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Distinct Pattern of Antibody Reactivity with Oligomeric or Polymeric Forms of the Capsular Polysaccharide of *Haemophilus influenzae* Type b

SUBRAMONIA PILLAI,* SHARON CICIRIELLO,† MAYA KOSTER, AND RONALD EBY

Praxis Biologics, Inc., Rochester, New York 14623

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The chain length of oligosaccharides required for antibody binding has been studied by using the capsular polysaccharide from *Haemophilus influenzae* type b or oligosaccharides derived from it. The concentration of competing antigens required to achieve a 50% inhibition of antibody binding by human polyclonal antisera in an in vitro competition enzyme-linked immunosorbent assay decreased progressively from $>10^{-3}$ to 5×10^{-7} M as the inhibiting saccharide chain length increased from 1 to 262 repeat units. Even small oligosaccharides (one or two repeat units) are potentially capable of competing to a significant level if a high enough concentration of saccharides is used. A similar pattern of reactivity was seen with a monoclonal anti-polyribosyl ribitol phosphate antibody, suggesting that the differences in the avidity of the antibody subpopulations in the polyclonal antisera do not contribute to the binding patterns observed. The binding reaction was specific as evaluated with pneumococcal saccharides. Furthermore, an oligosaccharide-protein conjugate binds antibody better than the free oligosaccharides do. Such a difference in binding was not observed between the polysaccharide and a polysaccharide-protein conjugate. Overall, the data suggest that identical epitopes are expressed by oligomeric and polymeric forms of the antigen and that a particularly more stable conformation in polysaccharides is preferred by antibodies. Covalent coupling of oligomers to protein increases the expression of stable conformation of epitopes. The data further suggest that this kind of antigenic analysis may be important for the design and synthesis of glycoconjugate vaccines.

The binding of antibody to polysaccharide antigen has been shown to depend on the recognition of relatively simple oligosaccharide epitopes consisting of no more than six or seven sugar residues (8, 17). However, the existence of more complex conformation epitopes has been suggested for a number of other capsular polysaccharides including those of type III group B streptococci (20), group B *Neisseria meningitidis* (13), *Escherichia coli* K1 (14), and type 14 *Streptococcus pneumoniae* (19). In all these cases, the affinity of antibody binding to oligosaccharide increased as the chain length of the antigen increased and conformational epitopes are believed to be fully expressed only in the high-molecular-weight form of the saccharide. If this is the case, the chain length of saccharides may have tremendous impact on the design of conjugate vaccines against a number of encapsulated bacteria. In fact, it has been suggested that conjugate vaccines containing saccharide fragments of shorter chain length may elicit a better T-cell-dependent antibody response than those containing high-molecular-weight polysaccharide. However, the chain must be sufficiently long to express the complete epitope of the native saccharide (2, 18).

In the present study, we have analyzed the binding characteristics of polyclonal and monoclonal antibodies to the *Haemophilus influenzae* type b polysaccharide (polyribosyl ribitol phosphate [PRP]) in an attempt to correlate the chain length with the antigenicity of saccharides. The data suggest that polysaccharide binds anti-PRP antibody better than oligosaccharides do. However, an increased antibody binding, in comparison to free oligosaccharides, was observed with oligosaccharide-protein conjugates. Overall, the data suggest that a stable conformational epitope resulting from the restriction in the rotational movement of individual sugar residues may be preferred by antibody. Such stable conformation epitopes are expressed by long-chain polysaccharides and, to a certain extent, by oligosaccharides when covalently coupled to a protein carrier.

* Corresponding author.
† Present address: Eastman Kodak Co., Rochester, NY 14652.

MATERIALS AND METHODS

Antigens. The purified *H. influenzae* type b polysaccharide was obtained from Praxis Biologics Inc. Oligosaccharides were made by oxidizing the polysaccharide with sodium periodate, and the resulting fragments were separated on a Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column to obtain materials with an average degree of polymerization (Dp) of 20 (Dp20). A conjugate vaccine was manufactured by covalently coupling Dp20 short-chain PRP of *H. influenzae* type b to a nontoxic mutant of diphtheria toxin CRM_{197} (3). *H. influenzae* type b polysaccharide coupled to a protein carrier (9) was obtained from Connaught Laboratories, Swiftwater, Pa. *S. pneumoniae* 6A polysaccharide was obtained from the American Type Culture Collection, Bethesda, Md.

