Induction of Functional Secretory IgA Responses in Breast Milk, by Pneumococcal Capsular Polysaccharides

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Capsule-specific secretory IgA (s-IgA) in breast milk may enhance protection against pneumococcal disease in infants. After immunization of 3 lactating mothers with 23-valent polysaccharide vaccine, specific s-IgA, but not IgG, increased by 2-fold in milk of at least 1 subject for 6 of 7 serotypes. The s-IgA was predominantly IgA1, in secretory form, and highly specific with avidity distinct from serum IgA and IgG. Milk whey from 2 immunized women supported dose- and complement-dependent killing of Streptococcus pneumoniae serotypes 19F and 14 by human neutrophils, as did purified s-IgA to serotype 19F. These data reveal that capsule-specific human s-IgA in breast milk can initiate killing of S. pneumoniae, providing proof of concept that vaccine-induced human mucosal s-IgA can support functional bactericidal activity. Determining the biologic role for s-IgA in killing and inhibiting adherence of S. pneumoniae in vivo will contribute to the development of mucosal vaccines against S. pneumoniae.

Rates of invasive infections (e.g., bacteremia and meningitis) with Streptococcus pneumoniae are highest in infancy, particularly in neonates from resource-poor nations [1, 2]. The currently available 23-valent pneumococcal capsular polysaccharide (PS) vaccine is not effective in infants, although a new 7-valent protein-polysaccharide conjugate vaccine is effective in infants [3] and others are under development [4]. However, such vaccines are initiated at age 2 months, precluding neonatal protection. The more-limited capsular serotype distribution of the conjugated vaccines, their multiple-dose regimens, and increased cost may limit general use in many countries in the near future. An alternative vaccine strategy is to immunize mothers either ante- or postnatally with the PS vaccine with the intention of providing passive protection to the infant by active transfer of IgG transplacentally and by transfer of secretory IgA (s-IgA) in breast milk [5]. This approach would have the potential advantages of protecting the infant in the critical neonatal period by use of the currently less expensive and antigenically more-diverse vaccine.

We previously demonstrated that specific mucosal s-IgA responses to 23-valent pneumococcal PS vaccine are shown in the saliva of children immunized at age 13 months after priming with 3 doses of conjugate vaccine in early infancy [6]. Moreover, capsule-specific polymeric serum IgA elicited by both natural infection and immunization supports killing of S. pneumoniae by complement and phagocytes [7, 8]. In 1986, Steinitz et al. [9] reported an anti-serotype 8 monoclonal antibody (MAb) IgA that increases the opsonization of pneumococci by mouse macrophages and induces direct (complement-independent) killing. Myeloid IgA receptors FcμRI (CD89) are important determinants in initiation of phagocytosis by PS-specific IgA derived from serum [10]. Here we extend an earlier report [5] that immunization of lactating women induces s-IgA reactive with pneumococcal capsular PS in breast milk.

Subjects and Methods

Subjects. Three women who delivered healthy infants 40, 43, and 96 days previously, had successfully established breast-feeding, and had ample milk production were given written information about the study by the community nurses providing their postnatal follow up. Breast milk (50–150 mL by electric breast pump or manual expression) and clotted blood were obtained prior to and 2 weeks after a single dose of 23-valent pneumococcal PS vaccine (Pneumovax; Aventis Pasteur-MSD) administered intramuscularly.
in the left deltoid muscle. Samples were transported at 2–8°C to our laboratory within 4 h. Milk samples were centrifuged at 10,000 g for 30 min at 4°C. Milk supernatant (whey) and sera were stored at −70°C until analysis.

