Objective: To evaluate distribution of IgG antibodies (Ab) in the airway, ear, and sinuses in association with inflammatory changes in a rabbit sinusitis model.

Design: We measured IgG Ab and lactate dehydrogenase levels in solutions from sinus, airway, and middle ear lavage and in serum, and determined interferon γ messenger RNA expression in sinus and ear mucosa at 1, 2, 3, and 4 weeks after inoculation with *Bacteroides fragilis*.

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

Intervention: *Bacteroides fragilis* was inoculated into the left maxillary sinus with ostium closed.

Results: IgG Ab was undetectable in all controls. IgG Ab (>50 µg/g protein) was present at 2, 3, and 4 weeks in most bilateral sinus lavage samples and in 2 of 6, 5 of 6, and 6 of 10 ear lavage samples at 2, 3, and 4 weeks, respectively, following inoculation. Inflammatory changes (histological and lactate dehydrogenase) were much greater in the inflamed sinus. IgG Ab (>50 µg/g protein) was also detected in most bronchoalveolar lavage samples after 2 weeks. Interferon γ mRNA was undetectable in all untreated and most sham-operated controls but was detected in the bilateral sinus mucosa at 1 to 2 weeks, and remained detectable up to 4 weeks in most rabbits. Serum IgG Ab levels positively correlated with those in lavage samples, with highest correlation with right sinus lavage IgG Ab levels (r = 0.56, P < .001).

Conclusion: IgG Ab levels in the upper airway mucosa likely increase within 2 weeks following bacterial inoculation as a part of mucosal immune responses independent of tissue necrosis.


Chronic sinusitis is a common but often debilitating disease, leading to diminished daily activity levels and consumption of substantial medical resources. Sinus inflammation may also cause increased airway responsiveness, especially in patients with asthma, and middle ear inflammation. In recurrent sinopulmonary infections, especially in children, poor antibody (Ab) responses are implicated with pathogenesis of chronic sinusitis. However, most patients with chronic sinusitis exhibit robust Ab responses against pathogens commonly cultured from the sinus fluid. It may be that local mucosal immune responses but not systemic immune responses are more closely associated with development of chronic sinusitis. In other words, intricate balance of inflammatory and immune responses in the sinus may determine the outcome of sinus inflammation. However, little is known about mucosal immune responses in chronic sinusitis that may be associated with difficulty in obtaining sinus lavage and biopsy samples from patients with sinusitis and healthy control subjects.

In a rabbit model of sinusitis induced by *Bacteroides fragilis*, we observed a rapid rise of serum IgG Ab levels against *Bacteroides*, characteristic for robust secondary humoral immune responses. Although sinus inflammation persisted in the inflamed sinus of bacterial inoculated rabbits, dissemination of infection was prevented in most animals in this model. Immune responses in the sinus and airway may have effectively encompassed the induced sinus inflammation and increase of serum Ab may be associated with more generalized airway mucosal immune responses in this model. In this study, we hypothesized that IgG Ab recruited or produced in the inflamed sinus and associated mucosal tissues partly determines the degree of pathogen dissemination and outcome of sinusitis.
Materials and Methods

Animals

Adult male New Zealand white rabbits (Birchwood, Red Wing, Minn) (weight, 2.5–3.5 kg) were used. Rabbits were kept in separate cages in the animal facility at the University of Minnesota, Minneapolis, and fed regular chow (Rabbit Chow, Complete Blend, Purina Mills, St Louis, Mo). The experiments were 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.17

