Antibody in Middle Ear Fluid of Children Originates Predominantly from Sera and Nasopharyngeal Secretions

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The human middle ear is devoid of any immunocompetent cells in normal mucosa. We sought to determine the source of antibody present in the middle ear. To address this question, we measured levels of immunoglobulin G (IgG) and immunoglobulin A (IgA) in serum, nasopharyngeal wash (NW), and middle ear fluid (MEF) samples of children with acute otitis media. The two-dimensional gel electrophoresis pattern of the entire array of IgA antibodies in the nasal wash (NW) and middle ear fluid (MEF) was compared from the MEF and NW samples using isoelectric focusing and Western blotting. The total IgG and IgA antibodies in the MEF and NW samples of 137 children were compared. The ratio of IgG to IgA in the MEF was significantly different (P < 0.008) compared to NW because IgA levels were higher and IgG levels lower in NW. The IgG/IgA ratio of MEF resembled serum consistent with transudation to the MEF. Small amounts of secretory IgA were detected in MEF but the electrophoresis patterns of the entire array of IgA antibodies in the MEF and NW were virtually identical in each child evaluated; thus, IgA in MEF derived predominantly from serum and the nasopharynx by reflux up the Eustachian tube. The IgG/IgA antibody levels in the MEF and the same composition of IgA antibody in the MEF and NW identifies the predominant source of antibody in the MEF as a transudate of serum combined with nasal secretions refluxed from the nasopharynx in children.

Colonization of the nasopharynx (NP) by respiratory bacterial pathogens produces both a systemic and a mucosal immune response (1, 10, 11, 15, 22, 26, 38). Local mucosal immunity in the NP plays a crucial role in the reduction of carriage and prevention of local disease (acute otitis media [AOM], sinusitis, and pneumonia) and systemic invasion by respiratory bacteria (4, 16, 20–22, 27, 36). Protective mucosal immune responses are most effectively induced by mucosal immunization through oral and nasal routes but the vast majority of vaccines in use today are administered by injection (19). Parenteral administration of Haemophilus influenzae type b and Streptococcus pneumoniae polysaccharide and polysaccharide-conjugate vaccines can induce a transient mucosal immune response but immunologic memory at the mucosal level is generally not induced (23, 24, 37).

Current pneumococcal conjugate vaccines for prevention of AOM and pneumonia are administered by injection and are considered to be effective by generation of antibody in serum and transudation of antibody to the NP, middle ear, and lung (3, 12, 25, 34, 37). New vaccines for the prevention of Streptococcus pneumoniae and nontypeable Haemophilus influenzae AOM and other mucosal infections are in development (2, 6, 11, 21, 22). Therefore, the sources of antibody at the site of infection (the middle ear) need to be understood.

Although the normal mucosa of the middle ear possesses neither immunocompetent lymphocytes nor associated lymphoid tissues, there are studies which have demonstrated the presence of secretory IgA (sIgA) in middle ear effusions and IgA plasma cells in the mucosa of the middle ear suggesting that a local immune response in the middle ear is possible and may contribute to resolution of or protection from infection (8, 9, 18). Previous studies have also shown severalfold more IgG antibody compared to IgA antibody in the middle ear, suggesting that antibodies in the middle ear arrive by transudation from serum (33). In the present study, we evaluated (i) simultaneous concentrations of IgG and IgA in serum, NW, and MEF samples in children with AOM; (ii) simultaneous concentrations of sIgA in NW and MEF samples; and (iii) the electrophoresis pattern of the entire array of IgA antibodies in the MEF and NW to determine whether the antibodies present in middle ear fluid were predominantly derived from serum and the nasal passageways by reflux up the Eustachian tube.

**MATERIALS AND METHODS**

**Patient population and sample collection.** Children with AOM included in the present study were 6 to 30 months of age, recruited as part of a prospective cohort as previously described (11, 22). The study was approved by the University of Rochester and Rochester General Hospital Research Subjects Review Board and written consent obtained from parents or guardians for the child to participate.

Nasopharyngeal wash (NW), middle ear fluid (MEF), and blood samples were collected from children at the time of diagnosis of AOM. For NW samples, 1 ml of sterile PBS was instilled and aspirated from each nares. For MEF, tympanocentesis was performed. NW samples varied from 0.5 to 2.0 ml of fluid recovered. MEF samples varied from 50 to 250 μl; the entire sample was added to 500 μl of phosphate-buffered saline (PBS). NP wash and MEF samples were inoculated into Trypticase soy broth, Trypticase soy agar with 5% sheep blood plates, and chocolate agar plates. Bacteria were isolated according to standard laboratory culture procedures to confirm infection.

