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Research Article

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A Mucosal Antibody Response following Systemic *Haemophilus influenzae* Type B Infection in Children

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ABSTRACT The possibility that mucosal antibody is produced as a host response to *Haemophilus influenzae* type b (Hib) infection was examined in this study. 17 of 18 prospectively evaluated children ranging in age from 2 mo to 7 yr developed a detectable level of anticapsular antibody in their nasopharyngeal secretions after systemic Hib infection. The mean concentration of nasal anti-capsular antibody of the 18 children was 554 ng/mg IgA (SD = 35–8,863) during the acute phase of illness and declined to 224 ng/mg IgA (SD = 19–2,688) in convalescence. Some children had mucosal antibody detectable at least 10 mo after infection. The mucosal antibody levels were not affected by the length of illness before diagnosis, type of disease, age of the patient, sex, or presence of detectable capsular antigen or viable bacteria in the nasopharynx. The mucosal antibody was predominantly of the IgA class and occurred independent of the serum antibody. Six of the children aged <1 yr who did not produce and/or sustain a serum antibody level correlated with protection demonstrated a persistent mucosal antibody response. These findings suggest that the mucosal immune system may have the ability to respond at an earlier age than the serum immune system and lead us to postulate that protective secretory antibodies to prevent systemic Hib disease may be inducible in young infants in spite of the poor serum antibody response occurring at this age.

INTRODUCTION

Since the work of Fothergill and Wright (1) in the 1930's, the prevailing assumption has been that specific resistance to invasive *Haemophilus influenzae* type b (Hib)[1] disease is mediated by serum antibody to the organism. Recognition of the importance of the capsule as a virulence determinant (2) and the demonstration of anticapsular antibody as therapeutic (3) was followed by the development of a purified capsular (PRP) vaccine by Schneerson et al. (4) and Anderson et al. (5). In a subsequent trial of more than 48,000 Finnish children, Peltola et al. (6) found that protection of older children was inducible by parenteral immunization with PRP. However, infants at highest risk for Hib disease—those under 18 mo of age—were not protected and produced variable and low levels of serum anti-PRP antibody after parenteral PRP immunization. Consequently, new approaches are needed to induce protection of young infants against Hib infection.

Nasopharyngeal colonization by Hib is thought to be an early, critical step in pathogenesis (7). The local immune response may play a key role in host defense against the colonization process and thereby may provide protection against Hib invasion. We have therefore undertaken a series of studies to gain an understanding of the mucosal immune response to Hib. Our initial report established the presence of secretory antibody to the capsule of Hib in human breast milk (8). The mode of induction and length of persistence of this secretory antibody is currently unknown.

The purposes of this current study were to determine if mucosal antibody to the capsular polysaccharide is produced in the nasopharyngeal secretions of children recovering from Hib disease, to characterize the kinetics of the response, and to evaluate the relationship of the mucosal response to the concomitant serum antibody response.

METHODS

Patient population. The patient population is presented in Table I. 18 patients ranging from 2 mo to 7.5 yr of age at the onset of their illness were prospectively enrolled during a 12-mo period in 1979-80. 8 children were <18 mo of age, 12 were male, and 17 were Caucasian. 13 children had meningitis, 4 had epiglottitis, and 1 had facial cellulitis. A positive blood and/or CSF culture (or in one case detection of PRP antigen by

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[1] *Abbreviations used in this paper*: ELISA, enzyme-linked immunosorbent assay; Hib, *Haemophilus influenzae* type b; PRP, purified capsule of Hib.

