Interferon Gamma Levels in the Sinus, Ear, and Airway in a Rabbit Sinusitis Model Induced by Bacteroides Inoculation

Harumi Jyonouchi, MD; Sining Sun, DDS; Cynthia A. Kennedy, MD; Frank L. Rimell, MD

Background: Previously, we found minimal bacterial dissemination and no evidence of systemic inflammation in a rabbit sinusitis model in which the left maxillary sinus was inflamed by Bacteroides inoculation with the ostium closed. However, we observed an increase in anti-Bacteroides IgG antibodies in the contralateral sinus, lower airway, and middle ear, with an apparent increase in interferon gamma (IFN-γ) messenger RNA expression in the ear and sinus mucosa.

Objective: To evaluate how IFN-γ production in the upper and lower airway is associated with localized bacterial sinusitis.

Design: Interferon gamma levels were measured in lavage solutions from the sinus, airway, and middle ear and in serum at 1, 2, 3, and 4 weeks following bacterial inoculation.

Subjects: The subjects were 6 rabbits at each time point. The controls were untreated (n = 5) and sham-operated (n = 4-5) rabbits at 2 and 4 weeks.

Intervention: Bacteroides fragilis (10⁸ plaque-forming units) was inoculated into the left maxillary sinus.

Results: Interferon gamma levels in the ear and sinus were less than 0.2 µg/g protein in controls. Following bacterial inoculation into the left sinus, IFN-γ levels increased up to 10-fold in both sinuses and even more in the middle ear at 3 weeks, independent of bacterial dissemination. Mean ± SD IFN-γ levels in the airway (0.3 ± 0.28 µg/g protein in controls) were not altered by bacterial inoculation into the sinus. Serum IFN-γ levels were very low (<0.05 µg/g protein) in most rabbits and were unchanged by bacterial inoculation.

Conclusions: Interferon gamma levels increase in the ear and contralateral sinus in response to localized sinus inflammation, indicating concerted mucosal proinflammatory immune responses in the upper airway. Such responses may lead to the aseptic middle ear inflammation often observed in patients with chronic sinusitis.

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In a rabbit sinusitis model induced by Bacteroides fragilis,1,2 we observed a rapid rise of serum IgG antibodies against Bacteroides, characteristic of robust secondary humoral immune responses.3 Although sinus inflammation persisted in the inflamed sinus, bacterial dissemination was prevented in most animals with Bacteroides inoculated into the sinus with the ostium closed.3,4 Our study revealed an increase in anti-Bacteroides IgG antibodies not only in the inflamed sinus, but also in the contralateral sinus, airway, and middle ears.3 We thus postulated that recruitment or local production of anti-Bacteroides IgG antibodies in the sinus, ear, and airway may have effectively prevented the dissemination of bacteria from the inflamed sinus. We also observed an apparent increase in interferon gamma (IFN-γ) messenger RNA expression in the sinus and ear mucosa as measured by semiquantitative reverse transcriptase polymerase chain reaction.3,5 Intracellular microbial infection induces predominant type 1 T-cell (T1) responses characterized by production of a large amount of T1 cytokines (IFN-γ, TNF-α [tumor necrosis factor α], IL-2 [interleukin 2], etc).5,7 The T1 responses promote phagocytic cell–mediated proinflammatory responses and effectively eradicate microbes.7,8 However, excessive T1 responses could cause excessive inflammation and tissue damage.6,8 Chronic sinusitis is often associated with middle ear inflammation and increased airway hyperresponsiveness, although microbial growth appears to be localized in the sinus.10,11 This localized sinus inflammation may be associated with reflex responses in the airway mediated by inflammatory mediators other than mi-
MATERIALS AND METHODS

ANIMALS

Adult male New Zealand white rabbits (weight, 2.5-3.5 kg) were used (Birchwood, Red Wing, Minn). Rabbits were kept one to a cage in the animal facility at the University of Minnesota, Minneapolis, and fed a regular laboratory chow (Rabbit Chow, Complete Blend; Purina Mills, St Louis, Mo). The experiments were conducted as approved by the Animal Care Committee at the University of Minnesota and conform to the guidelines of the International Association for the Study of Pain.14

