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Functional Capacities of Clonal Antibodies to *Haemophilus influenzae* Type b Polysaccharide

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***Haemophilus influenzae* type b (Hib) is an important pathogen for young children, and children can be protected with antibodies (Abs) to Hib polysaccharide (PS) capsule, a linear polymer of ribosyl ribitol phosphate. The structure of anti-Hib-PS Abs has been well characterized at the molecular level; about two-thirds of anti-Hib-PS Abs use a V_κ gene named A2, and the remaining anti-Hib-PS Abs use one of many other VL genes. In order to understand the structural basis for the variability in the function of these Abs, we prepared 18 clonally pure Abs from adults and studied their affinity, avidity, bactericidal potency in vitro, and ability to reduce bacteremia in newborn rats. Affinities and avidities were determined as the inverse of the concentrations of short (3 repeating units) and long (20 repeating units) ligands which could bind 50% of anti-Hib-PS Ab in solution, respectively. No significant correlations between the protection of newborn rats and affinity ($r = 0.02$) or avidity ($r = 0.16$) were observed. The amount of Ab required to kill 50% of bacteria in vitro decreased with avidity ($r = -0.32$), as expected. However, Abs with high affinity were unexpectedly found to have less bactericidal activity ($r = 0.38$). This suggests that avidity may be a better predictor of Ab function than affinity. Affinity and avidity results were negatively correlated ($r = 0.76$, $P = 0.0022$), and Abs that had A2 V_κ gene products had higher avidity ($P < 0.05$) and lower affinity ($P = 0.06$) than Abs that had other VL genes. A possible explanation of these observations is that the epitope for Abs with the A2 gene is within the Hib-PS chain itself, whereas the epitope for Abs with a non-A2 gene is the terminus of Hib-PS.**

Haemophilus influenzae type b (Hib) is a gram-negative bacterium with a polysaccharide (PS) capsule, which is composed of a linear polymer of ribosyl ribitol phosphate. It is an important pathogen for young children, and antibodies (Abs) to its capsular PS protect the children from Hib infection. For these reasons, the structure of Abs to Hib-PS has been studied extensively, and their structure has been well characterized at the molecular level. Study of the constant regions showed that immunoglobulin G1 (IgG1) and IgG2 Abs account for most of anti-Hib-PS Abs, although IgM and IgA Abs are made in small amounts. Studies of Ab V regions have found that anti-Hib-PS Abs use a limited number (two genes in the V_HIII family) of VH genes, whereas they use a VL selected from a large number (more than eight) of VL genes (reviewed in references 7 and 32). Yet most (about two-thirds) anti-Hib-PS Abs have one particular V_κ gene, named A2. Anti-Hib-PS Abs with A2 are referred to as A2 Abs. The remaining Abs have the other VL genes and are referred to herein as non-A2 Abs.

Anti-Hib-PS immune sera have been shown to be functionally heterogeneous. About 1 out of 10 polyclonal antisera is poor in bactericidal capacities (2, 20) or opsonizing capacities (8). Attempts to associate the Ab structure with its function showed that IgG1 and IgG2 Abs, which account for most of the Abs, are similar in function. Although IgA Abs are not functional (28), IgA Abs account for only a small amount of anti-Hib-PS Abs and do not account for the observed functional variability. Thus, despite the detailed understanding of anti-Hib-PS Abs, the structural basis for the functional heterogeneity is still unknown.

Murine studies indicated that the V regions can influence Ab function (1, 5, 21, 25). The bactericidal capacity of anti-Hib-PS Abs from children was found to be associated with either Ab affinity (14) or avidity (27). However, the studies examined polyclonal anti-Hib-PS Abs, and the V region structures influencing the antigen (Ag)-binding strength are unknown. Elucidation of the structural basis for the functional variation would be useful in improving vaccine designs. Here, we studied clonally pure Abs to identify the V region structures important for their function, i.e., bactericidal potency, in vivo protection, and Ab affinity and avidity.