**ELISA method.** Microtiter plates (Costar; Corning) were coated at 37°C for 5 h and then overnight at 4°C with 7 separate pneumococcal capsular PS (American Type Culture Collection [ATCC]) at concentrations producing optimal signals (5–10 μg/mL) in PBS (pH 7.3). After washes, 10% fetal bovine serum (FBS; Life Technologies) in PBS was added for 1 h at 37°C, which was shown previously to effectively block nonspecific binding. Each sample supernatant was diluted 1:100 in PBS containing 10% FBS and incubated for 30 min at room temperature with pneumococcal common cell wall PS (CPW; 50 μg/mL) (Statens Serum Institute) and added to triplicate wells. Serially diluted reference sera (89SF; gift of C. Frasch, National Institutes of Health, Rockville, MD) was treated the same way and added at the same time for each plate.

For the IgG assay, the plate was then incubated for 2 h at 37°C. After several washes, alkaline phosphatase-conjugated anti-human IgG (Sigma) diluted in PBS containing 10% FBS was added and incubated at 37°C for 2 h. After washes, p-nitrophenyl phosphate (PNPP; Sigma) substrate dissolved in diethanolamine buffer (pH 9.8) was added and incubated for 1 h at 37°C. We measured OD at 405 nm by using a plate reader (Dynex) and concentrations of IgG were calculated against a standard curve (with use of 89SF) constructed with Delta Soft software (BioMetallics).

For anti-pneumococcal PS IgA, after addition of samples, the plates were incubated at room temperature (to reduce nonspecific binding) on a horizontal rotator for 2 h. Murine MAbs to human IgA (Skybio) diluted in PBS containing 10% FBS was added and incubated at 37°C for 2 h. After washes, alkaline phosphatase-conjugated goat anti-mouse antibodies (Jackson Immunoresearch Laboratories) diluted in PBS containing FBS were added and incubated overnight at room temperature. Subsequent procedures were the same as for the IgG assay. For anti-pneumococcal PS IgA measurements, a plate coated with PBS only and otherwise processed identically with samples and detection reagents was used to control background for each assay. The OD values of samples and references on the PBS blank plate were subtracted from those coated with pneumococcal PS antigen. The sensitivities of the IgA and IgG serotype-specific immunoassays (defined as the mean + 2 SD of the ODs of the PBS background) were 40–62 and 16–48 ng/mL, respectively.

**Measurement of specific s-IgA and IgA1 and IgA2 subclasses.** Measurement of serotype 19F PS-specific secretory component (s-IgA) and IgA1 and IgA2 in breast milk was done as for the IgG assay except that murine MAbs to the secretory component (clone GA-1; Sigma), IgA1 (clone A89-036; Nordic), or IgA2 (clone A89-038; Nordic) were used instead of murine MAbs to human IgA. Titers are expressed as OD values.

**Measurement of antibody avidity.** Antibody avidity was measured as described by Goldblatt et al. [11] but modified for assessment of pneumococcal PS-specific antibody. In brief, Costar plates were coated with serotype 19F pneumococcal PS, washed, and blocked with PBS containing 10% FBS. Breast milk or serum samples diluted in PBS containing 10% FBS were incubated with pneumococcal CPS (50 μg/mL; Statens Serum Institute) for 30 min at room temperature before being added to the plate and incubated for 2 h at 37°C (for IgG) or at room temperature on a shaker (for IgA). After a wash, we added sodium thiocyanate diluted in PBS containing 10% FBS in duplicate at concentrations of 0–1 M, which was incubated at room temperature for 15 min. The subsequent procedures were as described for the ELISA method for IgG and IgA, respectively. Antibody avidity was expressed as avidity index corresponding to the molar concentration of sodium thiocyanate required to produce a 50% reduction in absorbance.

**Competitive inhibition assays.** To confirm the specificity of the IgA immunoassay in breast milk, inhibition assays were done. We used postimmunization milk whey diluted 1:100 in PBS containing 10% FBS. Six 10-fold dilutions of serotype 19F pneumococcal CPS diluted in PBS containing 10% FBS were prepared starting at a highest concentration of 200 μg/mL. An equal volume of each PS dilution was added to aliquots of each diluted milk sample tube and subsequently incubated on a horizontal rotator for 1 h at room temperature. The mixture was then added in duplicate to a microtiter plate and a standard immunoassay procedure was followed. The milk sample was likewise adsorbed by use of serotypes 4 and 9F PS, E. coli lipopolysaccharide (Sigma), and tetanus toxoid (Avantis Pasteur).