Anti-polysaccharide antibodies. Human polyclonal hyperimmune serum (OBRR serum; 70 μg/ml) was obtained from the Bureau of Biologics, U.S. Food and Drug Administration, Bethesda. This serum was also used as a standard in the Farr assay to quantitate anti-PRP antibodies in various sera. Human monoclonal anti-PRP antibody (16M3C8) was kindly provided by Richard Insel, University of Rochester, Rochester, N.Y. Rabbit anti-6A pneumococcal polysaccharides antibody was obtained from Dako Corp., Santa Barbara, Calif.

Fractionation of oligosaccharides. Oligosaccharides of different chain lengths were obtained by cleavage of the

polysaccharide with sodium metaperiodate followed by fractionation of the individual fragments by ion-exchange gel filtration chromatography. Briefly, lypohilized polysaccharide was dissolved in distilled water at 3.6 mg/ml. Either 0.5 or 0.2 mol equivalence of sodium metaperiodate in distilled water was added with vigorous stirring. This mixture was allowed to stand at room temperature in the dark for 3 h.

After the incubation period, the mixture was applied to either a strong-anion-exchange column (Accell Plus QMA; Waters) for the separation of low-molecular-weight fragments (1 to 14 repeats) or a gel permeation column (Sepharose CL-6B [Pharmacia] or Bio-Gel P-100 [Bio-Rad]), for the separation of higher-molecular-weight fragments (>14 repeats). The anion-exchange column was eluted with a sodium chloride gradient from 0 to 0.15 M. The gel permeation columns were eluted with 0.9% saline. Fractions were collected and analyzed for their total PRP content by the orcinol assay (5) and for the reducing end groups by the Park-Johnson assay (15). Ribose was used as the standard in both assays. The average number of repeats or Dp for each of the fractions was calculated by the following equation: Dp = [(moles of PRP)/(moles of reducing groups)] × 2.

Farr assay. Antibody to PRP was determined by a standard radioantigen-binding assay (RABA/Farr) (1). Briefly, various dilutions of sera, OBRR standard, and assay controls were prepared in fetal calf serum and allowed to react with [^{3}H]PRP with $^{36}Cl^-$ ions as fillers (volume maker). Samples were precipitated with 50% saturated ammonium sulfate and counted in a liquid scintillation counter. The amount of radiolabel precipitated was quantitated in comparison with the OBRR standard sera.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed by using the modification of the method described by Insel and Anderson (11). Ninety-six-well ELISA plates (NUNC) were coated with tyramine-conjugated PRP at 100 ng per well in phosphate-buffered saline (PBS [pH 7.2]). Plates were washed and then blocked with 0.1% gelatin in PBS, and various dilutions of anti-PRP antisera were added and incubated for 60 min at room temperature. Plates were washed and incubated with a predetermined concentration of alkaline phosphatase-coupled goat anti-human or goat anti-mouse antisera, washed, and developed with *p*-nitrophenolphosphate in diethanolamine buffer. The optical density (OD) was measured in an ELISA reader.

Competition ELISA. The anti-PRP ELISA as described above was modified to estimate the relative amount of anti-PRP antibody absorbed by different antigens. NUNC plates (96 wells) were coated with tyramine-conjugated PRP and blocked as described in the previous section. An appropriate concentration of anti-PRP antibody and various concentrations of antigens were mixed, and this mixture was added to the plate wells. After a 90-min incubation at room temperature, the plates were washed and reacted with appropriate alkaline phosphatase-conjugated antibodies as described above. We obtained 100% binding on wells with no additional antigens. The percent inhibition of binding for each inhibitor was calculated by using the following formula: % inhibition = 100 − [(average OD with competing antigen/average OD with no antigen) × 100]. A similar procedure was used for pneumococcal polysaccharide competition ELISA, except that the plates were coated with native 6A polysaccharide at 1 μg/ml. Rabbit anti-6A antibody was used in all these experiments.

