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Prolonged and Preferential Production of Polymeric Immunoglobulin A in Response to *Streptococcus pneumoniae* Capsular Polysaccharides

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***Streptococcus pneumoniae* is an invasive mucosal pathogen for which host defense is dependent on capsular polysaccharide-specific antibody. Capsule-specific immunoglobulin G (IgG), IgM, and IgA are produced following pneumococcal vaccination and infection. Serum IgA has two molecular forms, polymeric and monomeric. These forms may modulate the avidity of antigen binding and evolve over time as the immune response matures. Therefore, we sequentially characterized the molecular forms of serum IgA to three serotypes of pneumococcal capsular polysaccharides (types 8, 12F, and 14) after pneumococcal vaccination and after natural infection with type 14 *S. pneumoniae*. Although typically the form of IgA in antigen-specific systemic responses to protein antigens is predominantly polymeric in sera of patients shortly after exposure and shifts to the monomeric form in sera obtained several weeks later, the form of IgA in response to each pneumococcal capsular polysaccharide remained predominantly polymeric 1 month after natural infection and up to 1 year following vaccination. In contrast, IgA to pneumococcal cell wall polysaccharide was both polymeric and monomeric. Moreover, the form of IgA in response to polyribosyl-ribitol-phosphate (PRP), the capsular polysaccharide of *Haemophilus influenzae* type b, was predominantly monomeric in the sera of 8 of 10 subjects tested 1 to 3 months after vaccination with either PRP alone or the diphtheria toxoid conjugate of PRP. We conclude that systemic responses to pneumococcal capsular polysaccharides are distinct in the production of predominantly polymeric IgA over time. The persistence of polymeric IgA may facilitate binding and clearance of pneumococci from the systemic circulation or reflect limited maturation of the immune response to pneumococcal capsular polysaccharides.**

Immunoglobulin A (IgA) is the predominant Ig on respiratory mucosal surfaces, the initial sites of *Streptococcus pneumoniae* infection (4, 34). Of the two molecular forms of IgA, polymeric and monomeric, locally produced polymeric IgA is selectively transported across epithelial cells to mucosal surfaces by receptor-mediated endocytosis via the polymeric Ig receptor (12, 34). In contrast, IgA in serum is primarily in a monomeric configuration and is not destined for mucosal secretion (4). Nevertheless, antigen-specific IgA in serum may preferentially reflect infection or antigenic exposure at mucosal sites (1, 2, 8, 11, 20).

Although 90% of serum IgA is monomeric, the initial IgA response to antigenic challenge is often polymeric and subsequently shifts to the specific monomeric form over time (3, 27). This shift has been noted following enteric bacterial and viral infections (11, 20, 23) and after direct parenteral antigenic challenge through immunization (18, 19). This shift from antigen-specific polymeric IgA to monomeric IgA in serum following infection with the enteric pathogen *Clostridium difficile* paralleled acquisition of neutralizing activity by IgA against the organism's major toxin (toxin A) (11). This correlated with a functional maturation of the IgA response to toxin A, as this structural shift did not occur in patients who failed to develop neutralizing serum IgA.

The acute IgA response to pneumococcal capsular polysaccharides after vaccination is also predominantly polymeric (33). However, whether the structural transformation from polymeric to monomeric IgA observed with protein antigens also accompanies responses to polysaccharide antigens is unknown. Therefore, we studied the molecular forms of IgA specific to several polysaccharides over time following *S. pneumoniae* infection and vaccination with pneumococcal polysaccharides and with the capsular polysaccharide of *Haemophilus influenzae* type b, another mucosal pathogen with invasive potential.