MATERIALS AND METHODS

Preparation of clonal anti-Hib-PS Abs. Clonally pure anti-Hib-PS Abs from adults were prepared as previously described in detail (33). Briefly, a series of affinity chromatography steps, first with Hib-PS-Sepharose and then with Sepharose-coupled monoclonal Abs (MAbs) specific for IgG1 or IgG2, along with preparative isoelectric focusing was used to purify the Abs to clonality (30). Non-V_κII-A2 anti-Hib-PS Abs were further purified by subsequent passage over a column of Sepharose to which the MAb LuC-9 was conjugated to remove any contaminating V_κII-A2 anti-Hib-PS Abs (16). The V_κ family of purified anti-Hib-PS Abs was defined by amino acid sequencing (29–31) or by a VL subgroup-specific enzyme-linked immunosorbent assay (ELISA) (9). The ELISA assay showed that less than 5% of the protein concentration of the clonal Abs could be due to the non-relevant VL subgroups.

Since aggregates of Ab molecules may alter the functional potency of an Ab (6), the aggregates of the Ig molecules in the clonal Abs were removed by centrifugation at 200,000 × *g* for 20 min in an Airfuge (Beckman, Palo Alto, Calif.). The clonal Abs were sterilized by filtration and stored at 4°C in a maltose buffer (139 mM maltose, 30 mM NaCl, 50 mM NaH_2PO_4 [pH 6]), which minimizes Ab aggregation (22).

Determination of Ab concentrations. The amount of anti-Hib-PS Ab was determined by sandwich-type immunoassays as previously described in detail (9).

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The wells of Immulon II plates (Dynatech, Chantilly, Va.) were coated with goat Abs specific for human IgG. The plates were washed and blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Samples were then added, titrated, and incubated overnight. The plates were subsequently washed, and goat anti-human Ig Ab labeled with alkaline phosphatase was added. The amount of the enzyme immobilized in the well was determined with *para*-nitrophenylphosphate substrate (Sigma, St. Louis, Mo.). The optical density at 405 nm was read with a microplate reader (model 7520; Cambridge Technology, Watertown, Mass.). The amount of Ab in the sample was determined by comparing its optical density with that of the standard, which was a mixture of five different purified IgG myeloma proteins.

Affinity measurement. Affinity was measured exactly as described previously (13). The wells of microtiter plates were coated with tyramine-conjugated Hib-PS, and 50 μl of oligosaccharide, ranging by twofold dilutions from 0.55×10^{-5} to 17×10^{-5} M, was added to successive wells of even-numbered columns. The oligosaccharide was prepared by alkali hydrolysis and had three repeating units (13). To these wells were added 50 μl of Abs, diluted to give 400 ng/ml. To the odd-numbered columns were added 100 μl of serial twofold dilutions of Abs, ranging from 25 to 400 ng/ml, without any oligosaccharide. After incubation, the plate was washed, and peroxidase-conjugated goat anti-human IgG was added. Later, the amount of bound peroxidase was determined by using *o*-phenylenediamine. Affinity was determined as the inverse of the concentration of the oligosaccharide that produced an optical density at 492 nm identical to that in the well containing 200 ng of Ab per ml.