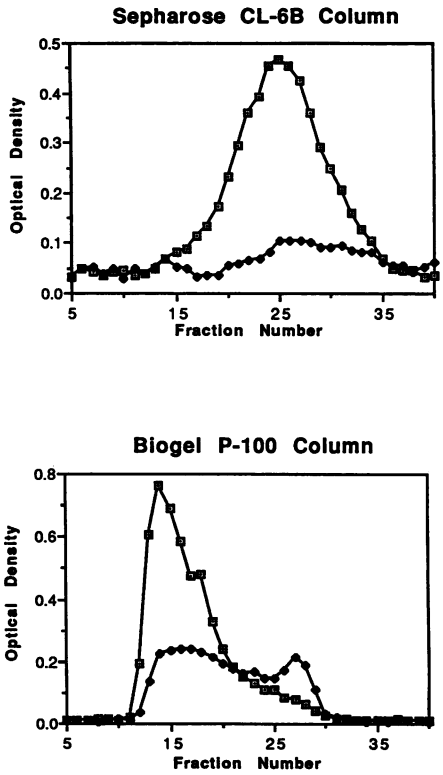

FIG. 1. Fractionation of *H. influenzae* type b oligosaccharides of different chain lengths by column chromatography. Polysaccharides were cleaved with sodium periodate and separated on either Sepharose CL-6B or Bio-Gel P-100 columns. Ribose (□) was estimated by the orcinol assay, and the reducing end group (◆) was analyzed by the Park-Johnson assay.

RESULTS

Preparation and purification of *H. influenzae* type b oligosaccharides of different sizes. To understand the role of the chain length of PRP in binding anti-PRP antibody, we cleaved *H. influenzae* type b polysaccharide into oligosaccharides by controlled oxidation with sodium metaperiodate. The reaction of the polysaccharide with periodate causes an oxidative cleavage of the carbon bond between adjacent free hydroxyl groups in the ribitol moiety. Periodate oxidation results in random cleavage of the main chain (polysaccharide fragmentation) and formation of two aldehyde groups, one at each end of the resulting saccharide fragment. The average size (Dp) of the oligosaccharide fragments formed is directly proportional to the molar amount of periodate used in the reaction. Since the amount of periodate is easily controlled, oligosaccharides having a desired molecular weight distribution can be obtained. The polydispersed oligosaccharides were fractionated by using either a strong-anion-exchange or gel permeation columns having the appropriate molecular weight cutoffs. For fragments in the Dp50 to Dp200 (15 to 75 kDa) range, a Sepharose CL-6B column was used, and for lower-Dp fragments (Dp10 to Dp50), a Bio-Gel P-100 column was used (Fig. 1). For very low-Dp oligosaccharide (Dp1 to Dp15), strong anion exchange with a saline gradient gave good resolution of the individual fragments. The average Dp for the oligosaccharides in each fraction was calculated as described in Materials and Methods.

Comparison of *H. influenzae* type b polysaccharide with

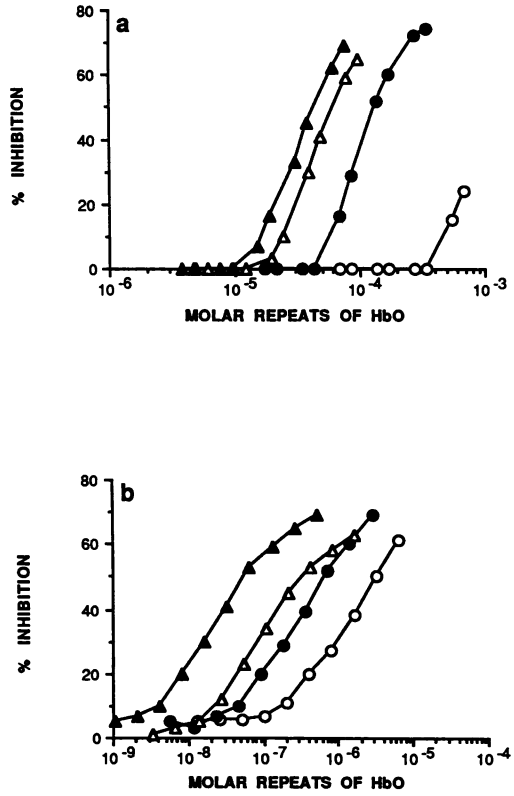

FIG. 2. Comparison of *H. influenzae* type b oligosaccharides (HbO) of different chain lengths in a competition ELISA with human polyclonal antisera. Plates were coated with tyramine-conjugated PRP at 100 ng per well, and the binding of antibody was inhibited with different concentrations of oligosaccharide. The data are presented as the percent inhibition observed with different molar repeats of PRP. Panel (a) symbols: ○, Dp1; ●, Dp2; △, Dp7; ▲, Dp9. Panel (b) symbols: ○, Dp21; ●, Dp47; △, Dp80; ▲, Dp262.