TABLE I
Patient Profile

Patient	Age at diagnosis	Sex	Race	Disease	Days ill prior to hospitalization	Blood culture	CSF culture	Nasopharyngeal or throat culture	PRP antigen in nasal secretions
	mo								
1	54	M	W	meningitis	1	+	+	−	−
2	93	F	W	meningitis and septic arthritis	4	+	+	−	+
3	9	M	W	meningitis	2	+	+	+	+
4	52	M	W	meningitis	2	+	+	NA	+
5	34	M	W	epiglottitis	1	+	NA	−	−
6	65	M	W	epiglottitis	1	+	NA	−	−
7	26	M	W	meningitis	1	−	+	−	−
8	27	M	W	meningitis	1	+	+	−	−
9	15	F	W	epiglottitis	1	+	NA	+	−
10	23	F	W	meningitis	1	+	+	−	−
11	24	M	W	meningitis	2	+	+	NA	+
12	2	M	W	meningitis	1	+	+	−	−
13	5	F	W	meningitis	1	+	+	−	−
14	10	F	W	meningitis	1	−	+	−	−
15	7	M	W	meningitis	2	+	+	−	−
16	6	M	W	cellulitis*	1	−	NA	+	+
17	36	M	W	epiglottitis	1	+	NA	−	−
18	8	F	B	meningitis	1	−	+	−	−

NA, not available. CSF, cerebrospinal fluid. +/−, present/absent.
* Patient had facial cellulitis diagnosed as Hib by positive urine counter-immunoelectrophoresis.

counter-immunoelectrophoresis of urine) and clinical disease were used as criteria for diagnosis.

Informed consent was obtained from the parents for participation of their child in the study, which was approved by the University of Rochester Committee on Investigations Involving Human Subjects.

Collection and handling of specimens. Serum was separated from clotted blood by centrifugation and stored at −70°C until assayed.

Nasal secretions were collected by instilling and immediately aspirating 5 ml of sterile phosphate buffered saline from each nostril of the child with a bulb syringe. Secretions were homogenized with a probe sonicator (model W220F, Ultrasonics Inc., Plainview, N. Y., 20 W for 30 s), centrifuged (Eppendorf 3200, Brinkman Instruments Westbury, N. Y., 8,000 *g* for 3 min), and the supernate concentrated fivefold (vol/vol) by pervaporation at 20°C. Secretions were processed and assayed on the day of collection when possible; otherwise they were frozen at −70°C, thawed once and assayed.

Assays. Nasal and serum antibody responses were quantitated by a radioantigen-binding assay using intrinsically-labeled ^{3}H-PRP with ^{36}Cl as a volume marker (lower limit of detection = 10 ng antibody protein/ml) (9). The antibody level was determined by comparing the percent antigen binding of a serum or nasal secretion to binding by a standard reference serum (S. Klein) (10)

Class-specific antibody responses were quantitated with an enzyme-linked immunosorbent assay (ELISA) (11) modified for use with PRP (manuscript in preparation). In brief, purified PRP was attached to wells of a microtiter plate (M. A. Bioproducts, Walkersville, Md.) (manuscript in preparation) and incubated with diluted serum or nasal secretions. Class-specific anti-human immunoglobulins (Atlantic Antibodies, Westbrook, Me.) conjugated to alkaline phosphatase by the method of Engvall and Perlmann (12) were added to the samples to determine the amount of class-specific antibody present. Myeloma proteins were used to assess the specificity of the enzyme-conjugated antisera. Class-specific antibody activity was quantitated by comparing the optical density generated by iodinated myeloma proteins of known specific activity with a post-PRP immunization serum, which was then used as an internal standard during each assay (manuscript in preparation). The data are expressed for each immunoglobulin class as the percentage of the total ELISA activity detected.

In preliminary experiments, using methods previously described (8), nonspecific antigen binding or inhibition of antigen binding by nasal secretions was not found in the radioantigen binding assay or the ELISA technique.

Assay for PRP antigen in nasopharyngeal secretions and serum was performed by latex agglutination (lower limit of detection, 0.1 ng PRP/ml sample) (13). Nasopharyngeal and pharyngeal cultures were tested for Hib by plating on chocolate agar.

Total IgA was quantitated by laser-nephelometry (Hyland Diagnostic Div., Travenol Laboratories, Costa Mesa, Calif.) (14). With this assay, quantitation is independent of the degree of IgA polymerization.[2] Standardization of this assay was accomplished with a reference serum (Hyland Laboratories, LAS-R reference sera, although results were comparable with secretory IgA from human colostrum (N. L. Cappel Laboratories, Cochranville, Pa.). We have reported the nasal antibody levels with respect to total IgA to facilitate comparison among washes in the same and other patients.

[2] Personal communication, Hyland Laboratories.