EXPERIMENTAL DESIGN

New Zealand white rabbits were inoculated with Bacteroides fragilis as described before.3,4 Five to 7 days prior to surgery, presurgery serum samples were drawn. At 1, 2, 3, and 4 weeks following inoculation, SL, BAL, and EL samples and post-surgery serum samples were obtained and IFN-γ levels were measured by enzyme-linked immunosorbent assay (ELISA). Three experiments were performed, and in each experiment, 2 rabbits were used at each time point. As controls, the same parameters were examined in the sham-operated rabbits at 2 and 4 weeks following the operation (n = 4 at each time point) and in 5 untreated rabbits. We have already reported the results of bacterial cultures, sinus mucosal histologic findings, cell numbers and cytologic findings, white blood cell count and differential white blood cell count, anti-Bacteroides IgG antibody levels, and mucosal IFN-γ mRNA expression in these experiments.3,5

SURGICAL PROCEDURES

Induction of Sinusitis

One colony of Bacteroides fragilis was grown overnight in Todd-Hewitt broth in an anaerobic chamber. The rabbits were anesthetized as reported before,3,4 the left maxillary sinus was opened, and the maxillary ostium was closed with a sterile cotton plug and cyanoacrylate ester (superglue) as detailed elsewhere.3,4 Bacteroides fragilis (108 plaque-forming units in 0.25 mL of sterile phosphate buffered saline [PBS]) was injected onto the cotton plug placed in the maxillary sinus.4 However, IFN-γ levels were comparable in right and left (inflamed) sinuses until 3 weeks after bacterial inoculation. At 4 weeks, IFN-γ levels declined in the contralateral sinus but remained high in the inflamed sinus (Figure 1).

RESULTS

Interferon gamma levels in the SL solutions increased gradually, reaching their highest levels 3 weeks after bacterial inoculation (Figure 1). As reported before, there was no growth of Bacteroides in the contralateral sinus in most rabbits, and mucosal inflammation was much greater in the inoculated left sinus than in the right sinus.4 However, IFN-γ levels were comparable in right and left (inflamed) sinuses until 3 weeks after bacterial inoculation. At 4 weeks, IFN-γ levels declined in the contralateral sinus but remained high in the inflamed sinus (Figure 1).

A significant rise in IFN-γ levels was also observed at 3 and 4 weeks in the SL solutions bilaterally (Figure 2), without positive culture of Bacteroides from the ear in most rabbits.5 These findings were paralleled by the rise of anti-Bacteroides IgG antibodies in the contralateral sinus and ears, as described before.3 In contrast, there was no growth of Bacteroides in the contralateral sinus.

ANALYTICAL METHODS

ELISA for IFN-γ

Rabbit IFN-γ levels were assessed by taking advantage of the cross-reactivity of rabbit and murine IFN-γ; that is, IFN-γ levels in lavage solutions and serum were measured by an ELISA that was originally developed to measure murine IFN-γ (Pharmingen, San Diego, Calif). Unconjugated anti-IFN-γ and biotinylated anti-IFN-γ were used as the first and second antibodies, respectively. The plate was coated with the first antibody overnight at 4°C and then the plate was treated with blocking buffer (PBS with 10% fetal calf serum, pH 7.4) at room temperature for 1 hour. The plates were then washed extensively, and samples (50 µL/well) were incubated for 2 hours at room temperature. Then the plates were washed and incubated with the second antibody for 1 hour at room temperature, and the color was developed by adding substrate solution (tetramethylbenzidine and hydrogen peroxide; Pharmingen) at room temperature. The color development was stopped by adding 2N sulfuric acid. The IFN-γ levels were calculated from a standard curve obtained using recombinant murine IFN-γ in each plate. The IFN-γ levels were expressed as micrograms per gram of protein. Total protein levels in lavage and serum samples were measured by the Lowry method.15

Statistics

Equality of 2 means was evaluated by Mann-Whitney test.16 Comparison of multiple values was done by Kruskal-Wallis test.16 Correlation of 2 parameters was assessed by the Kendall τ-b test.16
no significant increase of IFN-γ in the BAL solutions (Figure 2). The BAL samples from both controls and Bacteroides-inoculated rabbits had positive cultures of mixed bacterial flora, indicating low-grade bacterial infection.3,4 Serum IFN-γ levels remained very low and did not change during the period of observation (Figure 2). These findings are in contrast to our previous observation that anti-Bacteroides IgG antibody levels increased significantly in both the SL and BAL solutions.5

**COMMENT**

Patients with chronic sinusitis often develop serous/mucoid otitis media without evidence of microbial dissemination.10 This study addressed whether proinflammatory T1 responses develop in the middle ear in a rabbit sinusitis model induced by Bacteroides inoculation.

