

OLIGOSACCHARIDE-PROTEIN CONJUGATE VACCINES
INDUCE AND PRIME FOR OLIGOCLONAL IgG ANTIBODY
RESPONSES TO THE *Haemophilus influenzae* b CAPSULAR
POLYSACCHARIDE IN HUMAN INFANTS

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Immunity to *Haemophilus influenzae* type b can be mediated by serum antibody to the capsular polysaccharide (CP)¹ (1). Immunization of human adults and older children with isolated CP induces antibody; however, immunization of young infants fails to induce an antibody response (2, 3). The antibody response is inconsistent, nonboostable, low in titer, and nonprotective in children younger than ~18 mo of age. Children above that age can mount a protective antibody titer, but its magnitude remains less than the adult's until ~4 y of age. The cellular basis of antibody unresponsiveness and the delay of high-magnitude responses is unknown. In an effort to convert the CP to a more thymus-dependent (TD) immunogen, oligosaccharides prepared from the CP (4-6) or the CP itself (7, 8) have been presented with protein carriers. When the oligo conjugate was injected sequentially at ages 2, 4, and 6 mo, a mainly IgM primary and IgG secondary anti-CP antibody response was induced (5). In infants thus primed, titers could be further boosted by immunizing with isolated CP, even at an age normally unresponsive to CP vaccine (5). To elucidate the basis, at the B cell clonal level, of the increased antibody titer induced by the conjugate, and of the accelerated age-acquisition of responsiveness to the isolated CP, we have analyzed IEF patterns of serum antibody of the IgG isotype.

Materials and Methods

Vaccines. The conjugate vaccine was composed of oligosaccharides 3-10 repeating units in length (derived by hydrolysis of the *H. influenzae* b CP), coupled by reductive amination to diphtheria toxoid or the antigenically related nontoxic protein, CRM 197 (4). The conjugates contained ~4% saccharide and 96% protein, wt/wt (4, 5). The dosage was 25 μ g protein injected s.c. Purified CP (2) was later given to some subjects (a 10- μ g s.c. injection). The age of the vaccinees and the intervals between vaccination are noted below.

Antibody Quantitation. Serum antibody to the *H. influenzae* b CP was measured in a Farr-type radioantigen binding assay using [³H]CP, and calibrated with standard antiserum SK (Office of Biologics, U.S. Food and Drug Administration) as described (9).

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¹ *Abbreviations used in this paper:* CP, capsular polysaccharide; TD, thymus dependent; TI, thymus independent.

IEF of Serum Antibody. Analytical IEF was performed in vertical slab gels that were cast and run exactly as described (10). Antibody was detected by incubating on the focused gel, which had been preincubated in 18% sodium sulfate as described (8), 25 μ Ci of 125 I-labeled CP (10 μ Ci/ μ g CP), prepared by radioiodination of tyramine-coupled CP. After exposure to the labeled antigen, the gel was repetitively incubated with 18% sodium sulfate, crosslinked with 18% sodium sulfate in 0.1% glutaraldehyde, desalted, and dried (10). The dried gels were exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY).

Results and Discussion

The anti-CP IgG antibody induced by primary immunization with the conjugate vaccine showed limited diversity on analytical IEF gels (Figs. 1 and 2). The patterns of most subjects were easily resolved, and as few as two clonotypes were visible from some serum samples. Most clonotypes had pI of 7.5–9.5. Clonotypes