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MATERIALS AND METHODS

Subjects. According to protocols approved by the Human Subjects Subcommittees, informed consent was obtained from all study participants at the Veterans Affairs Medical Center, Minneapolis, Minn., the University of Minnesota, and Denver Disease Control. Sera were obtained from 10 healthy volunteers (ages, 24 to 36 years) at the time of vaccination and 1, 2, 4, or 8 weeks later, and at 33 to 52 weeks for selected subjects, following intramuscular immunization with pneumococcal 23-valent capsular polysaccharide vaccine (PNU-IMUNE; a generous gift from Lederle-Praxis Biologicals, American Cyanamid Company, Pearl River, N.Y.). Four subjects (ages, 21 to 48 years) were immunized with *H. influenzae* type b capsular polysaccharide (polyribosyl-ribitol-phosphate; PRP) vaccine (HIB-IMUNE, a generous gift from Lederle Laboratories), and sera were collected at 4 and 24 weeks. Six subjects (ages, 21 to 38 years) were given PRP-diphtheria toxoid conjugate vaccine (ProHIBit, a generous gift from Connaught Laboratories, Inc., Swiftwater, Pa.), and sera were collected at 1, 4, 12, and 24 weeks following immunization. Sera were also collected from two patients (34- and 35-year-old men who were HIV seronegative) who developed pneumo-

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nia and bacteremia due to *S. pneumoniae* (capsular serotype 14) within 6 days of the onset of symptoms and 4 weeks later.

ELISA for polysaccharide-specific IgA. As previously described (35), 96-well microtiter plates (Maxisorp; Nunc InterMed, Naperville, Ill.) were coated with purified pneumococcal capsular polysaccharide types 8, 12F, or 14 (5 μg/ml; Lederle Laboratories) or with the 23-valent capsular polysaccharide vaccine (11.5 μg/ml; Lederle Laboratories). Sera or gel filtration elution fractions of IgA, purified as described below, were added to the wells in serial dilutions and incubated at 25°C for 2 h. Specimens tested for pneumococcal capsular polysaccharide-specific IgA were preadsorbed overnight at 4°C with purified pneumococcal cell wall polysaccharide (50 μg/ml; Statens Seruminstitut, Copenhagen, Denmark). IgA to the capsular polysaccharide of *H. influenzae* PRP was measured with PRP (2 μg/ml) linked to poly-L-lysine (a gift from C. Meschwitz and P. McVerry, Connaught Laboratories, Inc.) to promote binding, as previously described (35). Antigen-specific IgA was detected with horseradish-peroxidase-conjugated affinity-purified goat anti-human IgA (Jackson Immuno-Research, Chantilly, Va.). Specific antibody concentrations were determined from serial serum dilutions by interpolation of absorbance values of standard and control sera that defined the linear part of a semi-log dilution curve. Levels in serum were calculated as nanograms of IgA per milliliter and fold rises (convalescent levels over preimmunization levels). Levels in elution fractions are reported as arbitrary enzyme-linked immunosorbent assay (ELISA) units, defined as the optical density derived from each 2.5-ml fraction.

IgA fractionation by molecular form. As previously described (11), sera were first depleted of IgG by affinity chromatography (Protein G Hi-Trap; Pharmacia LKB Biotechnology, Piscataway, N.J.) and fractionated by molecular sieve chromatography (Sephacryl S-300 HR; Pharmacia). Although precise quantitation of polymeric IgA may be problematic (5), in this study we assumed that both IgA species would react in the same manner on a molar basis in the ELISA system. The column was calibrated with polymeric and monomeric IgA standards (generous gifts of J. Mestecky, University of Alabama, Birmingham).

To exclude the possibility that the antigen-specific polymeric IgA actually represented immune complexes, we attempted to dissociate the complexes under acidic conditions (15, 30) from the fractions of IgG-depleted sera or IgA subsequently purified on an anti-IgA affinity column (11). Selected samples were acidified by buffer exchange with 0.1 M sodium acetate (pH 4.1) and then fractionated on the Sephacryl S-300 HR column, which had been equilibrated with the 0.1 M sodium acetate buffer and recalibrated. Elution fractions were neutralized with 1.0 M Tris-HCl (pH 9.0) prior to the ELISA. As additional confirmation of the polymeric structure of the specific IgA detected, fresh aliquots of the original specimens were subjected to partial reducing conditions to dissociate polymeric IgA into IgA monomers. The specimen was incubated with dithiothreitol (final concentration, 0.005 M) for 1 h at 37°C and then incubated with iodoacetamide (final concentration, 0.05 M) for 1 h at 25°C prior to fractionation on the Sephacryl S-300 HR column (23, 30).