**Purification of IgA from breast milk.** s-IgA from breast milk was purified as described previously by Gregory et al. [12]. In brief, cream from breast milk was removed by centrifugation at 10,000 g for 40 min. The skim milk was then adjusted to pH 4.7 with 0.1 M HCl and casein was precipitated by heating at 40°C for 30 min and removed by centrifugation at 10,000 g for 20 min. The pH was adjusted back to 7.0 with NaOH. After overnight dialysis against veronal-HCl buffer (pH 7.4) containing 0.05 M NaCl, samples were added onto a heparin-Sepharose CL-6B column (Pharmacia) to remove lactoferrin and lysozyme. The void volume was collected and dialyzed against 0.1 M potassium phosphate buffer (pH 7.2) overnight. The sample was then passed through a CNBr-activated Sepharose 4B column (Pharmacia) conjugated with anti-human IgA (Dako). The column was washed with phosphate buffer, and s-IgA was eluted with 0.1 M glycine-HCl (pH 2.5).

The content of IgA1 and IgA2 from purified IgA was estimated by passing the purified IgA through a jacalin-agarose column (Vector Laboratories) in 10 mM HEPES buffer (pH 7.5) containing 20 mM galactose. IgA2 was collected in the void volume and IgA1 was eluted with the same buffer containing 0.8 M galactose. The protein content in the fractions was monitored at an OD of 280 nm. From the chromatogram, we estimated that >80% of the purified IgA was of the IgA1 subclass.

**Antibody-dependent killing of S. pneumoniae by complement and phagocytes.** Killing of log-phase S. pneumoniae types 19F (ATCC 6319) and 14 (ATCC 6314) by serial dilutions of immune milk whey and purified s-IgA was done in the presence of a convenient complement source (10% baby-rabbit complement; Cedarlane Laboratories) screened for toxicity to cells and organisms and fresh human neutrophils (neutrophil:bacteria ratio, 500:1), exactly as described [7]. Assays of killing were performed with the indicated concentrations of capsule-specific IgA in milk whey or purified s-IgA fractions in the presence or absence of complement and with increasing concentrations of complement. The specificity of the capsule as the target for killing activity against each organism was determined by preincubation of undiluted whey with 100 μg/mL...
of soluble PS (types 14 and 19F capsules and CPS) for 1 h prior to addition of whey to organisms, complement, and phagocytes.

**Results**

**Concentrations of capsule-specific antibodies in breast milk.** Concentrations of capsule-specific s-IgA in breast milk increased by >2-fold after immunization in most instances (figure 1). Of 21 possible responses (3 subjects; 7 serotypes), >2-fold rises were noted in 15 cases (71%). At least 1 of 3 subjects generated such responses to all serotypes except serotype 23F, to which changes were most limited. The highest levels of capsule-specific s-IgA in breast milk were achieved to serotypes 14, 9V, and 19F.

Capsule-specific s-IgA concentrations in milk were 0.01–1.2 μg/mL prior to immunization and 0.2–8.4 μg/mL after immunization. Although total IgG was detectable in milk (50–87 μg/mL) and concentrations of capsule-specific IgG increased in serum in some instances (table 1), no specific IgG to any of the 7 serotypes tested was detected after immunization in breast milk from any subject. Concentrations of IgA to the pneumococcal PS also often increased in serum (table 1), but neither serum serotype-specific IgG nor IgA responses showed any consistent association with those of specific s-IgA in breast milk.

**Structural features of capsule-specific IgA in breast milk.** Consistent with reports with serum IgA reactive with capsular PS, the majority of specific s-IgA to serotype 19F in breast milk was IgA1, rather than IgA2. The ratio of specific IgA1 and IgA2 was 4.5:1 in the postimmunization whey sample from subject C. The immunoassay ODs in assays for anti-serotype 19F in all 6 samples (2 from each subject), when anti-IgA and anti-s piece detection antisera were used, correlated closely ($r = 0.96; n = 6$; data not shown), indicating that the specific IgA detected was largely or entirely s-IgA.