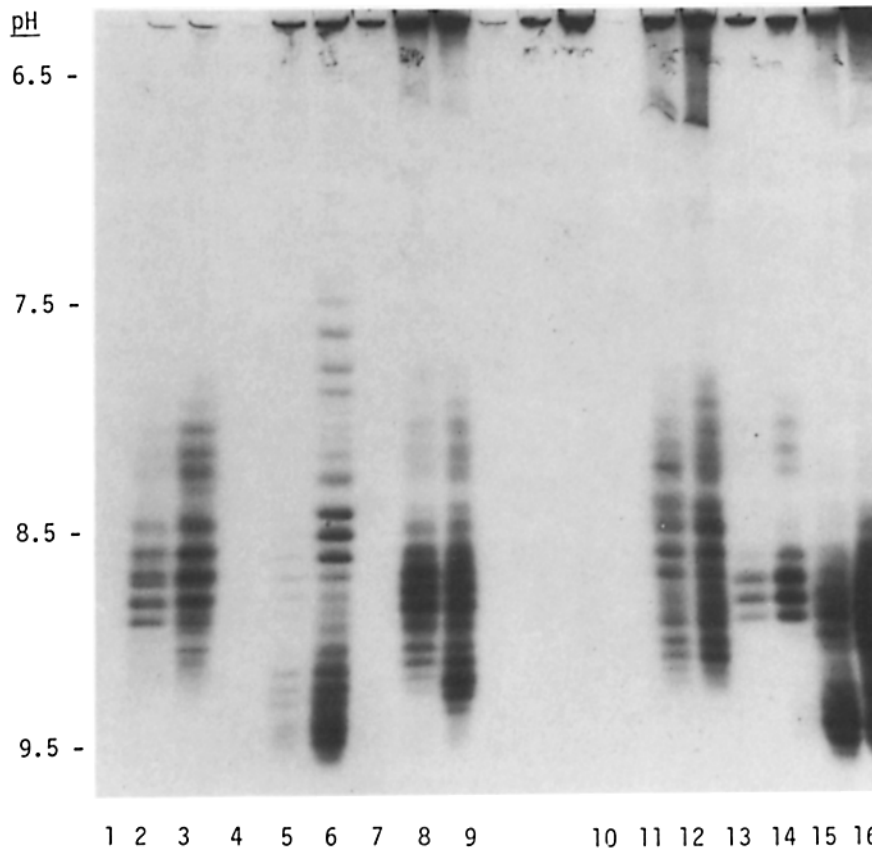


FIGURE 1. IEF patterns of serum antibody to the *H. influenzae* b capsular polysaccharide of children of designated ages before, and ~4 wk after primary or secondary (post 1°; post 2°) immunization with the conjugate vaccine. Antibody titers (μ g/ml) are given. A volume of 30 μ l was applied to the gel except where noted. (1–3) Subject 1, 15-mo-old: pre, 0.07; post 1°, 1.9; post 2°, 4.7. (4–6) Subject 2, 16-mo-old: pre, 0.05; post 1°, 1.1; post 2°, 4.6. (7–9) Subject 3, 30-mo-old: pre, 2.2; post 1°, 28 (20 μ l); post 2°, 50 (10 μ l). (10–12) Subject 4, 29-mo-old: pre, 0.13; post 1°, 22 (20 μ l); post 2°, 180 (5 μ l). (13, 14) Subject 5, 27-mo-old: post 1°, 10 (20 μ l); post 2°, 58 (10 μ l). (15, 16) Subject 6, 18-mo-old: post 1°, 1.7; post 2°, 22.