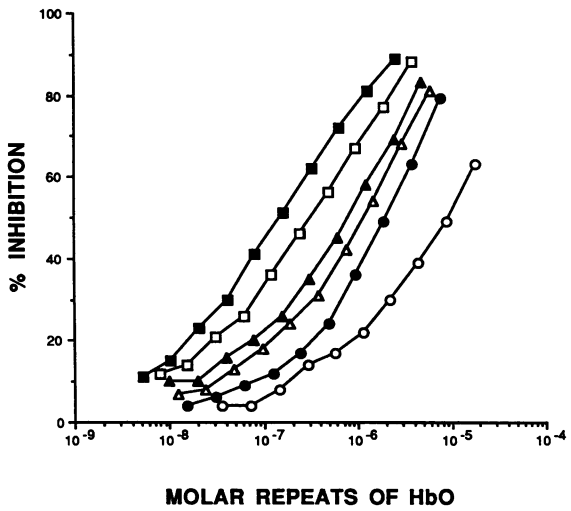

FIG. 3. Comparison of *H. influenzae* type b oligosaccharides (HbO) of different chain lengths in a competition ELISA with human monoclonal antisera. Plates were coated with tyramine-conjugated PRP at 100 ng per well, and the binding of antibody was inhibited with different concentrations of oligosaccharide. The data are presented as the percent inhibition observed with different molar repeats of PRP. Symbols: ○, Dp3; ●, Dp7; △, Dp9; ▲, Dp11; □, Dp14; ■, Dp21.

oligosaccharides of different sizes. The competition ELISA developed to determine the ability of conjugate vaccines to absorb antibody depends on the quantity and quality of antibody present in the immune sera. Polyclonal antisera may vary in their avidity, and therefore it is important to determine the initial binding capacity. The half-maximal OD (0.5 to 0.6) was obtained with a serum dilution of 1:3,000 with OBRR serum which contains 70 μg of anti-PRP antibody per ml. A similar half-maximal OD was obtained with a monoclonal antibody dilution of 1:20,000. This particular batch contains 69.3 μg of anti-PRP monoclonal antibody per ml as estimated by the Farr assay. Different amounts of antibody did not compromise the outcome of the results, as all antigens were tested against a constant dilution of any particular serum.

To determine the minimal size of the *H. influenzae* type b oligosaccharide that would bind any antibody, we performed competition experiments with oligosaccharides of different sizes. The percent inhibition observed was plotted against the molar concentration of repeat units used in the assay as depicted in Fig. 2. Competition curves obtained with polysaccharide are not included, for simplicity of presentation. However, for the purpose of calculations, the polysaccharide has been assumed to have 500 repeat units. Each increment in the molecular size of the *H. influenzae* type b oligosaccharide was associated with a shift to the left of the resulting inhibition curves. The concentration of inhibitor required for 50% inhibition decreased progressively with increasing saccharide chain length from $>10^{-3}$ M for a single repeating unit of oligosaccharide to 5×10^{-7} for a 262-repeat-unit polymer. Even the smallest oligosaccharide was potentially capable of attaining a significant inhibition if high enough concentrations of oligosaccharides are used in the assay.

This kind of absorption and the gradual decrease in the concentration of antigen required for a 50% inhibition may be due to the presence of different populations of antibody in the polyclonal antiserum used in this study. To analyze this possibility, we used a human anti-PRP monoclonal antibody in the competition ELISA in parallel with OBRR sera. The data are presented in Fig. 3. The inhibition curves obtained were similar to that obtained with polyclonal antiserum (Fig. 2). There was a gradual increase in the avidity of antibody binding as the chain length of the oligosaccharide was increased.