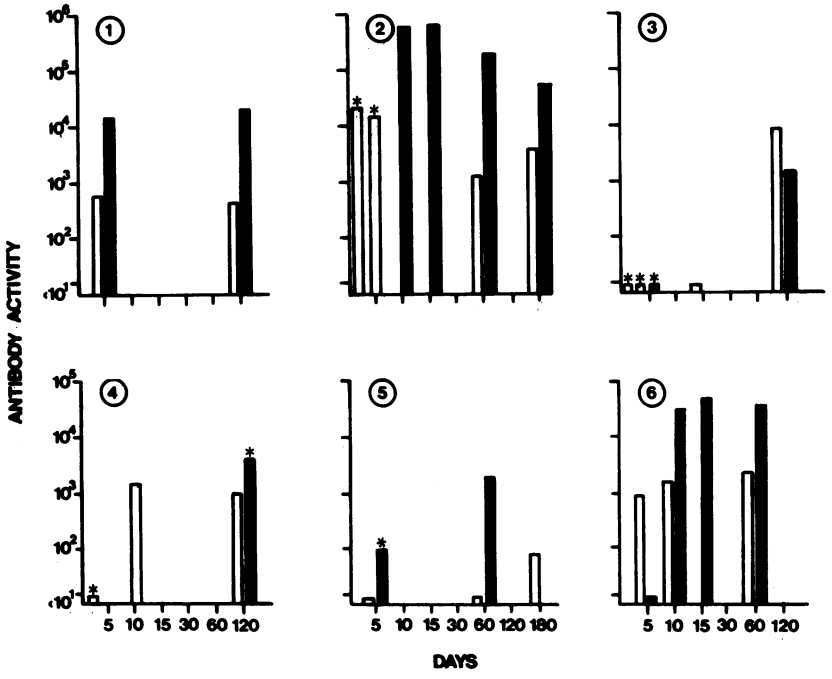

FIGURE 1 Nasal and serum antibody response pattern I. Antibody activity is expressed as nanograms of anti-PRP antibody protein per milligram total IgA for nasal secretions (□), and as nanograms of anti-PRP antibody protein per milliliter for serum (■). Days, days after admission to the hospital; *, presence of detectable PRP antigen or presence of viable bacteria in designated secretion or serum. Each number at the top left of the histogram refers to a single patient, also profiled in Table I. All samplings are shown.

Statistical Analysis. Comparison of antibody concentrations was made using Student's two-tailed *t* test. Correlations were determined by the use of Pearson's *r* (15). All statistical analyses were performed on the log of the data. Geometric mean antibody concentrations were calculated using the lower limit of detection value for the respective assay when a sample had no detectable antibody activity and are presented in the results as the mean±1 SD of the mean. When more than one antibody level was obtained of multiple samples collected during a defined time interval, the higher value was used in statistical analysis.

RESULTS

Mucosal antibody response. A nasal anti-PRP antibody response was detected in 17 of 18 children studied. There was no correlation between mucosal and serum antibody levels during either the acute or recovery phases of infection. However, four patterns of nasopharyngeal and serum antibody responses were observed: pattern I (Fig. 1), persistent mucosal antibody in association with persistent, high-magnitude serum antibody (six children); pattern II (Fig. 2), transient mucosal antibody in association with detectable serum antibody (four children); pattern III (Fig. 3), nondetectable mucosal antibody in association with detectable serum antibody (one child); and pattern IV (Fig. 4), persistent mucosal antibody in association with low-magnitude serum antibody (seven children). The onset of the mucosal anti-PRP antibody response occurred in the first 2 wk after the onset of apparent

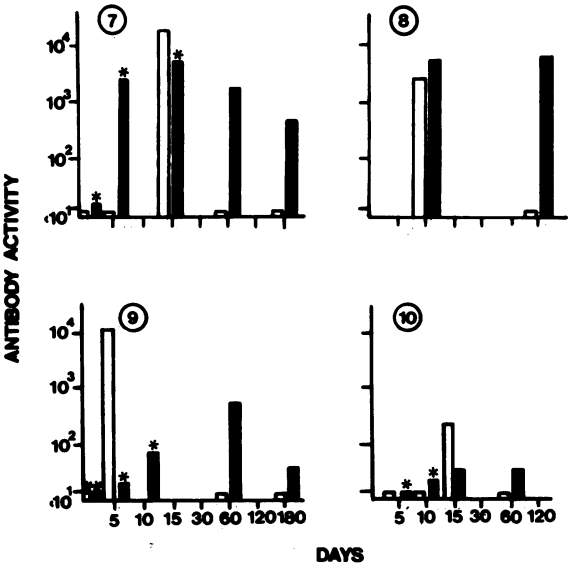

FIGURE 2 Nasal and serum antibody response pattern II. Legend as in Fig. 1.