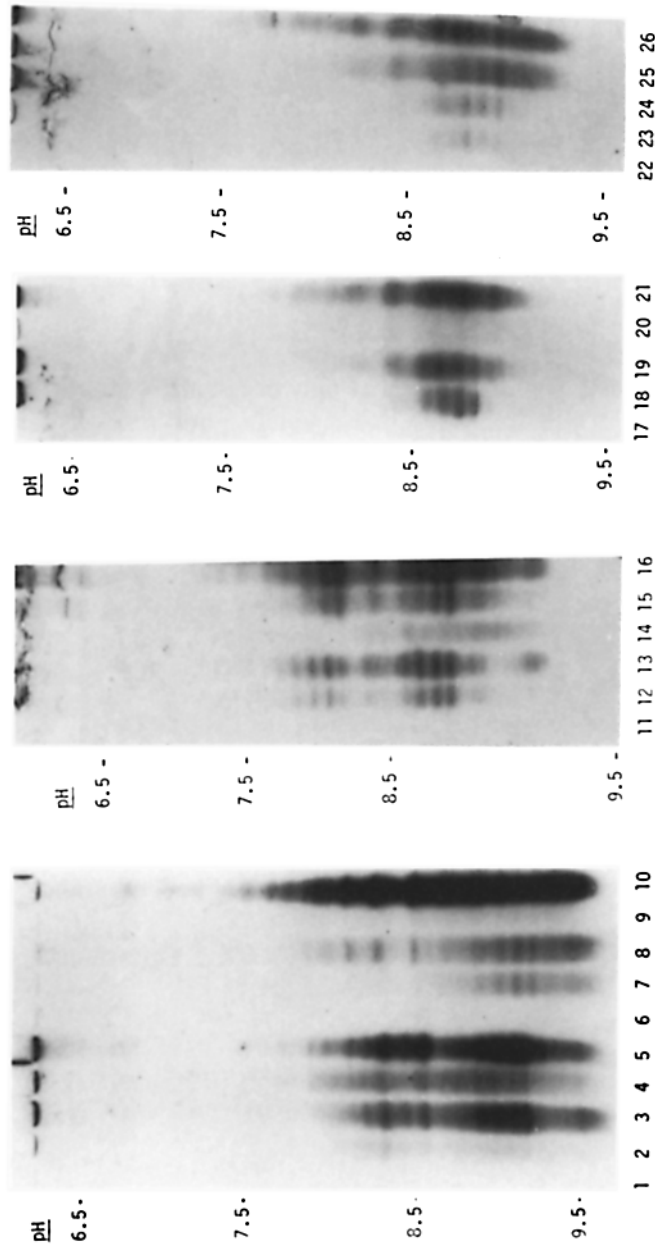


FIGURE 2. IEF patterns of serum antibody to the *H. influenzae* b capsular polysaccharide (CP) of children immunized with the conjugate vaccine and boosted with the CP vaccine. An exception is subject 8, who had a late rise in titer before a CP booster, presumably due to colonization. Designations are as in Fig. 1: (1-5) Subject 7, 2-mo-old; post 1° conjugate, 0.03; post 2°, 0.15; post 3°, 3.3. 9-mo-old; pre CP, 4.7; post CP, 100 (5 μ). (6-10) Subject 8, 2-mo-old; post 1° conjugate, 0.04; post 2°, 1.1; 1 mo post 3°, 4.2; 3 mo post 3°, 0.54 (10 μ); 5 mo post 3°, 70 (10 μ). (11-16) Subject 9, 14-mo-old; pre conjugate, 0.08 (20 μ); post 1°, 4.3 (20 μ); post 2°, 4.7 (20 μ). 20-mo-old; pre CP, 0.92 (20 μ); post CP, 1.4 (4 μ); post CP, 14 (20 μ). (17-21) Subject 10, 9-mo-old; pre conjugate, 0.06; post 1°, 1.8; post 2°, 10. 18-mo-old; pre CP, 1.6; post CP, 280 (2 μ). (22-26) Subject 11, 10-mo-old; post 1° conjugate, 0.10; post 2°, 0.74; post 3°, 2.2. 17-mo-old; pre CP, 4.2; post CP, 98 (2.5 μ).

were usually not visible before immunization (Figs. 1 and 2), and were sometimes not visible until after secondary immunization in the youngest children (Fig. 2). The antibody expression was no more diverse than that observed in serum of older children after immunization with the isolated CP (Fig. 3). Children of increasing age immunized with the isolated CP showed more diverse antibody expression (Fig. 3), but their patterns remained relatively restricted in spite of their increasing antibody titers, similar to the response of adults (10). The adult response also showed a lack of correlation between the magnitude of the antibody titer and the degree of clonal heterogeneity, with persistence of dominant clonotypes for years after immunization (10).

The response to secondary immunization with conjugate vaccines was accompanied by increased expression predominantly of the same IgG antibody clonotypes that had been expressed after the primary dose of vaccine (Figs. 1 and 2). Reimmunization with a third dose of conjugate vaccine also predominately increased expression of the same clonotypes expressed after earlier immunizations (Fig. 2). New clonotypes were detected only occasionally after the second (Fig. 1, lane 6) or third immunization (Fig. 2, lane 8); and, if expressed, they