Determination of avidity. To measure Ab avidity, we performed a modified ELISA as described by Hetherington (13). The wells of Immulon II plates (Dynatech) were coated with tyramine-conjugated Hib-PS (0.1 μg/ml) for 5 days, washed, and blocked with PBS containing 1% nonfat milk powder (Carnation). A clonal Ab containing 200 ng of Ab per ml was added to the wells in the presence of an oligosaccharide inhibitor at various concentrations (ranging from 0.06 to 960 nM) and then incubated for 2 h at 4°C. The inhibitor was a fragment of Hib-PS with 20 repeating units, obtained from Lederle-Praxis Biologics (Rochester, N.Y.). To other wells, samples containing 200, 100, 50, or 0 ng of Ab per ml were applied without any inhibitors. The plates were subsequently washed, and alkaline phosphatase-conjugated goat anti-human IgG Abs (product number 62-8422, lot number 21212172; Zymed Laboratories, San Francisco, Calif.) diluted in 1% nonfat milk in PBS were added to every well. After several hours of incubation, the wells were washed, and the amount of enzyme immobilized to the well was determined with *para*-nitrophenylphosphate substrate (Sigma). The optical density at 405 nm was read with a microplate reader (Cambridge Technology, Watertown, Mass.). The avidity was defined as the inverse of the concentration of the oligosaccharide that produced an optical density identical to that in the well containing 100 ng of Ab per ml.

Bactericidal assay. The bactericidal capacity of the Abs was assessed with the Eagan strain of Hib (4). Ab-free complement source (precolostral calf serum; 7.5 μl), 7.5 μl of PCM diluent (PBS with 0.14 mM $CaCl_2$ and 0.5 mM $MgSO_4$) containing 750 CFU of bacteria in the exponential phase of growth, and 15 μl of anti-Hib-PS Ab diluted in PCM were mixed in the wells of microtiter plates; each Ab was tested in twofold dilutions, giving final concentrations ranging from 5 to 0.156 μg/ml. After 30 min of incubation at 37°C, the number of surviving bacteria was determined by plating 10 μl of the reaction mixture on BHI-DB agar plates. Percent killing was determined in reference to a negative control containing complement but no Ab. A human serum pool provided by the U.S. Food and Drug Administration as an anti-Hib-PS Ab standard was used as a positive control.

Rat protection assays. The in vivo protective capacity of the Abs was determined by a well-established newborn rat protection assay with the Eagan strain of Hib (3). The bacteria were passaged twice through the rats as described below in order to increase pathogenicity, grown into a large batch, aliquoted, and stored frozen at −70°C until use. An aliquot was used only once for each experiment. Three-day-old infant Sprague Dawley rats (Charles River, Wilmington, Mass.) from different litters were randomly mixed before various amounts of Ab were given subcutaneously. Six to 10 rats were used for each experimental group. The following day, about 3,000 CFU of bacteria in the log phase of growth were given to each rat intraperitoneally. In preliminary studies, we examined inoculation doses of bacteria ranging from 300 to 30,000 CFU per rat and chose an inoculation dose of 3,000 CFU per rat because that dose of bacteria could be reproducibly prepared and induced a high degree of bacteremia with no obvious ill effect on the rats. Twenty-four hours later, blood was obtained from the rats by cardiac puncture, and serial dilutions of the blood sample were cultured on chocolate agar plates (Becton Dickinson, Cockeysville, Md.). To obtain the average number of colony-forming cells per 50 μl of blood sample from each rat, a statistical weight (Poisson distribution) was assigned to the number of colonies per plate for each serial dilution. The geometric mean numbers of colonies per 50 μl of blood were calculated for the experimental groups.

In all experiments when 100 ng of control serum was given to each rat, the bacteremia became 200- to 400-fold less than in rats not given the serum. To minimize the variability among the data from different experiments, the protective potency at 100 ng of Ab per rat was normalized as follows: log (normalized fold reduction by an Ab) = log (fold reduction by an Ab) × log (fold reduction by the control serum)/log (400), where fold reduction is defined as the ratio of bacteremia with no Ab protection to bacteremia with Ab protection.