Specificity of the competition reaction. The specificity of the competition reaction obtained with *H. influenzae* type b polysaccharide or oligosaccharide was analyzed in a heterologous system by using pneumococcal polysaccharide competition ELISA to ensure that the previous inhibition was specific to PRP. Plates were coated with native type 6A pneumococcal polysaccharide, and the binding of rabbit anti-6A antibody to the saccharide was inhibited with oligosaccharides or polysaccharides from the capsule of *S. pneumoniae* or *H. influenzae*. The data show that pneumococcal saccharides but not *H. influenzae* type b polysaccharides or oligosaccharides compete with the binding rabbit antibody to 6A polysaccharide (Fig. 4). Because of the presence of the ribitol moieties in type 6A pneumococcal saccharides, this may be an ideal saccharide for this kind of specificity study.

Comparison of reduced and nonreduced *H. influenzae* type b oligosaccharide. *H. influenzae* type b oligosaccharides used

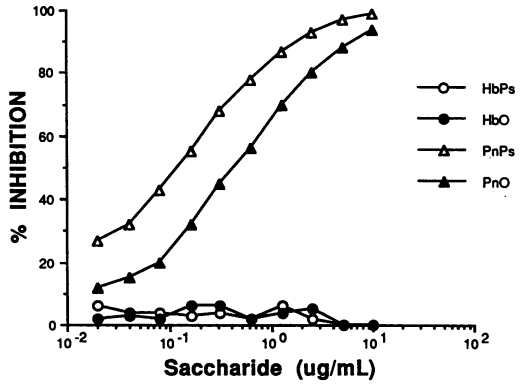

FIG. 4. Specificity of the competition ELISA. Plates were coated with pneumococcal 6A polysaccharide at 1 μg/ml and reacted with rabbit antibody directed against this antigen. Competition experiments were performed with pneumococcal polysaccharide and oligosaccharide (PnPs and PnO) and *H. influenzae* type b polysaccharide and oligosaccharide (HbPs and HbO).

in the competition experiments were obtained by cleaving polysaccharide with sodium periodate. The free aldehyde group formed as a result of the oxidative cleavage of the adjacent hydroxyl group of the ribitol moiety was not reduced. It is possible that free aldehydes interact and bind antibody and thus affect the binding avidity of the antibody. Therefore, the oligosaccharides were reduced with sodium borohydride and both reduced and nonreduced oligosaccharides were compared in a competition ELISA with monoclonal anti-PRP antibody. Oligosaccharides of Dp2, Dp13, and Dp24 were used. The data presented in Fig. 5 show that the competition observed for nonreduced saccharides is no different from that obtained with reduced saccharides. Therefore, the presence of free aldehyde groups does not adversely affect the binding of antibody to sugar residues.

Comparison of *H. influenzae* type b polysaccharide and conjugate vaccines. Having shown that *H. influenzae* type b oligosaccharide absorbs less antibody than the polysaccharides do, conjugate vaccines consisting of oligosaccharides or polysaccharides coupled to proteins were tested for their

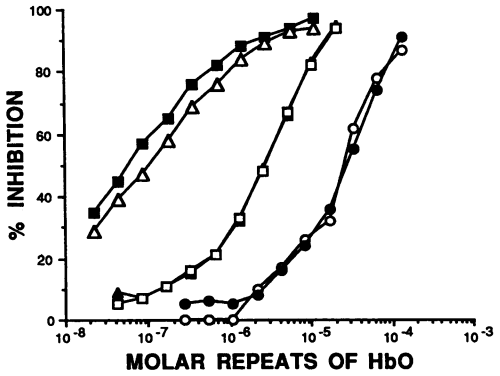

FIG. 5. Comparison of reduced and nonreduced *H. influenzae* type b oligosaccharide (HbO) of different chain lengths in a competition ELISA with human monoclonal antisera. Plates were coated with tyramine-conjugated PRP at 100 ng per well, and the binding of antibody was inhibited with different concentrations of oligosaccharides. The data are presented as the percent inhibition observed with different molar repeats of PRP. Symbols: ○, Dp2; ●, reduced Dp2; □, Dp13; ▲, reduced Dp13; ■, Dp24; △, reduced Dp24.