Because of anatomical resemblance of sinus structures, rabbits have been used as a model for human sinusitis. In previous studies, we have adapted a rabbit sinusitis model induced by inoculating a large amount of Bacteroides into the left maxillary sinus with the ostium closed.1,3–5 Although this model produced localized bacterial growth in the inflamed sinus, our analysis of immunoparameters indicated generalized immune responses involving the contralateral sinus, ears, and airways, as summarized below.

Bacteroides is a common pathogen in the rabbit gut mucosa, and most rabbits had low levels of anti-Bacteroides IgG antibodies prior to bacterial inoculation. Thus, inoculation of Bacteroides into the sinus induced a rapid rise of serum anti-Bacteroides IgG antibody levels (>100-fold), indicating robust secondary antibody responses against Bacteroides.6 Our previous study also showed increased anti-Bacteroides IgG antibody levels in the sinus, ear, and lower airway at 2 to 4 weeks following bacterial inoculation, which may indicate generalized mucosal immune defense in the upper and lower airways.3

In these studies we observed an apparent increase in IFN-γ mRNA in the ear and sinus mucosa. Mild inflammatory changes were found in the ear mucosa by histologic evaluation in spite of negative Bacteroides growth.3 Since IFN-γ is a proinflammatory T1 cytokine that promotes phagocytic cell–mediated proinflammatory responses,6,9 we speculated that T1 responses in the ear mucosa can be partly responsible for development of middle ear inflammation in this model. However, IFN-γ mRNA was measured by semiquantitative reverse transcriptase polymerase chain reaction, which did not permit us to evaluate the time course of IFN-γ production quantitatively parallel with changes in other immune and inflammatory parameters.

We found recently that rabbit and murine IFN-γ are cross-reactive; that is, we can assess protein levels of rabbit IFN-γ in serum and lavage solutions by using a commercially available murine IFN-γ ELISA kit. That finding prompted us to evaluate IFN-γ levels in the SL, EL, BAL, and serum samples in this model. Since IFN-γ has a short half-life and systemic increase of IFN-γ may cause significant systemic illness,17,18 we hypothesized that increase of IFN-γ may be localized to the adjacent mucosal tissues of the inflamed sinus, unlike anti-Bacteroides IgG antibody levels.

Our results revealed a significant increase of IFN-γ levels in the inflamed and contralateral sinuses as well as in the middle ear. This finding is consistent with our previous observation of detectable IFN-γ mRNA in sinus and ear mucosa.13 In the contralateral sinus and ears, IFN-γ levels began to decrease at 4 weeks after inoculation, although they remained high in the inflamed sinus. At 4 weeks, bacterial cultures remained positive in the inflamed sinus.3 Thus, our finding may indicate a negative feedback mechanism of immune defense in the contralateral sinus and ears, since excessive T1 cytokines could cause detrimental tissue damage.3–5,9,17,18

In contrast to anti-Bacteroides IgG antibody levels, IFN-γ levels in the serum remained low and did not change during the observation period. Interferon gamma levels in the BAL solutions tended to be slightly higher.
than those in the sinus or ear in control rabbits. This may be owing to preexisting low-grade infection in the bronchial airway in these rabbits; these animals were housed in a conventional animal care facility and were not treated with antibiotics. Interferon gamma levels in the BAL solutions were not altered following bacterial inoculation into the left sinus, unlike anti-Bacteroides IgG antibody levels. This may be associated with a dilution factor at the time of sampling. However, IFN-γ levels were standardized with protein concentrations in the samples. It is also possible that negative feedback may have occurred in the lower airway to prevent excessive rise of IFN-γ levels. Had rabbits been housed in a specific pathogen-free environment, we might have observed an increase in IFN-γ levels in the BAL solutions. Alternatively, the fact that IFN-γ levels were not altered following bacterial inoculation may be associated with difference of anatomical structures of upper and lower airways between rabbits and humans; postnasal dripping is less likely to occur in rabbits.11

In summary, this study revealed a rise in levels of IFN-γ, a proinflammatory T1 cytokine, in the middle ear and contralateral sinus in a rabbit sinusitis model in which inoculated Bacteroides was localized in the inflamed sinus. Our results suggest proinflammatory T1 responses in the middle ear and contralateral sinus as part of a mucosal immune defense in response to localized microbial infection in the sinus. Interferon gamma production is positively and negatively regulated by multiple cytokines, including IL-10, IL-12, IL-18, and TGF-β (transforming growth factor β).19-21 Excessive T1 responses in the middle ear may lead to serious otitis media in a subset of patients with chronic sinusitis. It may be rewarding to analyze these cytokines in EL fluid from patients with chronic sinusitis and/or chronic otitis media.

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