TABLE 1. Summary of clonal Abs

Ab	Vaccine[a]	VL[b]	Heavy-chain isotype	Affinity (10^3 M)	Avidity (10^7 M)
A2					
G-G1	CHO	V_κII	IgG1	670	43
DT4G1	DT	V_κII	IgG1	330	140
B-G2	CHO	V_κII, J_κ2	IgG2	163	110
M-G1	CHO	V_κII	IgG1	163	430
B-G1	CHO	V_κII, J_κ1	IgG1	163	56
C-G1	CHO	V_κII, J_κ1	IgG1	163	250
D-G1	CHO	V_κII, J_κ1	IgG1	80	720
D-G2$_\kappa$	CHO	V_κII, J_κ1	IgG2	40	430
C-G2	CHO	V_κII, J_κ1	IgG2	<10	300
A-G1	CHO	V_κII	IgG1	—[c]	150
Non-A2					
H-G2	CHO	V_κI	IgG2	670	42
CRM1G1	CRM	V_κIII	IgG1	670	58
D-G2$_\lambda$	CHO	V_λ	IgG2	670	—
DT4G2	DT	V_λ	IgG2	330	24
R-G2$_\lambda$	CHO	V_λ	IgG2	163	130
K-G2	CHO	V_κI	IgG2	—	8.7
DT6G2	DT	V_κI	IgG2	—	200
E-G2$_\lambda$	CHO	V_λ	IgG2	—	240

[a] Serum donors were immunized with a PS vaccine (indicated as CHO), ProHibit vaccine (Connaught Laboratories, Swiftwater, Pa.) (indicated as DT), or HibTiter vaccine (Lederle-Praxis, Rochester, N.Y.) (indicated as CRM).

[b] V_κI, V_κIII, and V_λ indicate that the Ab has a VL in the V_κI subgroup, V_κIII subgroup, or V_λ, respectively. V_κII indicates that the Ab has the A2-V_κII VL gene. Among the Abs with the A2-V_κII gene, when the J_κ gene present is known, the J_κ gene used for VL is indicated.

[c] —, not determined.

RESULTS

Affinity and avidity of anti-Hib-PS Abs. To determine the affinity and avidity of clonal anti-Hib-PS Abs, we prepared 18 clonally pure Abs by various affinity chromatography steps. Although most of the Abs were obtained from donors vaccinated with a PS vaccine, many non-A2 Abs were obtained from donors vaccinated with conjugate vaccines (Table 1), which elicit non-A2 Abs better than PS vaccines (9). Their concentrations were determined by ELISAs which detect the Abs by their CH or CL regions in order to determine the concentrations of these Abs independent of their Ag-binding abilities. Their affinities and avidities were measured by determining the concentration of short and long ligands, respectively, which can bind 50% of anti-Hib-PS Ab in solution, as described before (13). One Ab had an affinity value of less than 10^4 M^{-1}, and we assigned its affinity to be 5×10^3 M^{-1} for the purpose of analysis. As shown in Fig. 1, the affinities and avidities of the clonal Abs ranged more than 50-fold. Interestingly, they had a strong negative correlation ($r = -0.76$ and $P = 0.0022$ by Spearman's rank sum test); Abs with high avidities have low affinities. The average avidity and affinity values of clonal Abs were comparable to those seen with polyclonal antisera (data not shown).

The strength of Ag binding was not influenced by CH, inasmuch as IgG2 Abs had affinities and avidities similar to those of IgG1 Abs ($P = 0.4$ and 0.48, respectively, by two-tailed t test). In contrast, the VL region influenced the binding strength of the Ag. The average avidity of 10 Abs with the A2 V_κ gene was about threefold higher than that of seven Abs with non-A2 VL genes (187×10^7 M^{-1} versus 61×10^7 M^{-1}; $P < 0.05$ by two-tailed t test). The average affinity of the Abs with the A2 V_κ gene was about fourfold less than that of non-A2-