Experimental Design

Rabbits were inoculated with Bacteroides fragilis as described in the “Surgical Procedures” subsection. Five to 7 days before surgery, presurgery serum samples were drawn. At 1, 2, 3, and 4 weeks following inoculation, SL, BAL, and EL samples, sinus and ear mucosa, and postsurgery serum samples were obtained. Bilateral sinus and ear samples were also obtained at the same time as the other samples. As markers of mucosal immune responses, we measured IgG Ab levels of EL, SL, and BAL samples and IFN-γ mRNA expression in the mucosa. These values were compared with serum IgG Ab levels and LDH levels in lavage samples at each time point. Three experiments were performed, and, in each experiment, 2 rabbits were used at each time point. Samples of BAL and mucosa were obtained in the latter 2 experiments. As controls, the same variables were examined in the sham-operated rabbits at 2 and 4 weeks following the operation; 4 to 5 rabbits were tested at each time point. Samples of BAL, EL, SL, sinus mucosa, and peripheral blood from 5 untreated rabbits served as additional controls. The results of bacterial cultures, sinus mucosal histology, cell number and cytology, white blood cell count and cell differential, and peripheral blood and SL cellular responses obtained in these experiments have been reported elsewhere (Sun et al10 and H.J.; S.S.; C.A.K; Anie Roche, PhD; James Miller, BS; Gregg Germaine, PhD; K.C.K.; and F.L.R., unpublished data, March 9, 1998).

To assess a role of IgG Ab in mucosal immune responses in this rabbit sinusitis model, we measured IgG Ab levels against Bacteroides in the sinus lavage (SL), ear lavage (EL), and bronchoalveolar lavage (BAL) samples at 1, 2, 3, and 4 weeks following bacterial inoculation. Upon pathogen invasion, resting-stage T cells are activated into type 1 and type 2 (T1 and T2) effector-stage T cells, and T1 cells produce a large amount of interferon γ (IFN-γ) that in turn mediates subsequent proinflammatory immune responses.12,14 T1 cell responses are commonly associated with intracellular viral and bacterial pathogens including Bacteroides.13,16 Tissue necrosis also occurs at the site of pathogen invasion, resulting in liberation of cytoplasmic enzymes such as lactate dehydrogenase (LDH). Thus, we also evaluated how IgG Ab levels were associated with changes in IFN-γ messenger RNA (mRNA) tissue expression (as a marker of T-cell–mediated proinflammatory responses) and LDH levels (as a marker of necrosis) in lavage samples, and serum IgG Ab levels. Our results indicate that IgG Ab may be recruited or produced not only in the inflamed sinus but also in the contralateral sinus, airway, and even in middle ears, independent of tissue necrosis.

Surgical Procedures

Induction of Sinusitis

One colony of B fragilis was grown overnight in Todd-Hewitt broth in an anaerobic chamber. Anesthesia was initiated using an intramuscular injection of diazepam (1.3 mg/kg), followed by an intramuscular injection of a mixture (0.25–0.3 mL/kg) of fentanyl citrate (0.315 g/L) and fluanisone (10 g/L) (Research Diagnostics, Flanders, NJ). The mixture was administered every 40 minutes (0.15 mL/kg). The left maxillary sinus was opened by drilling through the bony ceiling of the nasal dorsum and the maxillary ostium was closed with a sterile cotton plug and cyanoacrylate ester (Super Glue). Bacteroides fragilis (10⁴ plaque-forming units in 0.25 mL of sterile phosphate-buffered saline [PBS]) was injected onto the cotton plug. The opening in the bony ceiling was closed with orthodontic resin. Occasionally, bleeding was controlled by applying a solution of cocaine hydrochloride (20%) with a cotton-tipped applicator. After the surgical procedure, each rabbit was examined every day for evidence of disseminated infection. In the sham operation, the ostium was closed in the same way after sterile PBS (0.25 mL) was injected onto the cotton plug placed in the sinus.

Sampling of SL, BAL, EL, and Sinus Mucosa

Under anesthesia, SL samples were obtained by gently flushing the left maxillary sinus with 2 to 3 mL of sterile PBS after removing the cotton plug. Serum samples were obtained by venipuncture through an auricular vein. The rabbits were then humanely killed with an overdose of pentobarbital sodium (100 mg/kg) placed in the sinus mucosa. Ear mucosa, EL, and BAL samples were obtained; BAL samples were taken by flushing sterile PBS through a tracheal tube placed into a right bronchus.