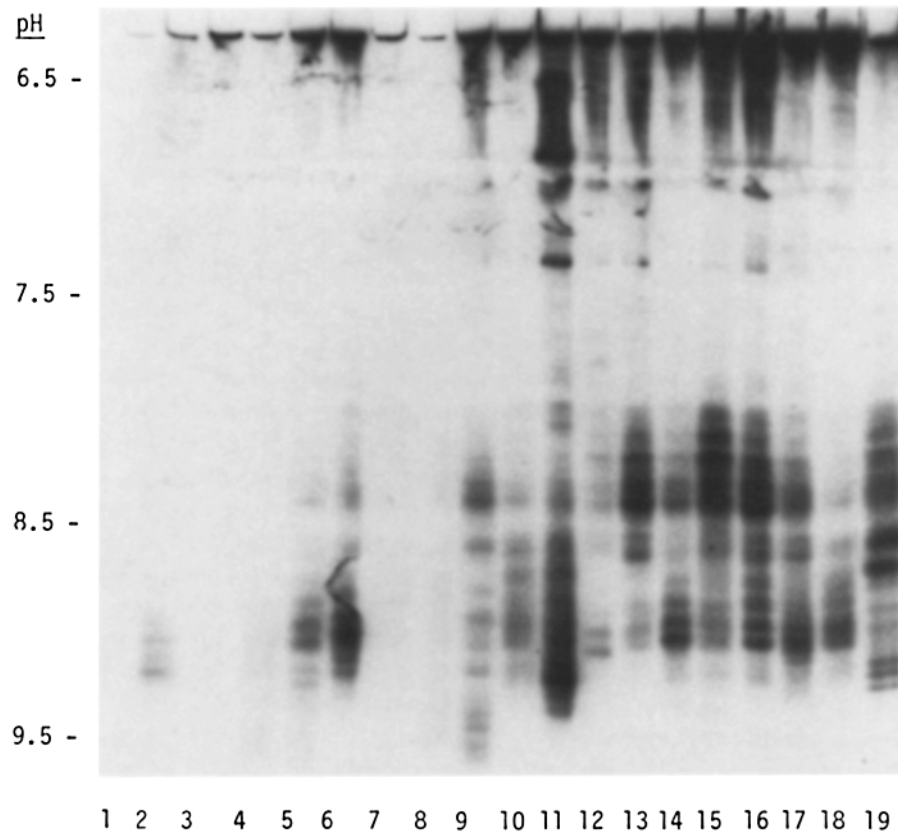


FIGURE 3. IEF patterns of serum antibody to the *H. influenzae* b CP of 18-47-mo-old children immunized with the purified CP vaccine and applied to the gel left to right in order of increasing age.

usually had lower pI than those previously expressed. Similar results following reimmunization have been observed in 26 subjects.

In contrast, the *H. influenzae* b CP and the CP of most other bacteria cannot prime by themselves for IgG memory responses in either man (2-4) or animals; in animals it is known that simultaneous activation of T cell help is required for priming (11). Presumably, the protein carrier is providing that effect. The ability of secondary immunization to activate the dominant clones previously primed, and the lack either of recruitment of more diverse antibody-secreting precursors or the lack of detectable somatic mutations in the Ig V region gene segments of previously primed B cells resemble the observations (12) of the permanence of the IgG clonal antibody patterns established by primary immunization with group A and A-variant polysaccharide after repetitive immunization in mice. This fixed, rather than expanding, repertoire of antibody-producing cells after repeated conjugate immunization, which to our knowledge has not been previously shown in man, contrasts with the maturation of the immune responses during secondary immune responses to most protein antigens and haptens (13).

Immunization of children with isolated CP after a series of conjugate vaccines was capable of further increasing the antibody titer (5). It was possible that the conjugate vaccine had stimulated an IgG memory response in two different B cell subpopulations; one that would be responsive and one that remained non-responsive to immunization with the CP itself. In mice, TD forms of an antigen can stimulate diverse precursors, not all of which can consistently respond to thymus-independent (TI) forms of the same epitope (14, 15), a finding that suggests a dual rather than linear pathway of development. Here, however, the isolated CP increased expression or reexpression of the same antibody clonotypes that had previously been induced by the conjugate vaccine, and all the clonotypes induced by the conjugate vaccine were restimulated by immunization with the isolated CP (Fig. 2). This result has been observed in seven of the eight subjects studied. In one subject, the IEF clonotype pattern at the time of CP immunization, which was 3 mo after the third conjugate immunization, differed from that observed at two and one-half weeks after conjugate immunization. The CP vaccine did, however, boost all the clonotypes apparent in the CP preimmunization serum. The finding that all the clones expressed after the conjugate vaccine were restimulated by CP suggests that, functionally, only one type of memory cell exists after multiple immunization with a conjugate vaccine, and it is a memory cell responsive to CP. We do not know whether the precursor cell induced by the conjugate to respond to CP initially belonged to a potentially CP-responsive subset and has the ability to respond to a TD form of the antigen earlier in ontogeny than to the isolated CP, or belonged to a subset that would normally be unresponsive or easily tolerized to the CP (11, 16, 17), and with exposure to the conjugate vaccine has become CP-responsive. A precedent for the former possibility has been described (16) in the earlier development, in mice, of an antidextran antibody response to immunization with isomaltohexaose coupled to KLH than to immunization with dextran itself. With either possibility, our findings suggest that the developmental pathway for this antibody response is occurring along a single common linear pathway with a changing requirement