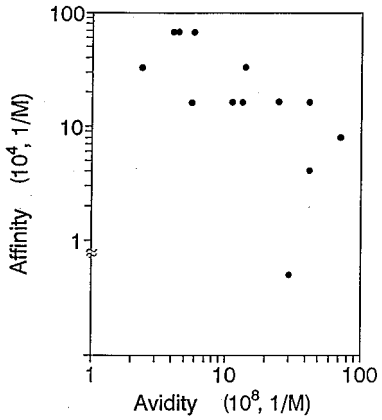

FIG. 1. Relationship between avidity (*x* axis) and affinity (*y* axis). Wavy lines on the *y* axis indicate the sensitivity limit of the assay. We assigned 5×10^3 M^{-1} for the sample with undetectable affinity for the purpose of analysis. The correlation coefficient is −0.76.

type Abs (111×10^3 M^{-1} versus 438×10^3 M^{-1}; $P = 0.06$ by two-tailed *t* test).

Most of the Abs with the A2 V_κ gene have an identical VL region; they do not have somatic mutations, and many of them use the Jk1 gene product joined to the A2 V_κ gene product by arginine (30), as indicated in Table 1. Consequently, by examining the avidities and affinities of A2 Abs with the $J_\kappa 1$ gene, the effect of the V_H region on Ag binding strength could be inferred. The affinities of clonal Abs C-G2 and B-G1 differed by more than 16-fold, and the avidities of clonal Abs B-G1 and D-G1 differed by more than 10-fold (Table 1). The V_H region thus also had a significant effect on Ag binding. It is known that two V_H genes are used for anti-Hib-PS Abs. Since it was not practical for us to distinguish them and the V_H may have somatic mutations, it is unclear at present how V_H would influence Ag binding.

Bactericidal capacity of anti-Hib-PS Abs. To identify the features of the Ab that affect the functional potencies of the clonal Abs, we determined the in vitro bactericidal potency of 10 Abs by measuring the Ab concentrations required to kill either 50 or 90% of bacteria compared with the number of CFU in the Ab-negative control. Two replicate experiments gave very similar results, and the 90% endpoints were either the same or twofold higher than the 50% endpoints. Consequently, the results from one experiment were used for analysis (Fig. 2). To examine the association between the bactericidal potency of an Ab and its Ag-binding ability, the amount of Ab required to kill 50% of the control was shown as a function of Ab affinity (Fig. 2A) or Ab avidity (Fig. 2B). One Ab did not kill 50% of bacteria even at 5 μg/ml, and 10 μg/ml was used as the concentration of the Ab needed to kill 50% for the purpose of analysis. Bactericidal potency did not show an expected correlation with affinity; Abs with high affinity actually had less bactericidal potency ($r = 0.38$ by Spearman's rank sum test), but this correlation became very small ($r = 0.12$ by Spearman's rank sum test) when the point with lowest affinity was removed from the calculation. In contrast, bactericidal potency and avidity showed only a fair degree of expected correlation ($r = -0.32$ by Spearman's rank sum test). Although the correlations are not very strong, these findings suggest that avidity is a better predictor of Ab function than affinity. The bactericidal potencies of A2 and non-A2 Abs were similar ($P = 0.36$ by two-tailed *t* test). Also, IgG1 and IgG2 Abs had similar bactericidal potencies ($P = 0.49$ by two-tailed *t* test).