Analytical Methods

Humoral Immune Responses

Bacterial cell lysate was prepared by centrifuging overnight cultures of stationary phase cells for 20 minutes at 14 000g,
washing twice with PBS (pH 7.3) with proteinase inhibitor (1 mmol/L) (Leupeptin, Boehringer Mannheim, Indianapolis, Ind.), and EDTA (1 mmol/L), and resuspending the pellet to the same washing buffer. IgG Ab levels against the whole bacterial extract were assessed by enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay plate (Nunc, Naperville, Ill.) was coated with bacterial whole extract (3 mg/L) in a coating buffer (sodium bicarbonate, 0.1 mol/L, pH 8.3) overnight at 4°C and incubated with PBS and fetal calf serum (10%) for 1 hour at 37°C. The plate was washed with PBS (pH 7.4) with polyborate 20 (Tween 20, 0.5 mL/L) and serum samples diluted with dilution buffer (0.05 mol/L Tris [pH 8.1], 1 mmol/L magnesium chloride, 15 mol/L sodium chloride, 0.05% polysorbate 20, 0.2 g/L sodium nitrate, and 10 g/L bovine serum albumin) up to 50 µL/well were incubated for 2 hours at room temperature. Then the plate was washed and incubated with anti-rabbit IgG Ab conjugated with alkaline phosphatase (1:4000, Harlan, Indianapolis) for 2 hours at room temperature. After washing, the color was developed by adding substrate solution (100 µL/well, 104 Phosphatase Substrate Tablet, 1 tablet/5 mL Sigma Chemical Co, St Louis, Mo) at room temperature. Optical density was measured at 450 nm with 630 nm as a reference value. The IgG Ab levels were quantified using known quantities of rabbit IgG captured by anti-rabbit IgG Ab as standards. IgG Ab levels in the lavage samples were measured using sequentially diluted samples starting from undiluted ones; typically 6 to 8 two-fold sequential dilution for each sample. As for serum samples, initial screening was performed using 1:10, 1:100, 1:1000, and 1:10000 diluted serum samples. Depending on the results, 2-fold sequentially diluted serum samples were used for quantification, starting at 1:10, 1:100, or 1:1000 dilution; 10 to 12 two-fold sequential dilution was used for each sample. IgG Ab levels that were above the undetectable level by enzyme-linked immunosorbent assay (>3.15 µg/L) were expressed as micrograms per grams of protein. Protein levels in lavage and serum samples were determined by the Lowry assay. IFN-γ mRNA Expression

Expression of IFN-γ mRNA was assessed by semiquantitative reverse transcriptase polymerase chain reaction (PCR) using glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA expression as an internal control. Primers were synthesized on the basis of complementary DNA sequence of rabbit G3PDH (GenBank data bank at National Institutes of Health, Bethesda, Md) and rabbit IFN-γ. Harvested sinus mucosal samples were kept in liquid nitrogen and homogenized with a reagent (TRI REAGENT, Molecular Research, Cincinnati, Ohio). RNA was extracted, and single-stranded DNA was synthesized as described previously. Semiquantitative PCR was done by adding 1 set of primers for IFN-γ and another set of primers for G3PDH at the same time: 1 cycle at 94°C for 5 minutes, 25 to 35 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 7 minutes, and 1 cycle at 6°C for 6 minutes. Quantities of the electrophoresed PCR products were analyzed by a densitometer and standardized to an internal control (Bioline Whole Band Analyzer, Millipore, Ann Arbor, Mich). The results were expressed as integrated optical density of IFN-γ PCR product divided by integrated optical density of control G3PDH PCR product.

LDH Levels

Lavage samples were centrifuged at 1500 rpm for 5 minutes and the supernatant was harvested and frozen at −20°C until the day of measurement. Serum samples were also kept frozen at −20°C. Levels of LDH were measured using an LDH kit (EC1.1.1.27 UV-test, Sigma Chemical Co) and results were expressed as units per gram of protein.