for T cell-dependent activation. Once a TD form of the antigen has induced memory cells, the TI form can restimulate a response from that memory cell.

New clonotypes were occasionally apparent (at the time of immunization with CP) that had not been detected 1 mo after conjugate immunization (Fig. 2, subject 11), a finding usually associated with an increase in the antibody titer during the interval between immunization, as well as with increased expression of the clonotypes primed by conjugate vaccine. These new clonotypes were also boosted by CP immunization. New clonotypes were occasionally apparent after CP immunization (Fig. 2, subjects 9–11), which may represent the product of new memory cells induced during the interval between conjugate and CP immunization, or which may represent induction by the CP of a different isotype switch of the conjugate-primed memory cell. In one subject (Fig. 2, subject 8), the antibody titer at 3 mo after receiving conjugate vaccine, which had decreased from the 1 mo postimmunization titer, increased >100-fold following presumably environmental antigenic exposure 3–5 mo after conjugate immunization. This marked boost in titer was also accompanied by increased expression of all the clonotypes expressed after conjugate immunization, as well as expression of new clonotypes. The finding that an increase in antibody titer after the peak post-conjugate vaccine antibody response was accompanied by increased expression of the conjugate-primed clonotypes suggests that environmental exposure to a TD form of the CP on a different carrier or to a TI form of the CP also has the ability to restimulate conjugate-primed memory cells.

These observations differ from the immunogenicity in animals of some CP and CP conjugate vaccines. The antibody response in mice and rabbits to immunization with the *H. influenzae* b CP is poor, whether administered either as a primary vaccine or as a secondary vaccine after priming with either conjugate vaccines or killed bacteria (2, 7, 8, and our unpublished observations). In contrast, immunization of rabbits and mice with killed pneumococci or TD forms of pneumococcal CP can prime for secondary responses to the isolated CP (18–21). However, the secondary response to the isolated CP is not produced until fairly long after primary immunization (19), and is produced later than observed for secondary responses to TD forms of the CP (21). Furthermore, secondary immunization of animals with the TI form of an antigen or a CP after priming with the TD form can, at times, produce exhaustive differentiation of the clones without proliferation and regeneration of memory (14, 18, 19). However, there is no evidence that exhaustive differentiation is occurring in these human B cells, as the children primed with conjugate vaccine and boosted with the isolated CP have had persistent high-magnitude antibody titers for >1 yr after the CP immunization.

Summary

The diversity of the IgG antibody induced by immunization of human infants and children with conjugate vaccines, composed of oligosaccharides prepared from the *Haemophilus influenzae* b capsular polysaccharide (CP) and covalently linked to diphtheria toxoids, was studied by analytical IEF. The antibody response was similar, in the degree of restriction, to that observed in the antibody response of older children to immunization with the CP alone. The booster responses

induced by reimmunization with conjugate vaccines were accompanied by increases predominantly in the IgG antibody clonotypes expressed after the priming dose of vaccine. After a series of conjugate immunizations, immunization with isolated CP boosted the antibody titer and increased expression from all the clonotypes that were expressed after conjugate immunization. These findings suggest that the conjugate vaccines are acting on a limited number of human B cell clones that are preferentially restimulated after reimmunization. Little evidence of antigen-specific B cell recruitment was found. In addition, the ability of isolated CP immunization to restimulate the same B cell clone indicates that the responding B cell has matured and suggests a linear rather than a dual developmental pathway for the B cell participating in this human antibody response.

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