Statistics. Mean levels of capsule-specific IgA following immunization were compared with preimmunization levels by Student's paired two-tailed *t* test. Data were analyzed with the StatView 4.0 statistical program (Abacus Concepts, Berkeley, Calif.).

RESULTS

Levels and time course of serum IgA responses to pneumococcal capsular polysaccharides after immunization. IgA specific to each of the three pneumococcal capsules tested was present at low levels in sera prior to immunization (means ± standard errors in micrograms per milliliter for the pneumococcal capsules were as follows: 0.3 ± 0.1 for type 8, 0.2 ± 0.3 for type 12F, and 0.6 ± 0.2 for type 14). Immunization with pneumococcal vaccine induced a substantial rise in the level of IgA specific for each capsular type by 2 weeks (means ± standard errors in nanograms per milliliter: 20.1 ± 9.2 for type 8, 1.4 ± 0.4 for type 12F, and 1.3 ± 0.4 for type 14; $P < 0.05$ for each type) (Fig. 1). Levels of type-specific IgA decreased only slightly 4 weeks following vaccination, with some levels still above baseline as late as 52 weeks after vaccination (data not shown). The relative levels by capsular type (type 8 > type 12F > type 14) were the same when measured by fold increase (data not shown) or by absolute values.

Molecular form of pneumococcal capsular polysaccharide-specific IgA. The predominant molecular form of pneumococcal capsular polysaccharide-specific IgA was polymeric in the serum of each of five patients drawn at 1 week following vaccination (Fig. 2a; Table 1). Similarly, 4 weeks after immunization, IgA specific for each of the three capsular types tested remained almost exclusively polymeric. In only very few instances was a smaller monomeric peak also identified (e.g., type 8 response from an immunized subject is shown in Fig. 2b).

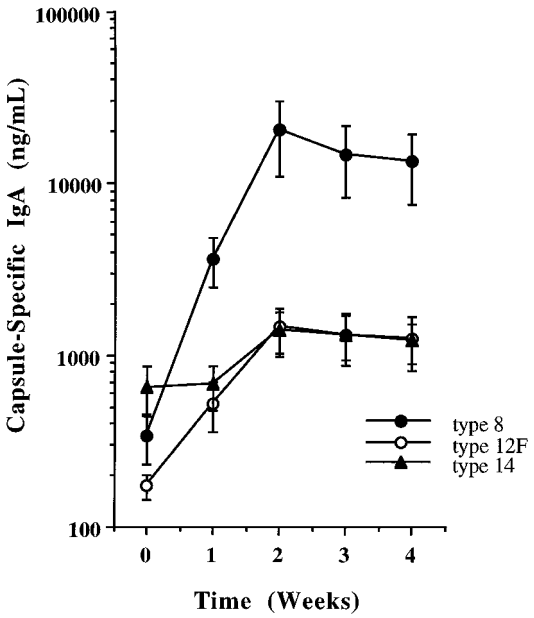

FIG. 1. IgA responses to specific pneumococcal capsular polysaccharides over time following pneumococcal vaccination. The mean levels (nanograms per milliliter ± standard errors) are shown for each of 10 subjects at 1, 2, 3, and 4 weeks following vaccination. Specific IgA levels peaked 2 weeks after vaccination for all types ($P < 0.05$, levels at 2 weeks versus the baseline).

To determine whether the shift from polymeric IgA to predominantly monomeric IgA, which has been described previously for protein antigens (18, 19, 23), occurred later than 4 weeks with pneumococcal capsular polysaccharides, we tested later specimens following immunization. In four additional subjects, the predominant molecular form of pneumococcal capsular polysaccharide-specific IgA 8 weeks after immunization was polymeric, as it was in sera from two of the original subjects tested at 33 and 52 weeks (Table 1).

To compare the molecular form of capsule-specific IgA following pneumococcal vaccination with that following natural infection, sera from two patients with *S. pneumoniae* (serotype 14) pneumonia and bacteremia were analyzed. Again, type 14 capsule-specific IgA in both patients was predominantly polymeric at 1 and 4 weeks following infection (Fig. 3; Table 1).