STATISTICS

Statistical analysis was performed using commercially available software (SPSS for Windows, SPSS Inc, Chicago, Ill). Equality of 2 means was evaluated by the Student t test or by the Mann-Whitney test depending on distribution pattern of samples (normal vs skewed). Comparison of multiple values was done by the Kruskal-Wallis test or analysis of variance using raw data or z score. Correlation of 2 variables was assessed by the Kendall τ-b test. Correlation of multiple variants was assessed by stepwise regression analysis using log-transformed data.
IFN-γ mRNA was detected in the sinus mucosa bilaterally as early as 1 week following inoculation of *B. fragilis* and remained detectable in the left (inflamed) sinus mucosa in all the bacterial inoculated rabbits up to 4 weeks (Figure 4). In the right sinus, IFN-γ mRNA was detected in 4 of 4, 3 of 4, and 2 of 4 inoculated rabbits at 2, 3, and 4 weeks, respectively (Figure 4). In sham-operated rabbits, IFN-γ mRNA was detected at 4 weeks in 2 of 4 rabbits that developed sinusitis by colonized *Pasteurella* but undetectable in those that did not develop sinusitis with colonized microbes. In the last set of experiments, IFN-γ mRNA expression was also examined in middle ear mucosa. Expression of IFN-γ mRNA was detected at 2 to 4 weeks following inoculation, while IFN-γ mRNA was detectable in none of ear mucosal samples from untreated or sham-operated rabbits (data not shown).

**CORRELATION OF LAVAGE IgG Ab LEVELS WITH LEFT SL AND SERUM IgG Ab LEVELS**

IgG Ab levels in the left sinus positively correlated with IgG Ab levels in the serum, right sinus, right and left EL, and BAL samples (Table). Right SL IgG Ab levels showed highest correlation with those of the left sinus (Table). Likewise, serum IgG Ab levels correlated with IgG Ab levels in most lavage samples (Table). Stepwise linear regression analysis was performed using log-transformed data of IgG levels in lavage and serum samples with left SL IgG Ab level as the dependent variable. Although la-
The study determined distribution of IgG Ab in the airway, middle ear, and sinus fluid in a rabbit model of sinusitis induced by *B. fragilis*. In this model, microbes were inoculated into the left maxillary sinus with the ostium closed. IgG Ab against *Bacteroides* were detectable in the inflamed and uninfamed sinuses, airway, and middle ear within 2 weeks following bacterial inoculation, although sinus inflammation was localized to the left inflamed sinus.10 Mucosal IFN-γ mRNA expression was also noted in both sinuses and middle ears. To our knowledge, this is the first report describing the distribution of IgG Ab against causative microbes, and our results indicate that changes in IgG Ab levels likely reflect mucosal immune responses independent of tissue inflammation.

In a rabbit sinusitis model with *B. fragilis*, we observed previously that sinus inflammation was persistent but localized in the bacterial inoculated sinus up to 4 weeks.10,11 In the inflamed left sinus, we observed marked cellular infiltration dominated by neutrophiles, increased apoptotic changes, and elevated LDH levels. In contrast, most inflammatory variables remained low and unchanged in the contralateral sinus and airway (BAL) in parallel to minimal dissemination of *Bacteroides*.10 Likewise, white blood cell and differential cell counts and serum LDH levels remained equivalent to those of control rabbits up to 4 weeks following inoculation. The only notable changes detected outside the inflamed sinus were increased goblet cells and glands in the contralateral sinus and ear mucosa and a rapid rise of serum IgG Ab against *Bacteroides*. Antibody deficiency has been implicated with treatment-resistant sinusopulmonary infection and dissemination of common bacterial pathogens.6,7 This may suggest that an increase of serum IgG Ab is associated with prevention of bacterial dissemination to adjacent organs.

IgG is a major immunoglobulin in the mucosal lumen and appears to play a role in first-line mucosal immune defense. However, IgG Ab recruited to the site of pathogen invasion also helps eradicate intracellular pathogens such as *Bacteroides* by activating phagocytic cell-mediated cell lysis and complement cascade.12-16 Thus, IgG Ab levels in the SL, EL, and BAL solutions are informative to address a role of IgG Ab in this rabbit model of sinusitis. To address proinflammatory immune responses induced by *Bacteroides*,12-15 we opted to measure IFN-γ mRNA expression by sinus mucosa that is more specific for T-cell-mediated proinflammatory responses.12,15 We were unable to measure rabbit IFN-γ protein levels and IgA Ab levels, since necessary reagents for the assay were not available. We could not detect IFN-γ mRNA expression by cultured SL cells either, probably due to poor viability of SL cells (H.J.; S.S.; C.A.K; Anie Roche, PhD; James Miller, BS; Gregg Germaine, PhD; K.C.K.; and F.L.R., unpublished observations, March 9, 1998).