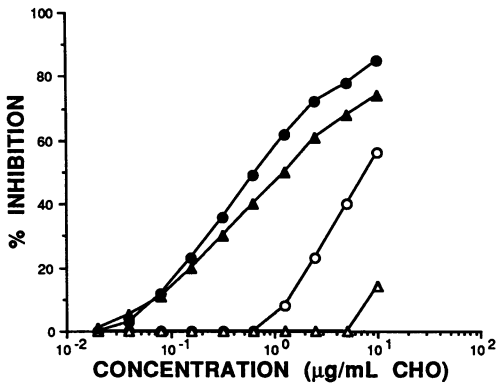

FIG. 6. Comparison of *H. influenzae* type b oligosaccharide or polysaccharide and conjugate vaccines based on these molecules in a competition ELISA with human polyclonal antisera. Plates were coated with tyramine-conjugated PRP at 100 ng per well, and the binding of antibody was inhibited with different concentrations of antigens. The data are presented as percent inhibition observed with different concentrations (micrograms per milliliter) of PRP. Symbols: ○, conjugated *H. influenzae* oligosaccharide; ●, conjugated *H. influenzae* polysaccharide; △, *H. influenzae* oligosaccharide alone; ▲, *H. influenzae* polysaccharide alone.

ability to bind anti-PRP antibody. Competition ELISA data from three different experiments are presented in Fig. 6. Polysaccharides and oligosaccharide of Dp20 were used as controls. The polysaccharide binds antisera very effectively as expected. The polysaccharide-protein conjugate binds slightly but not significantly more antibody per unit weight of carbohydrate than does polysaccharide itself in all these experiments. On the other hand, the oligosaccharide-protein conjugate vaccine binds two- to threefold more antibody than do unconjugated free oligosaccharides. Overall, the data suggest that anti-capsular antibody binds with higher avidity to oligosaccharide that is coupled to a protein carrier, whereas the avidity of polysaccharide binding to anti-PRP antibody is not significantly different from that of polysaccharide-protein conjugate vaccine.

DISCUSSION

The results of the ELISA inhibition studies with capsular polysaccharide of *H. influenzae* type b and oligosaccharides derived from it indicate that the avidity of antibody to polysaccharide is determined by the chain length of the saccharides. The polysaccharide and the longer oligosaccharides compete better than the shorter oligosaccharides. The oligosaccharide-protein conjugate competes better with PRP than free oligosaccharides do. However, the polysaccharide-protein conjugates compete as well as the free polysaccharide. Thus, antibody binding to the repeated antigenic epitopes is determined, in addition to other factors, by the chain length of the sugar molecules.

The inhibition of binding polyclonal antibody to *H. influenzae* type b polysaccharide on the solid phase by oligosaccharide was specific (Fig. 4) and was 10 times less efficient than that obtained with the corresponding polysaccharide (Fig. 6). The mechanism for the increased binding of antibody by high-molecular-weight forms of these antigens is not known. It is possible that the hyperimmune serum used in these studies contains subpopulations of anti-*H. influenzae* type b polysaccharide antibody of high and low affinities and

thus may preferentially bind to these different forms of saccharides. However, inhibition curves obtained with a human monoclonal anti-PRP antibody were no different from those obtained with polyclonal antibody (Fig. 3). Therefore, the affinity differences of different types of antibody in the polyclonal antiserum do not explain the observed phenomena. Another possibility is that in the polymeric form of the antigen, the antibody-binding epitope, is stabilized in a particular conformation which may be favorable for antibody recognition and binding. The same epitope in the oligomers may not possess a similar conformation, or the favored conformation may be present at low frequency and thus supports only low-affinity interactions. Such an interpretation has been offered for similar experimental data obtained with other charged (13, 14, 20) and neutral (19) polysaccharides. A third possibility is that the binding of one immunoglobulin molecule to a site on the polysaccharide leads to a marked increase in the avidity of interaction between adjacent determinants and the antibody and thus confers a stable conformation. This concept is supported by data showing that the binding of one monoclonal antibody to group A streptococcal carbohydrate antigen greatly enhanced the binding of a second monoclonal antibody to the same antigen (9).