The polymeric configuration of the specific IgA detected was confirmed first by acid dissociation. The size of specific IgA did not shift following sodium acetate buffer exchange, as would have been expected if the high-molecular-size IgA represented immune complex formation (data not shown). Next, specific IgA did decrease in size following incubation of the specimen under partial reducing conditions, consistent with the dissociation of polymeric IgA into monomeric IgA (data not shown) (23, 30).

Molecular form of IgA specific for polysaccharides other than the pneumococcal capsule. To determine whether the preferential and persistent polymeric IgA response to pneumococcal capsular polysaccharides was common to IgA responses to other polysaccharide antigens, we characterized IgA responses to pneumococcal cell wall and *H. influenzae* type b capsular polysaccharides. In contrast to the IgA response specific to the pneumococcal capsule, which demonstrated almost exclusively polymeric IgA, the IgA response specific to pneu-

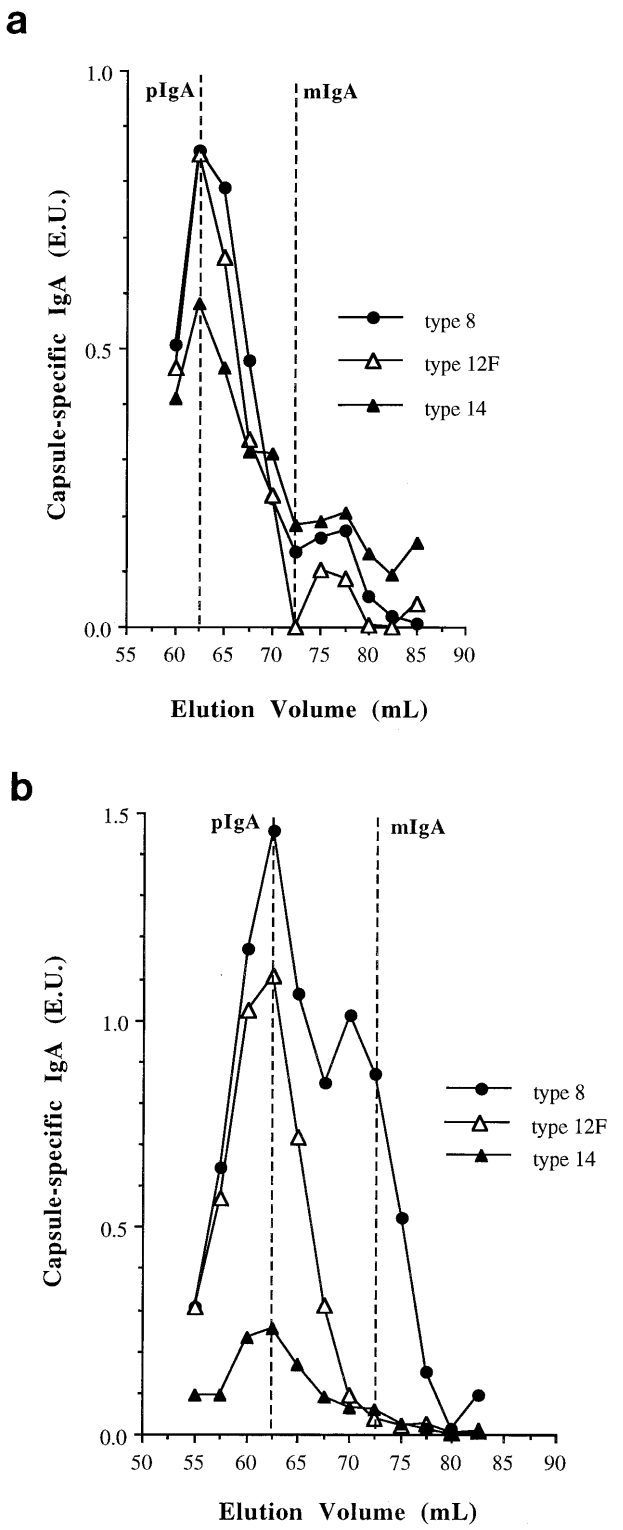

FIG. 2. Molecular form of pneumococcal capsular polysaccharide-specific IgA in sera obtained 1 week (a) and 1 month (b) after pneumococcal vaccination of a representative subject. Specific IgA levels are reported as arbitrary ELISA units (E.U.) in the elution fractions of these sera separated by Sephacryl S-300 HR gel filtration. Dashed lines indicate the elution peaks of the polymeric IgA (pIgA) and monomeric IgA (mIgA) standards.