IgG Ab was detectable at 2 weeks following bacterial inoculation and remained detectable up to 4 weeks in both sinuses, BAL, and about two thirds of EL samples. IgG Ab levels were equivalent in bacterial inoculated and contralateral sinuses with approximately 2-fold higher protein levels in the left sinus than in the right sinus. IgG Ab levels in BAL samples were about half that of the levels found in the SL samples. However, LDH levels in BAL and contralateral SL samples were much lower than those in the inflamed sinus. Histological changes in the right (uninfammed) sinus were far less remarkable than those in the inflamed left sinus.10,11 Moreover, there was no correlation between IgG Ab and LDH or protein levels in the lavage samples. These results make it unlikely that tissue destruction and subsequent infiltration of plasma caused a rise of IgG Ab in the SL and BAL solutions. Instead, it is reasonable to postulate that IgG Ab against *Bacteroides* was actively recruited to the sinus and airway lumen as a part of mucosal immune responses that involve not only the inflamed left sinus but also the contralateral sinus, airway, and possibly the middle ear. Since there was little evidence of bacterial dissemination in adjacent organs,10 a rise of IgG Ab levels in the adjunct airway mucosal tissues may be mediated by putative inflammatory and immune mediators such as cytokines and neuropeptides other than bacterial antigens.12,15

We detected the rise of serum Ab levels at 1 week,10 but IgG Ab in the SL, BAL, and EL samples was only detectable at 2 weeks (not at 1 week), although their levels were positively correlated. Pathogen invasion generally induces rapid IgG Ab production by memory B cells in the lymph node and recruitment of memory T cells at the site of inflammation, followed by subsequent antigen-specific responses.12,15 Our results suggest that a rapid rise of serum IgG Ab levels may effectively prevent hematogenous dissemination of *Bacteroides*. Serum IgG Ab may also be mobilized into the mucosal lumen of the sinus, airway, and possibly middle ear as a consequence of sinus inflammation induced by *Bacteroides*. Alternatively, IgG Ab in lavage fluid may be produced by plasma...
and activated memory B cells in the mucosa or regional lymph nodes.

Expression of IFN-γ mRNA was detected at 1 week following bacterial inoculation before the appearance of IgG Ab in the SL, EL, and BAL solutions. To our knowledge, this is the first report determining IFN-γ mRNA expression in rabbit models. Interferon γ mRNA that was undetectable in control rabbits became detectable bilaterally in the sinus mucosa following inoculation, although Bacteroides was inoculated into the unilateral (left) sinus. Because production of proinflammatory cytokines including IFN-γ occurs rapidly as a part of first-line mucosal defense, our finding may not be surprising. However, our results indicate that proinflammatory immune responses in the mucosa occurs not only in the inflamed sinus but also in the adjunct mucosal tissues including sinus and middle ear. To our knowledge, this is the first report to demonstrate such generalized mucosal immune responses against localized inflammation in a rabbit sinusitis model.

At 4 weeks, IFN-γ mRNA was undetectable in the right sinus of 2 of 4 bacterial inoculated rabbits and we observed a decline of IgG Ab levels in the serum, although lavage IgG Ab levels remained elevated. In response to intracellular pathogens, the body generates proinflammatory immune responses mediated by so-called proinflammatory cytokines (IFN-γ and interleukin 12). To prevent detrimental effects of excessive proinflammatory responses, the actions of these cytokines were counter-regulated by other cytokines, including interleukin 10, transforming growth factor β, and interleukin 4. Such counter-regulatory immune defense may be associated with an apparent decline of mucosal immune responses at 4 weeks, in spite of the persistent inflammation and positive bacterial culture in the left sinus at 4 weeks.

In summary, this is the first report to demonstrate generalized mucosal immune responses against localized bacterial inflammation in a rabbit sinusitis model that might have minimized bacterial dissemination. Nevertheless, sinus inflammation persisted. Further studies to examine mucosal immune responses at later than 4 weeks in this model may help address roles of mucosal immunity, cytokine regulatory mechanisms, and microenvironmental factors for the development of chronic sinusitis.

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