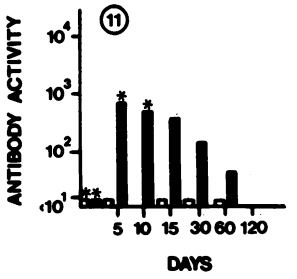

FIGURE 3 Nasal and serum antibody response pattern III. Legend as in Fig. 1.

disease in the majority of children and persisted in seven children (patients 1–5, 12, and 13) evaluated at ≥120 d and in two children (patients 12 and 13) evaluated at 300 d after diagnosis (Fig. 1–4).

In the 18 children studied, the mean concentration of nasal antibody during the acute phase of their illnesses was 554 ng anti-PRP antibody/mg IgA (SD = 35–8,863). The mean concentration of mucosal antibody in the early convalescent phase (30–60 d after diagnosis) was 170 ng anti-PRP antibody/mg IgA (SD = 14–2,040) which was similar to the mean concentration of 224 ng anti-PRP antibody/mg IgA (SD = 19–2,688) in the late convalescent phase (≥120 d after diagnosis) of recovery. The decline in nasal antibody concentration from the acute level to the levels in early and later convalescence was not statistically significant.

Acute and convalescent nasal antibody concentrations were not correlated. In addition, the length of illness prior to diagnosis, type of disease, age of the patient, sex, and presence of detectable nasopharyngeal PRP antigen or viable bacteria when independently evaluated showed no correlation with the levels of the mucosal antibody during the acute or convalescent phase of disease.

Comparison of mucosal and serum antibody response. Six children (patients 1–6 and grouped as pattern I) had high levels of both nasal (1,182 ng/mg IgA, SD = 237–5,915) and serum (11,186 ng/ml, SD = 1,600–78,360) anti-PRP antibody in the convalescent phase of their illness (Fig. 1). Four of the six (patients 1, 2, 4, and 6) had high levels of this mucosal antibody in the first 2 wk after disease onset (2,137 ng/mg IgA, SD = 427–10,685). In each of these cases, the antibody persisted at all subsequent sampling intervals. Two children (patients 3 and 5) developed detectable mucosal antibody only late in the recovery phase (120 and 180 d after diagnosis). We cannot exclude the possibility of intervening colonization with Hib or cross-reacting bacteria as an explanation for the development of detected nasal antibody at these times. For this group of six patients, the serum and nasal antibody concentrations did increase between the acute (serum = 3,454 ng/ml, SD = 34–331,338; mucosal = 357 ng/mg IgA, SD = 18–7,145) and convalescent phases (serum = 11,186 ng/ml, SD = 1,600–78,360; mucosal = 1,182 ng/mg IgA, SD = 237–5,915). However, this increase was not statistically significant.

Four children (patients 7–10, pattern II) developed a transient nasal antibody response although detectable serum antibody persisted (Fig. 2). The acute mucosal anti-PRP antibody level was 3,365 ng/mg IgA (SD = 481–23,556). The serum anti-PRP antibody level