Whatever the mechanism, the idea that higher-molecular-weight forms of polysaccharide develop unique conformational epitopes is not unlikely. When compared with protein antigens which have unique conformational epitopes based on secondary and tertiary folding structures (6), polysaccharides have less flexibility to form a similar conformational folding. However, the repetitive nature of the linkage pattern of polysaccharides results in a regular, periodic structure of the polymer as described for a β(1-3) glucan, which forms a hollow helix, and glucans with β(1-4) linkages, which have an extended ribbon conformation (16). Such a prediction of conformation based on the linkage pattern is not yet possible for complex polysaccharides. Recent experimental data based on crystallographic and X-ray diffraction studies have suggested a regular helical shape for several complex polysaccharides (7, 12, 21). Such crystallographic data are not available at present for *H. influenzae* type b polysaccharide.

The estimation of the minimum chain length of oligomers that would compete with polysaccharides is very critical to our understanding of the epitopes expressed by *H. influenzae* type b polysaccharide. A progressive increase in the avidity of anti-polysaccharide antibody to bind to these oligomers was observed as the saccharide chain length increased from 1 to 262 repeating units. The logarithmic curve fit analysis of individual inhibition curves (Fig. 2b) supports the idea that this pattern of antibody reactivity is not due to an increase in the affinity of antibody binding. The analysis showed that the slopes of the curves (mean S = 33.962 ± 0.495; r = 0.9945 ± 0.003) are not different from each other. These analyses suggest that the affinity of binding is not different for each saccharide. Therefore, a shift in the inhibition curves may be due to the avidity of antibody binding to saccharides rather than to the affinity differences. The free aldehyde groups of *H. influenzae* type b oligosaccharides do not affect the antibody binding and avidity, as shown by the identical inhibition curves (Fig. 5) obtained with reduced and nonreduced oligosaccharides.

The increase in antibody-binding avidity between Dp1 and Dp2 oligomers is especially dramatic when compared with that of Dp2 and Dp7 or other oligosaccharides used in this study. This difference may be explained by the complete filling of antibody-binding sites by Dp2 oligomers because the binding site of an anti-polysaccharide antibody has been shown to be filled optimally by an epitope corresponding to no more than six or seven monosaccharides (10, 17). Dp2 oligomers of PRP, although having only four monosaccharides, may fill in the binding site of the antibody because of the presence of phosphate groups (4) in addition to ribose and ribitol moieties.

The increase in avidity from Dp2 to Dp7 is possible, to a certain extent, because the antigen chain length increases sufficiently to allow simultaneous binding of both Fab regions of the antibody molecule to separate epitopes of the oligomer. However, further increases in antibody-binding avidity as the antigen chain length increases beyond that expected to accommodate bivalent binding is difficult to explain (Fig. 2). Another possibility is that high-molecular-weight forms of polysaccharides have a conformation that is suitable for high-affinity binding of anti-polysaccharide antibody. The data also suggest that this conformation is not unique to high-molecular-weight polymers but also is expressed by oligomers of one or two repeating units. The oligomers of one or two repeating units are able to inhibit antibody binding to the native polysaccharides at a high oligosaccharide concentration (Fig. 2), suggesting that the same epitope is expressed by oligomers and polymers. It is possible that oligomers have unlimited rotational movement of individual sugars and that only a certain conformation is favorable for antibody binding, and therefore a large number of oligomers are needed for effective competition. Such a rotational mobility may be stabilized by the polysaccharide in a favorable conformation for antibody binding. Two-dimensional nuclear magnetic resonance structural analysis of *N. meningitidis* group B polysaccharide by Yamasaki and Bacon (22) supports this idea. These authors suggest that smaller oligosaccharides are more flexible and may not form an ordered structure as the polysaccharide does. The data presented in Fig. 6 comparing the competing ability of *H. influenzae* type b oligosaccharides alone and coupled to protein carriers supports this interpretation. The oligosaccharides of Dp20, when stabilized by coupling onto a protein, bind with higher avidity to anti-PRP antibody than do the oligosaccharides alone. Such differences in the binding pattern were not observed when polysaccharide and polysaccharide-based conjugates were compared (Fig. 5). Thus, the stability of the antibody-binding site in a preferred conformation may be the reason for the differences in binding observed with various sizes of polysaccharide and its fragments. The data obtained in this study provides some supporting evidence for the hypothesis that the shorter-chain saccharide-protein conjugates, in addition to eliciting T-cell-dependent antibody response (2, 18), express the complete antigenic epitope of the intact polysaccharide.

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