These mucosal antibodies to serotype 19F were also specific for that serotype by immunoassay. Competitive inhibition assays, in which reactivity with plate-bound antigen is or is not inhibited by preincubation with homologous or heterologous antigens, confirmed the specificity of the assay and the antibodies. Binding was inhibited only by soluble serotype 19F capsule and not by other pneumococcal capsules or more-diverse antigens (figure 2). These data indicate that these locally produced antibodies are derived neither from cross-reactivity nor polyreactivity, but rather are generated in response to direct and discrete antigen challenge. The functional affinity or avidity of milk s-IgA reflects the strength of antigen-antibody binding. Avidity indices (expressed in moles of thiocyanate required to dissociate binding to the solid-phase antigen by ≥50%) for the serum IgG and IgA and milk IgA antibody to serotype 19F after immunization were 0.54, 0.46, and 0.81 in subject A and 1.0, 1.0, and 0.34 in subject C, respectively. Although the avidities, which often correlate with the functional activity of antibodies, of IgG and IgA to serotype 19F in serum were comparable, both appeared to differ from that of capsule-specific s-IgA in milk.

**Functional activity of capsule-specific s-IgA in breast milk.** Milk whey from the 2 subjects tested showed dose-dependent killing of serotype 19F S. pneumoniae when coincubated with complement and phagocytes (figure 3). No killing or decrement in bacterial numbers was observed in the absence of any individual component. s-IgA purified from each milk sample also
Table 1. Serum concentrations of anti–capsular IgA and anti–capsular IgG, against 7 vaccine serotypes, immediately before and 2 weeks after immunization with a 23-valent pneumococcal vaccine, in 3 breast-feeding mothers.

<table>
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<th>IgG, ng/mL Before immunization</th>
<th>IgA, ng/mL After immunization</th>
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NOTE. No preimmunization serum was available from subject C.

Our findings demonstrate that s-IgA reactive with surface PS of a Gram-positive organism can mediate killing of the organism by phagocytes with complement. Immunization with capsular PS has been effective at reducing nasopharyngeal carriage of both _S. pneumoniae_ [14, 15] and _Haemophilus influenzae_ [16]. In addition, children exposed to breast milk with other pathogen-specific antibodies have derived protection against those mucosal pathogens [17–19]. Thus, the ability of mucosal antibodies, particularly s-IgA, to mediate killing of _S. pneumoniae_ in vitro may provide a biologic basis for the beneficial clinical effects of immunization on respiratory carriage of invasive mucosal pathogens, effects that may limit subsequent disease, although other mechanisms, including interference with bacterial adhesion to epithelium, are also possible. This study extends previous observations that IgA is a prominent component of the response to pneumococcal PS [7, 20–22]. Although both specific IgG and IgA were detected in serum, only specific s-IgA, and not IgG, was detected at the mucosa in response to this parenteral vaccine. Anti-capsular IgG has been reported previously in human breast milk [23], and low levels of specific IgG may have been detected with more-sensitive assays or by removing competing s-IgA. In 2 of 3 subjects, we found >2-fold rises in breast milk-specific s-IgA responses for 5 of 7 serotypes studied. Only responses to serotype 23F were uniformly low. Appreciable increases in IgA to serotype 19F in breast milk, but only more-modest changes in antibodies to serotype 6B, have been described [5].