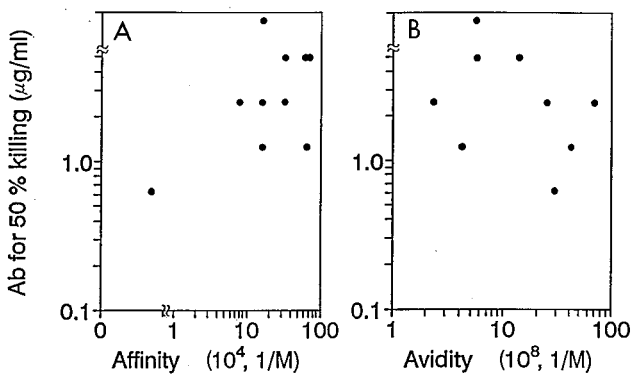

FIG. 2. Relationship between the amount of Ab required to kill 50% of bacteria (*y* axis) and (A) Ab affinity or (B) Ab avidity. The concentration of Ab necessary to reduce the number of bacteria by 50% compared with the number without Abs was determined. Wavy lines indicate the detection limits of assays. We assigned 10 μg/ml as the concentration required to kill 50% of bacteria for the sample that required more than 5 μg/ml for the purpose of analysis. The correlation coefficient between the Ab concentration and affinity was 0.38, but it became 0.12 when the datum point with the lowest affinity was removed from the analysis. The correlation coefficient between Ab concentration and avidity was −0.32.

Comparison of in vivo protective potency of anti-Hib-PS Abs. To identify the features of the Ab that affect the functional potencies of these Abs in vivo, we studied the abilities of these Abs to protect newborn rats from Hib infection. When protective effects were examined at various Ab doses in preliminary experiments, it was found that 50 to 100 ng per rat was optimal in discriminating Abs for their protective effects (Fig. 3). At these two Ab doses, the protective potency of 13 clonal Abs was determined in three separate experiments (Fig. 4). In these experiments, rats received 3,600 to 3,900 CFU of bacteria each and had bacteremia ranging from 3×10^5 to 4×10^6 CFU/50 μl of blood in the absence of any protective Ab. When rats were passively immunized with a serum pool containing 100 ng of anti-Hib-PS Ab, the protected rats had 200- to 400-fold-lower bacteremia (200-fold in one and about 400-fold in two experiments) than unprotected rats.

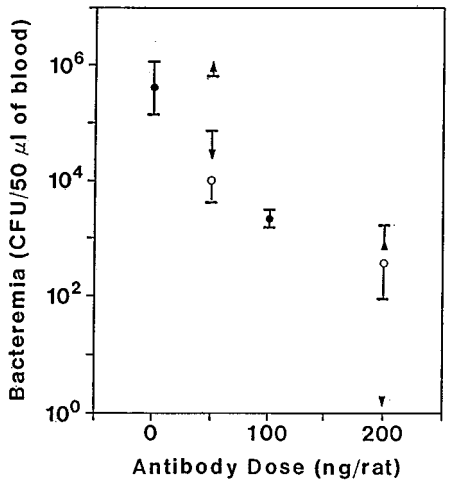

FIG. 3. Relationship between the number of CFU of bacteria per 50 μl of blood in newborn rats (*y* axis) and the doses of several Abs (*x* axis). Bars indicate the standard error. Solid circles indicate data for rats protected with a serum pool, used as the control. Open circles, triangles, and upside-down triangles indicate clonal Abs B-G1, D-G1, and G-G1, respectively.

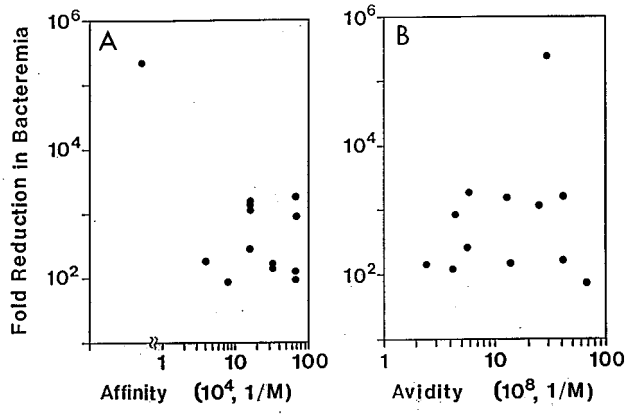

FIG. 4. Relationship between reduction in bacteremia in the presence of Ab (*y* axis) and (A) Ab affinity or (B) Ab avidity. A total of 100 ng of Ab was given for each rat. Wavy lines indicate the sensitivity limit of the assay. The correlation coefficient between bacteremia reduction and affinity is −0.23, but it was only 0.02 when the datum point with the lowest affinity was removed from analysis. The correlation coefficient between bacteremia reduction and avidity is 0.16.