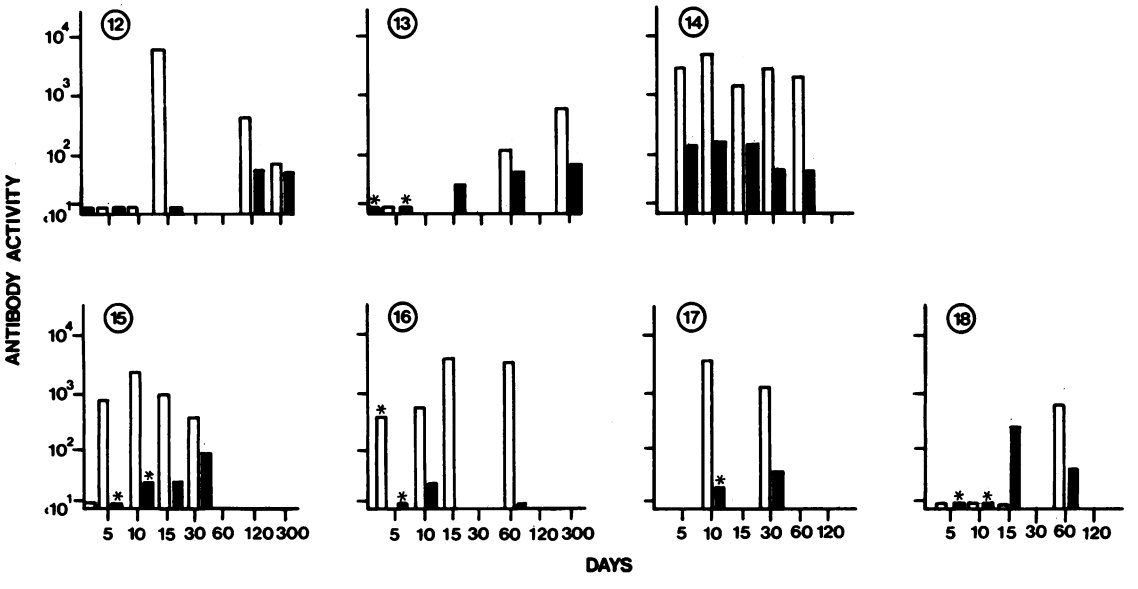

FIGURE 4 Nasal and serum antibody response pattern IV. Legend as in Fig. 1.

was 570 ng/ml (SD = 52–6,272) during the acute phase and 575 ng/ml (SD = 71–4,600) during convalescence. To evaluate the possibility of later redevelopment of mucosal antibody, two of the patients (7 and 9) were recalled 6 mo after illness. Both children still had no detectable mucosal antibody though they continued to have detectable levels of serum antibody.

No mucosal antibody was detected in one child (patient 11, pattern III) who had a sustained serum antibody response (Figure 3).

Last, seven children (patients 12–18, pattern IV) developed an early nasal anti-PRP antibody response (511 ng/mg IgA, SD = 32–8,168), which persisted at all subsequent sampling times (convalescence 967 ng/mg IgA, SD = 484–1,932), while developing only a low serum antibody level (acute = 25 ng/ml, SD = 8–75; convalescent = 50 ng/ml, SD = 25–100) (Fig. 4). These serum antibody levels are below the often quoted protective level (6). Six of these seven children were <12 mo of age. Four children, three from this group and one from pattern I, (patient 6, day 5; patient 12, day 15; patient 15, day 5, and patient 16, days 5 and 60) had a high concentration of mucosal anti-PRP antibody at a time when serum antibody was not detectable.

The acute nasal antibody concentrations were not significantly different among the three groups of patients with detectable levels (Figs. 1, 2, and 4). The concentration of nasal antibody during convalescence in the two groups of children with persistent antibody (Figs. 1 and 4), did not differ significantly. The serum antibody concentrations of pattern I and II patients were significantly higher than those of pattern IV patients during both the acute ($P \leq 0.02$ for patients in pattern I and pattern II compared to pattern IV) and recovery period after illness ($P \leq 0.0001$ for patients in pattern I and $P \leq 0.05$ for patients in pattern II compared to pattern IV).

Although the nasal antibody levels were not age-related, the antibody response patterns were affected by age. Six of the seven children less than 12 mo of age developed antibody responses which placed them in pattern IV (Fig. 4). The occurrence of an individual pattern was not related to type of disease, length of illness nor presence of detectable PRP antigen or viable bacteria in the nasopharynx.

Evaluation of possible contributing factors to non-detection of mucosal antibody. To evaluate the possibility of poor antibody recovery in the nasal aspirate as an explanation for failure to detect anti-PRP mucosal antibody in some specimens, the total IgA in the nasal washes with detectable anti-PRP antibody was compared to the total IgA in the washes without detectable antibody. No statistically significant difference was found between the two groups.

