Role of Immune Responses in Pathogenesis of nontypeable Haemophilus influenzae in Acute Otitis Media in Young Children

Bethany Kopin, 2006

Advised by Michael Pichichero, M.D.

Department of Microbiology and Immunology, University of Rochester

Accounting for approximately one out of three pediatric visits for children two years of age and under, acute otitis media (AOM) is one of the most common illnesses in young children. Most children have their first encounter with AOM before their first birthday. This illness has the potential to have devastating effects on a child’s speech, academic achievement, and social development. Several factors that identify a child at risk for recurrent disease include having the first episode of otitis media early in life, having other family members with the recurrent disease, enrollment in day care, and bottle feeding. In order to minimize the adverse effects of this disease, it is necessary to identify characteristics that may predispose a child to serious AOM infection.

The overall cause of these infections is a bacterium commonly known as nontypeable Haemophilus influenzae (NTHI). This gram negative bacterium colonizes the nasopharynx of children, and then spreads through the Eustachian tube (ET) to the middle ear where an inflammatory response occurs. The success of this organism as a colonizer and pathogen is due to its lack of reliance on any single method of attachment and its ability to respond rapidly to host defense mechanisms by antigenic variation of proteins and enzymes. Another unique characteristic that separates this bacterium from the closely related H. influenza type b is that NTHI lacks a polysaccharide capsule. Since the current vaccine against the H. influenzae type b strain is designed to target the recognized polysaccharide role of immune responses in pathogenesis of nontypeable Haemophilus influenzae in acute otitis media in young children

Fig. 1 Number of Episodes of AOM as a Function of Age

![Graph showing the number of AOM episodes as a function of age in months. The x-axis represents age in months with values ranging from 3 (47) to 15 (56). The y-axis represents the number of AOM episodes with values ranging from 0 to 12. The graph shows a peak around 8 months (50) with a lesser peak around 7 months (63).]
One possible method of intervention is the development of a vaccine that targets specific outer membrane proteins (OMP's). In other studies, the surface protein P6, among others, has shown particular promise for this purpose. P6 is a highly conserved OMP found in all strains of NTHI and is expressed on the surface of NTHI throughout all stages of infection. Therefore, the use of P6 as a vaccine component appears to be the most effective way to inoculate otitis-prone children against NTHI.3

Consequently, one proposed mode of protection against this bacterium is the possibility of an immunologic antibody response to P6, thought to be initially generated by the ET, which is the proposed mucosal effector site of this pathogen. This immuno-competent organ can be activated with appropriate antigenic stimulation and in turn bring about the proliferation of effector molecules known as immunoglobulins.4 Throughout various stages of infection, the dominant immunoglobulin class of antibody produced in humans against the surface protein P6 is IgG.5

Otitis-prone children experience an immunologic abnormality that causes them to experience recurrent infections. Several lines of evidence suggest that antibody levels to P6 are higher in non-otitis-prone children compared to otitis-prone children, implying that antibodies to P6 are relatively protective against otitis media. Moreover, the levels of IgG anti-NTHI antibody decline to the lowest level at six months of age and remain relatively low until two years of age. This range corresponds to the statistical values that show the greatest incidence of otitis media in the first few years of life. This insufficient quantity of IgG in otitis-prone children may account for the failure to recognize P6 as a specific immunogen and for recurrent infections,6 suggesting that otitis-prone children may not respond adequately to a vaccine containing P6.

Another potential method of protection from NTHI is the body's use of bactericidal activity, which describes the body's natural ability to prevent infection by inhibiting the growth or action of microorganisms by the use of specialized immune system components such as complement. This study utilizes these basic body components to further analyze the overall goal of determining a mechanism of intervention against NTHI.

The purpose of this study is to determine specific factors that play a role in immunologic protection against NTHI. These hypothetical factors include antibody against whole cell bacteria, antibody against the surface protein P6, and high levels of bactericidal activity against this organism. The sera of both non-otitis prone and otitis-prone pediatric patients were used to evaluate these aspects of host immunity. Throughout the course of this experiment, various methods and assays, such as ELISAs (NTHI whole-cell, P6), and measurement of bactericidal activity using acute and convalescent phase serum from pediatric subjects with varying otitis media experience, were employed. One of the overall goals of this experiment is to play a role in bringing the current medical community one step closer to the final development of an effective vaccine against NTHI.

### Methods

#### Collection of sera

The samples used were part of an existing collection of sera and matched organisms belonging to Dr. Michael Pichicero. Bacteria were isolated from middle ear fluids of children with AOM during acute phase (day 1) and convalescent phase (day 30) of infection, along with matched sera. At the time of sample collection, a blood draw and tympanocentesis (removal of fluid from behind the eardrum) were performed in compliance with current human subject research regulations. Each subject was given a unique identification that remained confidential.