The data from the three different experiments were normalized as described before by assigning the degree of bacteremia reduction by the serum pool to be 400-fold. Clonal Abs reduced bacteremia by 550-fold on the average. The protective potencies of Abs measured by the reduction in bacteremia were similar whether Abs had the A2 $V_\kappa 2$ gene (760-fold reduction) or were non-A2 Abs (330-fold reduction) ($P = 0.5$ by two-tailed *t* test). The protective potencies of IgG1 and IgG2 Abs were also similar ($P = 0.82$ by two-tailed *t* test). The normalized protective potencies correlated with the affinities in an unexpected manner ($r = -0.23$ by Spearman's rank sum test), but the correlation became insignificant ($r = 0.02$) when the outlier point with the lowest affinity was removed from the analysis (Fig. 4A). The normalized protective potencies did not correlate with avidity ($r = 0.16$ by Spearman's rank sum test) (Fig. 4B), even though the affinities and avidities of these 13 Abs range almost 50-fold (Fig. 4).

With most clonal Abs, when the amount of Ab given per rat was reduced from 100 to 50 ng, bacteremia increased by about 10- to 100-fold (data not shown). An exception was C-G2, which consistently reduced bacteremia much more (>10-fold) than other Abs at a dose of 100 ng per rat. Interestingly, C-G2 reduced bacteremia only as well as other Abs at a dose of 50 ng per rat. This finding indicates that the enhanced protective potency of C-G2 at 100 ng per rat is unlikely to be due to the wrong assignment of its Ab concentration.

DISCUSSION

The functional variability of anti-Hib-PS Abs induced by different vaccines has not been fully explained at the level of Ab structure. In order to examine which of the two Ag-binding parameters, affinity or avidity, better predicts the protective capacities of anti-Hib-PS Abs, we examined the abilities of clonal Abs to protect rats from bacteremia as well as to kill bacteria in vitro. We found that the in vivo protective potency was associated with neither avidity nor affinity, even though their values ranged more than 10-fold. The in vitro bactericidal potency of an Ab was associated with its avidity in an expected manner. However, affinity was negatively associated with bactericidal potency. Our study therefore suggests that the biologic function of anti-Hib-PS Abs was better correlated with the strength of binding of a long PS chain (avidity) than binding of a short PS chain (affinity). This conclusion was also supported by the findings for Ab C-G2, which had the lowest affinity yet had one of the highest avidities and was one of the best in function.

Our results indicate that monoclonal anti-Hib-PS Abs from adults can bind Ag sufficiently strongly that Ag binding may not be the limiting factor for their function in vivo. However, the binding strength is still sufficiently marginal that bactericidal potency is dependent on avidity. Furthermore, older individuals (21) and young children produce Abs whose V regions are different from those of Abs produced by young adults. For instance, newborn mice mostly produce Abs with no N regions (12, 18), and the absence of N regions should place a significant limit on the production of V_H regions with high avidity for Ags. A similar situation may exist in humans. Consequently, despite our results with adult Abs, some anti-Hib-PS Abs from children may have avidities so low as to be ineffective in vivo.

Previously, affinity was shown to predict the bactericidal capacity of polyclonal anti-Hib-PS antisera (14). Why does affinity fail to predict the bactericidal capacities of monoclonal anti-Hib-PS here? Two explanations are possible. First, the previous study examined antisera from children. While most of our clonal Abs from adults had measurable affinities, many samples from children in the previous study had Abs with unmeasurable affinities and may actually have had low avidities as well. Second, MAbs to the native Hib-PS differ not only in their strength of binding the Ag but also in the epitopes that they recognize. To avoid multivalent binding between Ab and its ligand, affinity is measured with a small (three repeating units) artificial ligand, which displays most of the epitopes of native Hib-PS unaltered but some epitopes altered from the native Hib-PS. The affinities of clonal Abs for a small ligand may represent binding to the altered epitopes rather than the strength of binding to native Hib-PS. In contrast, polyclonal Abs recognize multiple epitopes, and their affinities may indicate the binding strength for the "average" epitope of Hib-PS.