To assess the possibility that mucosal anti-PRP antibody was not detected because it was complexed with antigen, the nasopharynx or oropharynx was cultured for viable bacteria and secretions were examined for PRP antigen (Table I). Three patients had positive cultures, this occurred only at the time of admission to the hospital (patients 3, 9, and 16). Patient 16 had detectable anti-PRP antibody in the initial nasal sample despite the presence of viable bacteria. The initial but not subsequent nasal wash specimens obtained from the other two culture positive patients were negative for anti-PRP antibody. Three additional children (patients 2, 4, and 11) had PRP antigen detected by a latex agglutination assay; antigen was present only in the initial nasal washes obtained the day after admission. One of these three children had detectable anti-PRP antibody in the initial nasal sample despite the presence of PRP antigen (patient 2), while two did not (patients 4 and 11). Thus, six children had either viable bacteria or detectable nasopharyngeal PRP antigen, but only in their early nasal samples. Five of the six (all except patient 11) developed detectable mucosal antibody at some time during the study. We conclude that antigen complexed to antibody possibly accounted for failure to detect anti-PRP antibody in four of the early nasal samples but this was not a demonstrable factor in the others. We would also point out that the presence of detectable PRP in the serum (by latex assay), which could potentially affect circulating precursor lymphocytes that could bind this antigen, did not influence the detection of the nasal antibody response.

In the course of preliminary experiments, we found that mucosal anti-PRP antibody activity decreased (5–35%) from repetitive freeze-thawing of nasal wash specimens. Hence, all specimens in the data reported were processed immediately or frozen and thawed a single time. We cannot discount the possibility that antibody loss during sample processing may have reduced the antibody levels of specimens with low concentrations of anti-PRP antibody to below our lower limit of detection.

Analysis for class-specific antibody. The class-specific nasal anti-PRP antibody responses of four representative children are presented in Table II. IgA was the predominant immunoglobulin class of anti-PRP antibody in the nasal secretions. In contrast, the predominant immunoglobulin classes of anti-PRP antibody in the paired serum of these four children were IgG and/or IgM (Table II). This finding supports the premise that the anti-PRP antibodies found in the nasal samples are secretory antibodies and are not the result of passive transudation from the serum.

DISCUSSION

Protection against infection by secretory immunoglobulins has been demonstrated in man (16, 17). Several studies have shown that secretory IgA antibody pre-

TABLE II

Class-specific Nasal and Serum Anti-PRP Antibody Response

Patient	Days after admission	Sample	IgA	IgM	IgG
			%	%	%
1	5	nasal*	39	47	14
		serum*	18	53	29
	120	nasal	55	ND‡	45
		serum	1	1	98
2	60	nasal	50	16	34
		serum	1.5	0.5	98
	180	nasal	58	18	24
		serum	12	4	84
6	5	nasal	36	49	15
		serum	5	35	60
	10	nasal	45	28	27
		serum	11	32	57
14	30	nasal	43	32	25
		serum	2	95	3

* Class-specific anti-PRP antibody is expressed as a percentage of the total antibody detected in the ELISA.
‡ ND, not detectable.

vents adherence of bacteria to mucosal surfaces, thereby reducing or preventing colonization (18–21), and in some cases subsequent disease in the host (20, 21). Our current understanding of the pathogenesis of Hib infection suggests that invasive disease is preceded by colonization of the nasopharynx (7). The presence of mucosal antibody to Hib in the nasopharyngeal secretions may be an important host defense against this bacterial infection.

In this report we provide evidence that children respond to systemic Hib infection with secretory antibody in their nasopharynx. 17 of 18 children prospectively evaluated had detectable mucosal antibody following disease. This antibody response usually developed in the first weeks of illness and antibody persistence was documented in some cases at least 10 mo after illness. The nasopharyngeal antibody levels attained by these children were higher than those seen in a group of healthy adults with naturally acquired high-magnitude serum anti-PRP antibody levels (adult nasal antibody mean = 93 ng/mg IgA; (manuscript in preparation).