TABLE 1. Predominant molecular form of pneumococcal capsule-specific IgA in serum over time after parenteral pneumococcal vaccination or natural infection with *S. pneumoniae*

Antigenic stimulus (*n*)	Capsular polysaccharide serotype	Occurrence of polymeric form in sera at indicated week after vaccination[a]			
		1	4	8	33 to 52[b]
Pneumococcal vaccine (5)	8	+	+	NT	+
	12F	+	+	NT	+
	14	+	+	NT	+
Pneumococcal vaccine (4)	8	NT	NT	+	NT
	12F	NT	NT	+	NT
	14	NT	NT	+	NT
Natural infection (2)	14	+	+	NT	NT

[a] The polymeric form was predominant for capsule-specific IgA as determined by gel sieve chromatography. NT, samples not tested.
[b] Sera from two of five subjects were available for testing 33 and 52 weeks after immunization.

mococcal cell wall polysaccharide in the same specimen tested for capsular antibodies demonstrated nearly equal levels of polymeric and monomeric IgA 4 weeks following vaccination (Fig. 4). Immune response to cell wall polysaccharide in these subjects was not unexpected, as the pneumococcal vaccine contains cell wall polysaccharide in addition to the 23 capsular polysaccharides (22, 31).

Also, in contrast to the form of IgA recognizing pneumococcal capsular polysaccharides, the form of IgA reactive with the capsular polysaccharide of *H. influenzae* type b (PRP) was

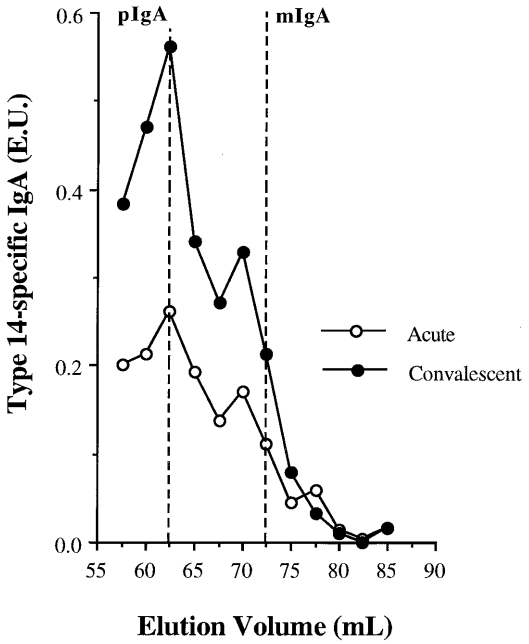

FIG. 3. Molecular form of type 14 pneumococcal capsular polysaccharide-specific IgA in sera obtained from a patient 1 week (Acute) and 1 month (Convalescent) after the diagnosis of bacteremic pneumococcal pneumonia due to type 14 *S. pneumoniae*. Fractionation was performed as described in the legend to Fig. 2, with specific IgA levels reported as arbitrary ELISA units (E.U.). Dashed lines indicate the elution peaks of the polymeric IgA (pIgA) and monomeric IgA (mIgA) standards.