**Enzyme-linked immunosorbent assay (ELISA).** (i) Total IgG, IgA, and sIgA determination. For total IgG and IgA antibody response, a saturating 1-μg/ml solution of IgG and IgA capture antibody (Rockland) stock was made in bicarbonate coating buffer (pH 9.4). The
performed. For serum samples the starting dilution was around 1:10^6 (in PBS–3% skim milk) was added to the wells, and 2-fold serial dilutions were performed. For serum samples the starting dilution was around 1:10^6 for IgG and 1:10^5 for IgA. The plates were allowed to incubate for 1.5 h at 37°C. After three washes, horseradish peroxidase (HRP)-conjugated goat anti-human IgG or IgA secondary antibody was added to the plates, followed by incubation at 37°C for 1 h. The plates were washed three times with PBST, and 20 μl of TMB microwell peroxidase substrate solution (KPL, Gaithersburg, MD) per well was added. The reactions were allowed to develop at room temperature for 20 min and stopped by the addition of 1.0 M phosphoric acid. Plates were read at 450-nm wavelength with a 630-nm reference. An ELISA titer of a mucosal antibody is defined in μg/ml as measured against a standard human reference serum (Bethyl catalog no. RS10-110-3). On each plate the reference standard and samples were run in duplicate, and the sample was evaluated again with a higher starting dilution of serum, and/or NW if that particular sample did not achieve the end-point. A negative control without serum was included on each plate to establish the background.

(ii) sIgA ELISA. An sIgA ELISA kit (Immunodiagnostik AG Bensheim) was used for the quantitative determination of sIgA in NP wash and MEF samples using standards and controls provided in the kit. Some of the serum samples were also tested along with NP wash and MEF samples collected from the same children.

2D gel electrophoresis of NW and MEF samples for IgA separation and Western blot detection. NW and MEF samples were characterized by two-dimensional (2D) gel electrophoresis. Briefly, 20 μl of each NW and MEF sample from children with AOM was subjected to isoelectric focusing on 7-cm, pH 4 to 7 immobilized pH gradient (IPG) strips on a Bio-Rad Protean IEF apparatus. After focusing, IPG strips were equilibrated, and further separation was achieved by gel electrophoresis on 12% polyacrylamide gels. Once the 2D separation was completed, proteins from each gel were transferred onto a nitrocellulose membrane using a Bio-Rad Transblot semidry transfer apparatus. Membranes were then washed repeatedly in Tris-buffed saline (TBS) buffer, followed by washes in TBS buffer plus 0.1% Tween 20 (TTBS), and blocked with milk protein (5% dried milk in TTBS). Membranes were then washed in TBS buffer and incubated for 2 h with HRP-conjugated antibody to IgA (Rockland Immunochemicals, 10 μg/15 ml of TBS). Antibody-treated antibodies were then washed as described above and treated with Lumiglo Reserve chemiluminescent reagent (KPL) for 1 min. The membranes were then blotted dry and imaged using a charge-coupled device camera-based imaging system.

**Results**

**Total IgG and IgA in NW, MEF, and serum.** Total IgG and IgA were quantitated in 137 NW samples, 137 MEF samples, and 34 serum samples from 137 children with AOM. The dilution of the NW and MEF samples was variable since the quantity of secretions recovered by the NW, and the quantity of the MEF was variable. Therefore, we compared the ratio of IgG/IgA since the ratio would not be effected by dilution, only the absolute quantitation. The ratio of IgG to IgA was not significantly different comparing the serum to MEF, suggesting no significant local production of IgA in the middle ear space at the acute onset of AOM (Fig. 1). In contrast, the IgA levels were significantly higher in NW samples compared to MEF samples (P < 0.008) and sera (P < 0.001), suggesting local IgA antibody production in the nasopharynx. Comparatively, the total IgG concentration was low in the NW samples relative to IgA, unlike the MEF and serum samples.

**sIgA detection.** We measured the total sIgA antibody levels in the NW and MEF of 15 children (Fig. 2A) and found a significantly higher amount in NW compared to MEF (P = 0.02). Serum samples had no detectable amounts of sIgA in our assay. The amount of sIgA in the samples was normalized with total IgA as a denominator since the quantity of secretions and MEF recovered was variable. sIgA was detected in both NW and MEF but the concentration of sIgA versus that of total IgA in
ance that the IgA in the MEF and NW samples are virtually identical.

NW samples was 7 times higher than in MEF once adjusted for total IgA (P = 0.004, Fig. 2B).

Isoelectric focusing to determine the pattern of the entire array of IgA antibodies in the MEF and NW. After induction of a mucosal antibody response at a mucosal site, the trafficking of B cells occurs to regional lymphoid tissue. After antigen processing, individual B cells mature, migrate to effector mucosal sites, and become antibody-producing plasma cells. Each B/plasma cell produces a unique clonotype of the antibody. The middle ear site is a part of the nasal associated lymphoreticular tissues (NALT), then unique antibody clonotypes should be present in the MEF compared to the NP. To assess the entire array of IgA antibodies in NW and MEF samples, we used 2D isoelectric focusing in 12 paired samples from children. Figure 3 shows the entire array of IgA antibodies of MEF (A) and NW (B) from one child. IgA polypeptides have been separated according to their isoelectric point with a dominant set of protein bands that falling between pH 5 and 6. Each pI band appears to have separated into a pair of peptides with differing sizes that preserve their respective pI values. Seven of the major pI bands are shared between both the MEF and nasal wash samples and account for nearly all detectable IgA in the child. This result shows that the IgA antibody found in MEF is indistinguishable from the NW, suggesting that the detected IgA in MEF originates predominantly from the NP by reflux up the Eustachian tube to the middle ear space.