The capsule-specific antibodies present in breast milk after immunization appear to be of mucosal origin. The antibodies captured by specific PS by ELISA reacted with antisera to secretory component, and neither levels, rates of response, nor the avidities of these antibodies in milk correlated closely with those in serum. Whether the specific s-IgA was induced locally in the breast or the cells homed to this site from primed lymphoid tissues at other mucosal sites is not known [24]. Other antigens delivered parenterally, such as tetanus toxoid, do not typically elicit such prominent IgA or mucosal responses. However, systemic antigenic challenge can elicit vigorous mucosal
Figure 2. Percent inhibition of immunoassay signal (OD at 405 nm) for anti–type 19F–specific IgA, by progressive dilutions of homologous antigen (type 19F capsular polysaccharide [PS]) and heterologous capsular antigens. PS and LPS (LPS–Escherichia coli) are expressed as μg/mL, tetanus toxoid as U/mL.

responses if the mucosa was previously primed by immunization or natural infection [25].

Our results are most significant for the demonstration that s-IgA in immune breast milk can support killing of S. pneumoniae. In this system, killing is dependent on the presence of both complement and phagocytes. Pathogen-specific IgA from serum mediates a range of immune and proinflammatory activities [13]. Moreover, pneumococcal capsule-specific IgA from serum mediates both killing [7] and phagocytosis [10] of the organism by an Fcα-related mechanism. Less well characterized until now has been the ability of mucosal s-IgA to initiate uptake and phagocyte-mediated killing of specific pathogens.

We propose that killing of S. pneumoniae by breast milk is dependent on capsule-specific s-IgA and not due to other antibodies or other components of breast milk (e.g., carbohydrates or other innate factors). In support of this hypothesis, we demonstrate that levels of capsule-specific IgA in milk increased after immunization. By ELISA, these antibodies were specific for the serotype tested. Killing of S. pneumoniae of 2 different serotypes by phagocytes was consistent in the presence of both immune s-IgA and complement, and both humoral components were required for killing. Killing was also dependent on the doses of both s-IgA and complement. The specificity of the effect was confirmed by elimination of killing, by preadsorption of milk with homologous, but not with heterologous, PS. The direct role of s-IgA was shown by dose-dependent killing by highly purified s-IgA in this system. The greater efficiency of killing by purified s-IgA than by milk whey (per microgram of IgA) could be due to the presence of inhibitory factors in milk or to modifications of the antibody, such as changes in glycosylation during purification, although preliminary data showed no evidence for the latter. Thus, s-IgA in milk can support killing of S. pneumoniae by complement and phagocytes.

Two issues that remain unresolved are the mechanism by which s-IgA activates complement and phagocytosis and the ability of s-IgA actually to support such functional activity in the mucosal milieu. IgA does not appear to bind C1q to activate the classical complement pathway [26, 27], but IgA, particularly polymeric IgA, can activate the alternative pathway [7]. However, interactions of IgA with secretory component have been proposed to interfere with the ability of s-IgA to interact with Fcα receptors on phagocytes [13]. One hypothesis to resolve these observations is that s-IgA may be able to initiate complement activation on the surface of the organism by the alternative pathway and that binding to and uptake by phagocytes are mediated in large part by complement receptors, rather than by those for IgA. Indeed, killing of S. pneumoniae by serum IgA was inhibited in part by complement receptor blockade [7]. Work is in progress to characterize this sequence.

A more clinically relevant point is whether specific s-IgA can support killing of S. pneumoniae at mucosal sites (e.g., nasopharynx, sinuses, auditory canal, and lung). First, IgA may be susceptible to enzymatic cleavage. The majority of specific s-IgA detected was of the IgA1 subclass, a form that is vulnerable to enzymatic cleavage. By ELISA, these antibodies were specific for the serotype tested. Killing of S. pneumoniae of 2 different serotypes by phagocytes was consistent in the presence of both immune s-IgA and complement, and both humoral components were required for killing. Killing was also dependent on the doses of both s-IgA and complement. The specificity of the effect was confirmed by elimination of killing, by preadsorption of milk with homologous, but not with heterologous, PS. The direct role of s-IgA was shown by dose-dependent killing by highly purified s-IgA in this system. The greater efficiency of killing by purified s-IgA than by milk whey (per microgram of IgA) could be due to the presence of inhibitory factors in milk or to modifications of the antibody, such as changes in glycosylation during purification, although preliminary data showed no evidence for the latter. Thus, s-IgA in milk can support killing of S. pneumoniae by complement and phagocytes.