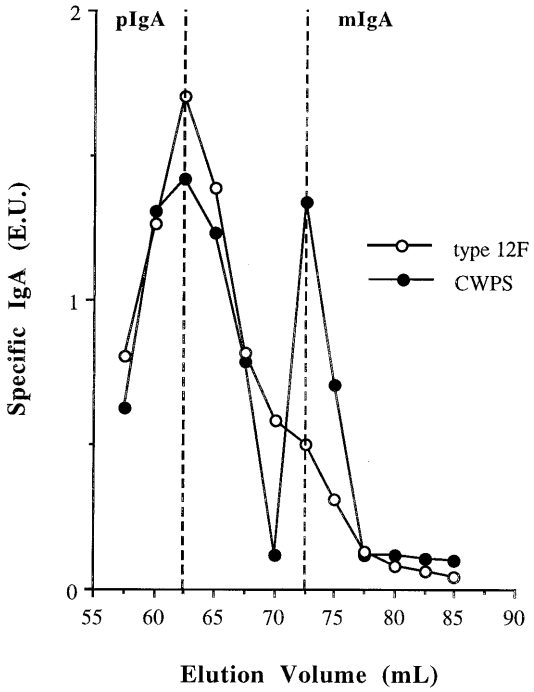

FIG. 4. Molecular form of pneumococcal cell wall polysaccharide (CWPS)-specific IgA and type 12F pneumococcal capsular polysaccharide-specific IgA in a serum specimen obtained 4 weeks after pneumococcal vaccination. The data are representative of three samples tested. Fractionation was performed by Sephacryl S-300 HR gel filtration with specific IgA levels reported as ELISA units (E.U.) as described in the legend to Fig. 2. Dashed lines indicate the elution peaks of the polymeric IgA (pIgA) and monomeric IgA (mIgA) standards.

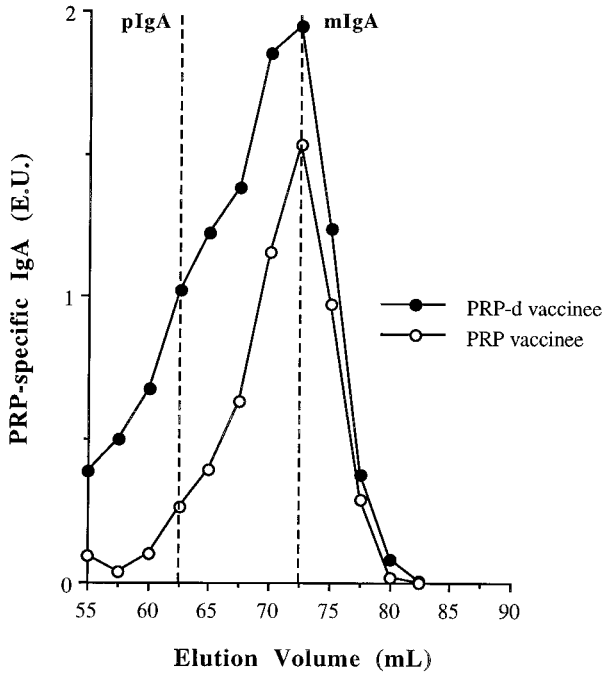

FIG. 5. Molecular form of *H. influenzae* type b capsular polysaccharide PRP-specific IgA in sera from two subjects obtained 6 months after vaccination with PRP and after vaccination with PRP-diphtheria toxoid (PRP-d). Fractionation was performed by Sephacryl S-300 HR gel filtration, with specific IgA levels reported as ELISA units (E.U.) as described in the legend to Fig. 2. Dashed lines indicate the elution peaks of the polymeric IgA (pIgA) and monomeric IgA (mIgA) standards.

predominantly monomeric in the sera from 8 of 10 convalescent patients obtained 4 weeks or later following vaccination (Table 2; Fig. 5). In three of four patients given the pure PRP vaccine, the predominant molecular form of PRP-specific IgA was monomeric by 4 weeks, and in one patient, specific IgA remained polymeric at 24 weeks. Similarly, in five of six patients given the PRP-diphtheria toxoid conjugate vaccine, PRP-specific IgA was also monomeric in specimens obtained 12 weeks or later. A shift from a predominantly polymeric to a monomeric IgA response was observed in the specimens from two patients in which very early (1 week) specimens were available. Neither molecular form of PRP-specific IgA was predominant in the late (24 weeks) specimen from one patient, although the specific IgA was predominantly monomeric at 4 weeks (subject 2, Table 2). Therefore, the molecular form of *H. influenzae* type b capsule-specific IgA in late (≥4 weeks) sera of immunized patients, independent of whether a pure polysaccharide or a polysaccharide-protein conjugate vaccine was used, was markedly different from the form of IgA recognizing specific pneumococcal capsular polysaccharides.