In 11 children, a rise in nasal antibody was detected during the study. Several children had detectable mucosal antibody in the first sample, which was collected in the initial week of apparent disease. These first samples were possibly obtained at sufficient time after nasopharyngeal bacterial colonization to allow the patients to produce nasal antibody. It is not uncommon to find anti-PRP antibody in the initially collected serum after the onset of apparent infection (22).

The mucosal immune response to PRP appears to occur independently of the serum antibody response. In four children (patients 6, 12, 15, and 16) mucosal antibody was found in the nasopharyngeal secretions at a time when no serum antibody was detectable, suggesting independent local production. In two of these children there was no detectable PRP in the serum; hence the absence of detectable serum antibody was not due to immune complex formation.

The predominant immunoglobulin class of anti-PRP antibody in the nasal secretions was IgA. In contrast, the predominant immunoglobulin classes of anti-PRP antibody in serum were IgG and IgM. The relative proportion of class-specific antibodies in the nasal secretions as compared to the serum supports our contention that anti-PRP mucosal antibodies result from secretion rather than transudation.

The most provocative results of this study pertain to the mucosal antibody responses of a group of young infants in whom very low levels of serum antibody were detected (Fig. 4). Six of the seven children in this group were <1 yr of age. While none of these children mounted and sustained a serum antibody level that has been correlated with protection following infection (6), all developed an early and persistent mucosal immune response. The kinetics and antibody class distribution of this response were comparable to older children in the other groups. The antibody concentrations achieved by these infants both in the acute and convalescent phases of illness were also comparable to the other children.

The detection of an independent mucosal immune response to PRP in very young infants may indicate that the functional maturation of the mucosal immune system precedes that of the serum immune system. Further, this finding provides evidence that a poor serum antibody response to a bacterial capsular polysaccharide does not preclude the capacity for a mucosal immune response. It is known that human secretory IgA can reach adult concentrations in saliva (23, 24), tears (25), feces (26), and notably in nasopharyngeal secretions (23) in the early months of life. However, that mucosal antibody to a specific antigen is elicitable earlier in ontogeny than serum antibody has not been shown in man.

It has been previously shown that parenteral immunization with PRP generally does not induce protective serum antibody in infants <18 mo of age (6). Even Hib infection in the young infant only induces a low titer of serum anti-PRP antibody (22), a result confirmed in this report. The effects of parenteral PRP immunization on secretory anti-PRP antibody production are cur-

rently under investigation in our laboratory. However, it is known that parenteral vaccines against cholera, influenza, and poliomyelitis are inefficient to completely ineffective in inducing secretory IgA antibodies (16, 27–30). One consequence of parenteral immunization can be boosting of previously existing secretory antibody titers (28, 31), and although the protection of older children and adults provided by parenteral PRP vaccination has been completely ascribed to serum antibody (4, 5), it is possible that nasopharyngeal antibody boosting was of importance.

Mucosal immunization is more effective than parenteral immunization at inducing secretory antibody and may result in a simultaneous serum antibody response (32). In 1975, Schneerson and Robbins (33) fed human volunteers live *Escherichia coli* that cross-reacts with PRP and showed that colonization with this bacteria boosted serum anti-PRP antibody levels. Mucosal antibody responses were not studied. More recently, Moxon and Anderson (34) found that rats fed cross-reactive *E. coli* prior to Hib challenge produced a more rapid clearance of Hib from the nasopharynx than control rats and displayed a decrease in the incidence of bacteremia and meningitis (34). Serum anti-PRP antibody could not be demonstrated following this *E. coli* colonization. Since it is known that antigen sensitization of the gut-associated lymphoid tissue can result in subsequent traffic of lymphoid cells to other mucosal sites with production of secretory antibody at those sites (35–37), these beneficial effects may have been promoted by a nasopharyngeal secretory IgA antibody response that resulted from traffic of IgA producing cells to the nasopharynx.

The possibility that secretory antibody in the nasopharynx could protect young infants against systemic Hib infection is worthy of investigation. The lack of protection of the most susceptible group of children—those under 18 mo of age—following parenteral PRP immunization has been disappointing. Our finding that children in this high-risk group develop a persistent mucosal antibody response while they are incapable of maintaining a protective serum antibody response provides impetus to the future exploration of mucosal immunization of children as an alternative approach to providing protection against Hib infection.

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