VL usage has been already associated with in vivo protective potency (5) and fine specificity for Ags (11, 33). This is the first example, as far as we are aware, that the VL region structure influences the strength of Ag binding. It was unexpected that Abs with non-A2 VL genes generally display higher affinity, although lower avidity, than A2 Abs. Avidity was measured by studying the binding of Ab to an Hib-PS ligand with 20 repeating units. In contrast, the affinity was measured with an Hib-PS ligand with only three repeating units. Consequently, an alternative interpretation of our finding is that the short ligand binds non-A2 Abs better than A2 Abs, whereas the long ligand binds A2 Abs better than non-A2 Abs.

Our finding is analogous to findings with murine antidextran Abs, which can also be divided into two types based on binding ligands of different lengths. Long-chain PSs can have epitopes that are dependent on conformation (19, 34), and long dextran ligands preferentially bind antidextran Abs by using V_H genes in the J558, 36-60, or J606 families, whereas short dextran ligands preferentially bind Abs by using V_H genes in the X24 or Q52 families (10, 17). Studies with dextran ligands of various lengths showed that Abs of the first type bind the internal Ag in the dextran chain, whereas Abs of the second type bind the nonreducing terminus of dextran, which is better accessible on the short polymer (10). These Abs are now called groove and cavity Abs, respectively, because three-dimensional modeling of their V regions showed that the Ag-binding site of the long-chain-favoring Abs is shaped like a groove, whereas the other is shaped like a cavity (23). The immunogenic conditions of the two types of Abs differ. The cavity-type antidextran Abs can be induced with dextran oligosaccharide conjugated on a

carrier protein but not with pure dextran itself (17), possibly because the oligosaccharide conjugate has more termini than the pure dextran.

This model could readily explain the observed inverse relationship between the avidities and affinities of anti-Hib-PS Abs. A2 Abs may preferentially bind the internal (or conformational) epitope of Hib-PS, and non-A2 Abs may bind its termini. Termini are more accessible in short chains, and the short ligand, used in the affinity measurement, can bind non-A2 Abs efficiently, whereas the ligand with the long chain, used in determining the avidity, preferentially binds the A2 Abs. In addition, this model is consistent with the presence of conformational epitopes for anti-Hib-PS Abs (24) and may explain why most non-A2 Abs cross-react with *Escherichia coli* K100 PS (32), which is identical to Hib-PS in its chemical composition except for one internal linkage (32). Because of the linkage difference, the tertiary structure of the *E. coli* K100 PS chain itself is different from that of the Hib-PS chain, but some of its termini are identical to Hib-PS termini (11a). Abs specific for the termini of Hib-PS could bind K100 PS termini equally well. It is interesting that, analogously to antidextran Abs, non-A2 Abs are expressed more following immunizations with conjugate Hib-PS vaccines than after immunizations with PS vaccines (Table 1) (9).

The model of two Hib-PS epitopes has been proposed before to explain the cross-reactivity of anti-Hib-PS antisera (15), but analyses of clonally pure Abs, as in these experiments, have clarified the model at the molecular level. Since conjugate Hib vaccines may have more Hib-PS termini than PS vaccines, the two-epitope model may provide a molecular basis for the different immunogenic behavior of Hib conjugate and PS vaccines (9). Abs to many PSs, like dextran, bind either their internal-chain (or conformational) epitope or terminal epitope (10, 19, 26). If further studies are able to support the two-epitope model for the immunogenicity differences among Hib vaccines, the model should be useful in developing conjugate vaccines of other bacterial PS capsules as well.

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