DISCUSSION

We have shown that IgA to pneumococcal capsular polysaccharides appears rapidly after antigenic challenge and that the predominant molecular form of this specific IgA in serum is polymeric, rather than monomeric, during the initial phase of

TABLE 2. Predominant molecular forms of *H. influenzae* type b capsule (PRP)-specific IgA over time after parenteral vaccination

Vaccine	Subject	Molecular form of PRP-specific IgA at indicated week after vaccination[a]			
		1	4	12	24
PRP vaccine	1		Poly		Poly
	2		Mono		Poly/mono
	3		Mono		Mono
	4		Mono		Mono
PRP-protein conjugate vaccine	5	Poly		Mono	
	6	Poly		Mono	
	7		Poly		Poly
	8		Mono		Mono
	9		Mono		Mono
	10		Mono		Mono

[a] Sera were tested by gel sieve chromatography. Poly, polymeric form of capsule-specific IgA; mono, monomeric form of capsule-specific IgA; poly/mono, similar proportions of monomeric and polymeric capsule-specific IgA in serum.

the immune response to vaccination. Moreover, we show that this predominance of specific polymeric IgA detected after immunization also characterizes the response to natural infection with *S. pneumoniae*. This observation is consistent with the findings of previous reports of acute responses to pneumococcal capsular polysaccharide vaccine as well as to several protein antigens (11, 17–19, 23, 27, 33). However, polymeric IgA responses to protein antigens are commonly transient and evolve to the monomeric form early in the convalescent phase (19, 20, 23, 27). A unique corollary in our study is that the IgA to pneumococcal capsular polysaccharides remained predominantly polymeric for a prolonged period, for 4 to 8 weeks and up to 33 to 52 weeks following vaccination.

The predilection for the persistence of the polymeric form of IgA to pneumococcal capsular polysaccharides was not shared by antibodies reactive with another pneumococcal polysaccharide (cell wall) or with another capsular polysaccharide (PRP from *H. influenzae* type b). Pneumococcal cell wall polysaccharide, like PRP, contains repeating phosphate motifs, features which could have an impact on the type of IgA response. Pneumococcal cell wall polysaccharide is a teichoic acid covalently linked to peptidoglycan in *S. pneumoniae*. Like PRP from *H. influenzae*, it contains ribitol phosphate, but choline phosphate is the main determinant of the antibody response to cell wall polysaccharide in humans and mice. Although cell wall polysaccharide and PRP are teichoic acids, the former is a highly branched polysaccharide which is structurally quite different from PRP. Thus, the switch to the monomeric form of IgA occurs with several distinct bacterial polysaccharides but not with those from pneumococcal capsules. Demonstration of these differences and the dissociation experiments suggest that the pattern of migration of the antibodies through the gel filtration column, upon which the designation of the molecular form is based, is not a technical artifact.

A recent study of serum IgA responses following enteric infections with *C. difficile* showed that the polymeric-to-monomeric shift was coincident with a functional maturation of the IgA response (11). The change was reflected by the acquisition of toxin-neutralizing activity by serum IgA. Whether the prolonged polymeric IgA response to pneumococcal capsular polysaccharides has adverse or beneficial functional consequences for clearance or killing of the organism is under investigation.

In this regard, the production of antigen-specific polymeric IgA early in the immune response has been proposed to convey a functional advantage to the antibody. Multivalent antibodies may bind antigens with a functional affinity (avidity) higher than those of their comparable monomeric forms (14, 27). As has been described for IgM, antibody-antigen interactions with low intrinsic affinity can be greatly enhanced through multivalency, primarily because of a decreased rate of dissociation (14). Persistence of the polymeric form of IgA may compensate for the proposed low intrinsic affinity and limited affinity maturation (32) that may characterize antibodies to pneumococcal capsular polysaccharides. Thus, both the rapid appearance of these antibodies after antigen challenge and the multivalency of polymeric IgA may contribute to the early control of *S. pneumoniae* infections in the systemic circulation.