#### Whole Cell Bacteria ELISA Assay

The Enzyme-linked-immunosorbant-assay (ELISA) assay was used to determine serum (acute and convalescent phase) antibody response to matched organisms isolated during acute phase of disease. Organisms were grown on chocolate agar (BBL Laboratories, MD) at 37°C for 18 hours in CO2 to ensure viability and purity. Colonies were then collected and placed in 10mL of phosphate buffered saline (PBS) until an optical density of 0.5 (OD600) was reached. Next, 100µl of bacterial suspension and 100µl of coating buffer (Kirkegaard & Perry Laboratories (KPL), Gaithersburg MD) were placed into 96-well plates. The plate was incubated at room temperature for one hour. After incubation, contents were tapped out and 200µl of bovine serum albumin working stock (BSA 1:1000 Dilution) (KPL, Gaithersburg MD) was added to each well. Incubation was carried out again for fifteen minutes. Contents were again tapped out after incubation and 100µl of human sera was added to the first 2 columns of the plate. With the exception of the last column (column 12), a ten fold serial dilution was then performed across the remaining columns of the plates. Column 12 was left as a negative control, and therefore no serum was added. After an hour of incubation, wells were tapped out and washed using a washing buffer working stock. 200µl of this solution was added to each well, and contents were tapped out of each well into a waste receptacle for a total of three times. 100µl of diluted secondary anti human IgG of goat origin (alkaline phosphatase conjugate [Biosource Laboratories, Camarillo, CA]) was added to each well. The plate was then incubated for one hour at room temperature. After incubation, the washing step was repeated. An ELISA kit (KPL) was then employed, and prepared according to manufacturer's instructions. Plates were then developed using P-nitrophenylphosphate substrate, also from KPL. After development, absorbance was read on a Dynatech ELISA reader at a wavelength of 405 nm. Absorbance values were divided by controls to yield an absorbance of response index value.

#### P6 assay

ELISA assays were also performed to determine the degree of patient sera immune response elicited against the OMP known as P6. This assay was performed identically to the Bacterial Whole Cell ELISA as described above with the exception of using sera at a consistent dilution of 1:1000. The purified P6 antigen used in this aspect of the experiment was a gift from Dr. Timothy Murphy, SUNY Buffalo, Buffalo, NY.
Bactericidal assay

The serum bactericidal assay is a functional measure of the ability of antibodies in conjunction with complement to kill bacteria and is consistent to measure functional antibodies in vitro. This method was engaged in this study for that purpose.

NTHI isolates were grown in BHI (brain heart infusion) broth (BBL) containing X factor (lysed huRBC), and V
factor (NAD). The bacteria were then incubated at 37° C with shaking until an OD_{490} of 0.8 (~ 4x10^6 CFU/ml) was reached. The log phase bacteria were suspended to 2 x 10^4 CFU/ml in 1 x PCMA buffer (PBS + CaCl_2 + MgCl_2 + 0.1% BSA). Precolostral calf serum was used as a complement source and was diluted to 20% in the bacteria - 1x PCMA mix. Immediately after dilution, the bacteria and complement mix were used in the assay.

The bactericidal assay was performed in a 96 well RB sterile plate. Test sera and control sera were serially diluted in 1x PCMA buffer.
PCMA buffer. Bacteria and complement were added to the wells containing diluted sample. Bacteria + complement alone and bacteria alone were run as controls in parallel with the samples. After incubation, an aliquot from each culture well was plated onto chocolate agar plates (BBL). The plates were then incubated overnight. Bacterial colonies were enumerated and bactericidal titers were expressed as percent killing versus the negative control (bacteria + complement without antiserum).

Results

There appear three groupings of results that allow for easy analysis of the collected data. Organizing the data in this manner facilitates clear understanding of the overall findings. These three groups consist of the immune response in comparison to age, otitis media index (OMI), and phase of infection. The OMI is defined and discussed in its relevant section below.

Group 1

Throughout the course of this experiment there were several underlying factors that contributed a great deal to the overall results and findings. Two of these factors were the subject's age and the subject's number of previous NTHI episodes. The patients range in age from 3-15 months and have different immunological histories and backgrounds. The progression of AOM can be divided into two major phases: An acute and a convalescent phase. The acute phase corresponds to the time period immediately following initial infection, while the convalescent phase corresponds to the time period 30 days after the initial point of infection. As seen in Figure 1, the patients with the highest number of previous NTHI episodes (otitis prone) are those in the age category of 5-8 months. This category correlates with the time period when IgG production is at its lowest point. Other subjects that are outside the 5-8 months range have fewer number of episodes, and are therefore non-otitis prone. This all-time low production is obviously a major contribution to the high number of OM episodes seen at this point.

Figure 2 also reiterates low IgG production between the ages of 5-8 months, showing the acute and convalescent phase bactericidal titer versus age in months. In this case the term titer refers to the reciprocal of the last dilution of a titration giving a measurable effect. Bactericidal activity is found to be weakest once again when IgG production is lowest. Moreover, this illustration also shows the ability to increase immune response and antibody production with age: bactericidal activity increases with age and maturity.