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Figure 4. Killing of *Streptococcus pneumoniae* by breast milk and neutrophils, which is shown to be complement dependent. Increasing concentrations of milk whey (capsule-specific IgA determined by ELISA) were incubated with *S. pneumoniae* serotypes 14 (A) and 19F (B), in the presence of human neutrophils (phagocyte:bacteria ratio, 500:1) with (●) or without (○) 10% baby-rabbit complement. Lower panels, Increasing concentrations of baby-rabbit complement incubated with *S. pneumoniae* serotypes 14 (C) and 19F (D) human neutrophils (phagocyte:bacteria ratio, 500:1), in the presence (●) or absence (○) of fixed concentrations of capsule-specific IgA in milk whey (type 14, 160 ng/mL; type 19, 250 ng/mL). Data are representative of 1 of 2 or 3 replicate experiments from different neutrophil donors.

to the lytic effects of pneumococcal IgA1 protease [28]. However, the protease may be less active against s-IgA, and its effects in vivo may be limited by the large amounts of nonpathogen-specific IgA present in milk. Second, levels of complement at the mucosae may be insufficient to effect killing. We have shown that s-IgA-associated killing by phagocytes is complement dependent, but levels of complement at most mucosal surfaces are typically quite low.

Two scenarios may pertain to this question. First, in the presence of active mucosal infection and inflammation, sufficient amounts of complement may exude from plasma into the mucosal site, through the inflamed mucosae, to support killing of the organism. Alternatively, in the presence of local inflammation, phagocytes drawn to the local chemokine gradients and further activated at the site may support killing by s-IgA in the absence of complement, as suggested in work with polymeric serum IgA [7]. In this regard, a number of lines of investigation with experimental pneumococcal pneumonia suggest that cytokines (e.g., tumor necrosis factor-α) [29] and chemokines serve a protective function [30, 31] and may enhance Fcγ receptor expression and affinity [32]. These conditions may pertain in the presence of a viral upper respiratory infection [33], which can predispose to secondary bacterial infection. Clinical observations on the protective effects of immunization with capsular PS on rates of colonization and mucosal disease will promote our understanding of the role of specific antibodies at the mucosa.

In summary, we found that immunization with the pneumococcal capsular PS vaccine can elicit antibodies specific for the pathogen and support killing of *S. pneumoniae* in vitro. Because of the tremendous impact of mucosal and invasive pneumococcal disease worldwide, an immunization strategy is required that reduces serious pneumococcal infections in early infancy, particularly in areas of poverty. Such a program should
Figure 5. Specificity of killing of *Streptococcus pneumoniae* by breast milk with complement and neutrophils. Milk whey diluted 1:2 was adsorbed with diluent (None), purified serotype 14 (T14 PPS) or 19F (T19F PPS) capsular polysaccharides, or cell-wall polysaccharide (CWPS) (each, 100 μg/mL), for 2 h at room temperature. Killing of serotypes 14 (A) and 19F (B) was determined as described in Subjects and Methods, with 10% baby-rabbit complement and neutrophils (phagocyte:bacteria ratio, 500:1). Data are representative of 1 of 2 or 3 replicate experiments with different neutrophil donors.

be both logistically feasible and affordable. Malnourished lactating women produce smaller volumes of milk but titers of secretory antibody in their milk are not reduced [34]. The 23-valent pneumococcal PS vaccine is less expensive to manufacture than current conjugate vaccines, includes a wider range of serotypes, and is reasonably immunogenic in adults. Prospective clinical trials to assess the efficacy that immunization of pregnant women by these vaccines has in the prevention of disease in children are currently in development and provide an accessible and potentially effective approach to protection against serious pneumococcal infections with PS and other promising protein antigens [35].

Acknowledgments

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References