Recent investigations have suggested that functional activities specific to polymeric IgA include promoting clearance of foreign antigens. Specific IgA in the intestinal lamina propria may bind and transport tissue antigens to the lumen, and polymeric IgA may participate in clearance of *S. pneumoniae* from the circulation in a similar manner (12). Humans lack the polymeric Ig receptors (secretory component) expressed on hepatocytes of many rodent species, which facilitate IgA-mediated clearance of antigen circulating in blood via the hepatobiliary system (26). However, human epithelial cells in the bile duct and gall bladder do express the receptor and appear capable of transporting small amounts of IgA (12). Nevertheless, a more viable hypothesis for *S. pneumoniae* is that polymeric IgA in serum enhances clearance of the organism early in the course of infection by promoting opsonization, phagocytosis, and killing of this invasive pathogen, perhaps by complement-dependent mechanisms.

Classically, the proposed roles of IgA have been to bind and deflect foreign antigens from adherence to tissue surfaces, to prevent tissue invasion, and to limit inflammation (21, 34). The first two activities pertain primarily to secretory IgA at mucosal sites. The proposal for the last role is bolstered by observations that IgA does not effectively initiate the complement cascade (28). Indeed, IgA was shown to inhibit complement-mediated killing of bacteria by IgG (10, 28). In contrast, direct complement-dependent killing of two gram-negative bacteria, *H. influenzae* and *Neisseria meningitidis*, can be induced by specific IgA in vitro (9). Nikolova et al. recently demonstrated that these dual functions of IgA, to inhibit IgG-associated complement-mediated phagocytosis and to promote opsonization and phagocytosis, were dependent upon the state of activation of the phagocytic cells (24). Inhibitory activities were associated with resting phagocytic cells, whereas the opsonizing activity of IgA occurred with cells activated by interleukin 8, which upregulated Fcα expression. Moreover, both earlier and recent data indicate that antigen-specific polymeric, but not monomeric, IgA can bind and activate complement and promote phagocytosis (6, 7, 13, 29). Thus, the rapid and preferential production of polymeric pneumococcal capsule-specific IgA following antigen exposure may promote clearance of this gram-positive bacteria by IgA-directed complement activation and deposition on invading *S. pneumoniae*. Such opsonization may subsequently initiate Fcα- and complement receptor-mediated uptake and killing by phagocytic cells in the blood, liver, and spleen in vivo.

In summary, the preferential and persistent production of polymeric IgA is unique to pneumococcal capsular polysaccharides among the polysaccharides studied. However, this distinction is shared by each of the three pneumococcal serotypes examined. Significant immunologic cross-reactivity (IgG) has been demonstrated between different pneumococcal capsular types and between capsular polysaccharides of *S. pneumoniae* and other bacterial species (e.g., 19F and *Klebsiella pneumoniae* (16, 25). The distinct structural and biochemical features common to pneumococcal capsular polysaccharides which influence the evolution of these responses by IgA-producing B cells are as yet undetermined. It is possible that a prolonged polymeric IgA response may not be seen with some pneumococcal serotypes (e.g., 6A and 6B) that share features with PRP (16), which elicits a polymeric-to-monomeric IgA shift. The multivalency afforded by polymeric IgA may compensate for the low intrinsic affinity of antibody to these antigens and contribute to the control of *S. pneumoniae* infections in the critical early stages of invasive disease by promoting clearance and killing. The significance of a prolonged polymeric IgA response, however, is uncertain but may be relevant in designing vaccines with local activity against this important mucosal pathogen.

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polysaccharides and HIB-IMUNE and ProHIBit vaccines; J. O'Brien for technical assistance; A. Dalmasso, University of Minnesota, Minneapolis, for supplying the monoclonal IgA1 standard; and J. Mestecky, University of Alabama at Birmingham, for the gift of polymeric and monomeric IgA standards.

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