Figures 3 and 4 illustrate the difference between the immune responses mounted against whole bacteria versus P6. Comparing the immune response values obtained, there is a relatively weak acute response and a stronger convalescent response to whole cell bacteria. Inversely, there is a stronger acute response and a relatively weak convalescent response to P6. Collectively, this data shows the overall difficulty to retain memory cells against the antigen P6.

Group 2

The OM index is defined as the number of previous OM episodes divided by the age in months. This factor allows us to consider the subject's age in relation to the prior number of episodes as a single variable. Overall, the lower the OM index, the more immunologically fit the individual.

There is an upward incline in the immune response as
OM index increases, as illustrated in Figure 5, implying an actual higher immune response in subjects who also have a relatively high OM index. Also, the difference in height between the acute and convalescent phases indicates successful immunological memory against whole cell NTHI.

The lower a patient's OM index, the higher the immune response generated, as represented by the downward shift in slope as the OM index increases. Figure 6 shows the OM index in relation to the immune response elicited against the surface protein P6. In this case the convalescent phase response is somewhat lower than that seen in the immune response to whole bacteria. These results most likely suggest that the immune system does not mount as strong of an immune response post-infection, indicating little or no immunological memory retention.

Children with low OM indexes mount a better immune response against the purified antigen P6 than against the whole cell bacteria, as shown in Figures 5 and 6. Inversely, subjects with a higher OM index seem to be better at mounting an immune response against whole cell bacteria than to purified P6.

The following various figures show the multiple relationships held between OM index and immunity against NTHI. For instance, Figure 7 illustrates the OM index in comparison to the bactericidal activity against NTHI. This figure differs from Figure 6 in that there is a significantly lower immune response mounted in the convalescent phase in comparison to the acute phase, implying that little immunological memory was formed against the pathogen. The graph also shows that with a higher OM index there is a lower convalescent immune response. This illustrates that otitis-prone subjects are less efficient in mounting a convalescent immune response.

Group 3
The third grouping allows for careful analysis of the trend seen between phase of infection and immune response. Overall, it demonstrates the dynamics seen between the microbe's pathogenesis and the reaction of the body's immune system. For example, Figures 8-10 comparatively examine the relationship between phase of infection and the average immune response mounted by the patients. Figure 8 shows each phase of the immune response elicited against whole cell NTHI. As seen from the graph, there is a significant difference in immune response between the acute and convalescent phase of infection. In this case the immune response in the convalescent phase is considerably higher than in the acute phase, implying successful immunological memory and more probable chance of clearing the pathogen. Figure 9 differs from Figure 8 in that there is almost no immunological memory retained against the surface antigen P6. Figure 10 depicts the immune response between the acute and convalescent phases to be relatively similar. These results indicate that there is very little memory gained between these two distinct phases of infection with regard to bactericidal activity.

Discussion
Experiments were performed in order to determine the relative immune response elicited against NTHI whole bacteria in otitis-prone versus non-otitis-prone subjects during acute and convalescent phases of infection. The acute phase corresponds to the time period immediately following initial infection, while the convalescent phase corresponds to the time period 30
days after the initial point of infection. The results obtained indicate a general increase in immunity between the acute and convalescent phase of infection, indicating a memory response generated after infection.

We also performed assays in order to find the average immune response elicited against the NTHI OMP known
as P6. The acute and convalescent phases were compared to one another to find the degree of immunologic memory. In addition, the patient’s vulnerability to this pathogen was taken into consideration by noting his or her number of previous episodes. The results obtained indicate that, in most cases, there is little or no immunologic memory generated after the 30 day period, and this memory differs a great deal from the memory formed against NTHI whole cell bacteria. The degree of response obtained from the acute phase of infection is virtually equal to that of the convalescent phase of infection, implying almost no immunologic memory to the OMP P6.

Finally, bactericidal assays were performed to measure the functional ability of antibodies along with complement to kill bacteria. As with the other types of experiments, the acute and convalescent phases were analyzed to find the underlying difference between the immune response of otitis-prone subjects versus non-otitis-prone subjects. The results illustrate the vulnerability of patients between the ages of 5 and 8 months. The bactericidal activities at these points were at an overall low, reflecting the known low production of IgG during this time period. This figure also shows the ability to increase immune response and antibody production with age.

Non-otitis-prone patients are less efficient than otitis-prone individuals in mounting an immune response against whole cell NTHI, but are more efficient in mounting an immune response against purified P6. Although there is a relatively strong immune response mounted against P6 in the acute phase by non-otitis-prone individuals, there is even less of an immune response mounted in the convalescent phase, indicating that there is little or no memory gained upon the primary infection. These observations indicate that the antibodies that recognize the P6 on the pathogen are not protective, and do not explain the non-otitis-prone patient’s intrinsic ability to fight off NTHI infection.

Collectively, the findings from these studies offer something new to the scientific and medical community. A majority of other investigators, such as Dr. Howard Faden, have conducted similar experiments and found P6 to only be a hypothetical vaccine target. The findings here take Faden’s work to the next level and show that P6 is not a protective antigen in the pathogenesis of nontypeable Haemophilus influenzae in acute otitis media in young children.

References