role of IFNγ in the observed differences between the groups.

The present results showed that IgG1 to IgG2a ratios (IgG1/IgG2a) were much higher in the IFNγ−/− group compared with the IFNγ+/− group (2.25 and 1.2, respectively). Furthermore, IgG1 levels were higher in the IFNγ−/− mice compared with the IFNγ+/+ animal. Th1 cytokines have been shown to support immunoglobulin isotype switching to IgG2a, while Th2 cytokines support switching to IgG1 (Mosmann and Coffman, 1989). Based on these markers, the present results suggest that in normal animals there is a balance between Th1 and Th2 responses. However, the lack of IFNγ in the knockout mice resulted in a shift toward a Th2 response, which is considered an anti-inflammatory response, due to the anti-inflammatory cytokines secreted by Th2 cells. The lack of IFNγ did not impede the total antibody response, so the humoral protective response was not compromised.

Recently, Baker et al. (1999) have shown that mice lacking IFNγ demonstrate decreased bone loss following infection of the oral cavity with P. gingivalis, suggesting that IFNγ is a central mediator in this process. However, this model does not allow for the quantitative assessment of the cytokines at the local site of the inflammatory disease. The present study used the subcutaneous chamber model, which allows for the investigation of host-bacteria interactions in vivo. Taken together, the results of the two studies suggest that the reduced bone loss in the IFNγ−/− mice may result from a reduced production of pro-inflammatory cytokines at the site of infection. Thus, it is reasonable to suggest that the blocking of IFNγ production could be a suitable pharmacological approach to the treatment of localized chronic inflammatory diseases such as periodontal disease. Assuma et al. (1998) showed that it is possible to block experimental alveolar bone loss by using a combination of antagonists to 2 inflammatory mediators, TNF-α and IL-1β, while each antagonist alone induced only partial protection. The present results demonstrated that suppression of IFNγ levels alone might be sufficient to control the levels of TNF-α and IL-1β at the inflammatory site, suggesting another possible therapeutic modality for the inhibition of bone loss.

ACKNOWLEDGMENTS

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REFERENCES