DISCUSSION

The pathogenesis of AOM involves NP bacterial colonization, followed by ascension of the pathogen up the Eustachian tube into the middle ear. Current pneumococcal conjugate vaccines diminish bacterial NP carriage and reduce the likelihood of developing AOM by stimulation of high antibody levels in serum that transudate into the NP and middle ear space. Here we have provided evidence that the antibody detected in the middle ear during AOM derives predominantly from serum transudation and reflux of nasal secretions containing antibody, with no evidence for independent, local antibody production.

Investigators published two elegant studies in the 1970s that strongly suggested local production of pathogen-specific antibodies was occurring in the middle ear space of children with AOM (7, 28). Our results are not inconsistent with their observations since they did not investigate the possibility that the origin of the pathogen-specific antibodies identified in the middle ear space of children with AOM were not from local production but rather from reflux of nasopharyngeal secretions. The mucosal immune system is divided into two parts: mucosal inductive sites and mucosal effector sites (13, 17). Effector sites are characterized by the predominance of polymeric IgA (pIgA)-secreting plasma cells in the lamina propria or glandular stroma, and the expression of polymeric immunoglobulin receptor on the basalateral surface of epithelial cells that take up and transport pIgA to form secretory IgA. Inductive sites are mucosal sites with organized lymphoid follicles and specialized epithelium that includes M cells, which sample antigenic material from the lumen and transport it to the underlying lymphoid cells. After the antigenic stimulation, IgA committed B cells and regulatory T cells emigrate and circulate until they relocate at effector sites where the B cells terminally differentiate into pIgA secreting plasma cells. Collectively the organized follicular inductive tissues are referred to as mucosal-associated lymphoid tissues and serve as the main source of mucosal pIgA secreting cells. In the nasopharyngeal area, the inductive sites include NALT, where they encounter environmental antigens and become activated.

In mouse model studies, the normal mucosa of the middle ear has only a few immunocompetent cells, but acute and chronic inflammation results in significant recruitment of T cells, B cells, macrophages, dendritic cells, and NK cells (8, 9, 30). Studies by Kodama et al. (14) have shown T cells in the middle ear mucosa of mice after intranasal immunization with vaccine candidate P6 protein of Haemophilus influenzae. Also, memory T cells, as well as P6-specific IgA-forming B cells, were detected in the middle ear mucosa. The findings of Kodama et al. suggested that antigen-specific IgA-forming B cells primed in NALT of mice might home to the middle ear mucosa and differentiate into IgA-producing plasma cells. Suenaga et al. (29) also examined the characteristics of the lymphocytes in the middle ear mucosa of mice at the single cell level after intranasal immunization. Their results suggest that the middle ear mucosa has the same function as the nasal mucosa as an effector site in NALT.

Our results in children suggest that if local production of antibody occurs in the MEF that the contribution is relatively small (and undetectable in 2D gel electrophoresis) compared to the contribution from serum and nasal secretions refluxed to the middle ear from the NP at the onset of AOM. If the middle ear is an effector site, the IgA antibodies produced in the middle ear should be of a different clonality than that produced in NP. Our comparison of NP fluid and MEF using isoelectric focusing showed that the middle ear IgA antibodies showed the same IgA bands, with identical pI values. The result suggests that antibodies detected in MEF predominantly originate from transudation of serum and by reflux of secretions from the NP. Reflux of secretions from the NP to the middle ear is possible because of the angle and immaturity of the Eustachian tube in children and infants, and the supine position in which infants are often placed. Studies have shown the presence of gastric pepsin/pepsinogen levels in the middle ear severalfold higher than serum, indicating reflux of gastric fluid via the Eustachian tube into the middle ear (5, 31, 32). In normal adults, Winther et al. (35) showed radiopaque contrast dye placed in the NP reaches the middle ear during swallowing and yawning.

Our isoelectric focusing study has limitations. The presence of two differently sized peptides at each pI value cannot be directly detected in the context of our experiments. The size differential was uniform across the entire isoelectric focusing range, which was consistently seen across all patient samples. This uniform difference in peptide...
mass, as well as the retention of pl values between larger and smaller peptides, suggests that the mass difference was a function of proteolysis somewhere within the constant region of the IgA heavy chain. When the larger and smaller peptides at each pl value were considered in sum, the density of IgA clonotypes at a given pl value were qualitatively equivalent. A small amount of antigen-specific and/or species-specific IgA antibody would not be detectable with this method. The total quantity of IgA in MEF in the samples we tested was very low, and efforts to isolate antigen- or species-specific sIgA and perform isoelectric focusing were unsuccessful.

In conclusion, we suggest that the antibody detected in the middle ear of children derives predominantly from transudation of serum and reflux of IgA from the NP.

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