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Immunology

A model of high-affinity antibody binding to type III group B *Streptococcus* capsular polysaccharide

(antigen–antibody binding/molecular mimicry/oligosaccharide/bacterial polysaccharide/neonatal bacterial meningitis)

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ABSTRACT **We recently reported that the single repeating-unit pentasaccharide of type III group B *Streptococcus* (GBS) capsular polysaccharide is only weakly reactive with type III GBS antiserum. To further elucidate the relationship between antigen-chain length and antigenicity, tritiated oligosaccharides derived from type III capsular polysaccharide were used to generate detailed saturation binding curves with a fixed concentration of rabbit antiserum in a radioactive antigen-binding assay. A graded increase in affinity of antigen–antibody binding was seen as oligosaccharide size increased from 2.6 repeating units to 92 repeating units. These differences in affinity of antibody binding to oligosaccharides of different molecular size were confirmed by immunoprecipitation and competitive ELISA, two independent assays of antigen–antibody binding. Analysis of the saturation binding experiment indicated a difference of 300-fold in antibody-binding affinity for the largest versus the smallest tested oligosaccharides. Unexpectedly, the saturation binding values approached by the individual curves were inversely related to oligosaccharide chain length on a molar basis but equivalent on a weight basis. This observation is compatible with a model in which binding of an immunoglobulin molecule to an antigenic site on the polysaccharide facilitates subsequent binding of antibody to that antigen.**

Oligosaccharides homologous to regions of the repeating structure of the type III group B streptococcal capsular polysaccharide [α-D-Neu*p*NAc-(2→3)-β-D-Gal*p*-(1→4)-[→4)β-D-Glc*p*-(1→6)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal-(1→] have been isolated from human urine and identified on human glycoproteins; yet, the type III polysaccharide is immunogenic in most human subjects, and cross-reactions with host antigens have not been seen (1–3). Our previous investigations suggested that the polymeric structure of this bacterial polysaccharide, in addition to its primary repeating-unit structure, might be a critical factor in the host's ability to discriminate between the bacterial polysaccharide and homologous host oligosaccharides. We have recently reported that a pentasaccharide complete single repeating-unit oligosaccharide derived from the type III group B *Streptococcus* (GBS) capsular polysaccharide is very weakly antigenic (4). A double repeating-unit oligosaccharide was more antigenic than the single repeating-unit oligosaccharide but less so than the native polysaccharide, suggesting that antigenicity depended on antigen-chain length (4).

The current studies were undertaken to define the relationship between antigenicity and molecular size in this model system. In particular, we sought to estimate relative affinities of antigen binding to type III GBS antibody for oligosaccharides of different sizes. Large binding-affinity differences between antibody and large-M_r versus small-M_r antigens of the same repeating structure might represent a mechanism for host immune recognition of a bacterial polysaccharide as distinct from homologous oligosaccharides on host glycoproteins. By measuring antigen–antibody binding over a range of oligosaccharide concentrations for each of four oligosaccharides, we generated saturation binding curves for each oligosaccharide. Analysis of these curves has confirmed large differences in affinity of binding of small oligosaccharides versus large oligosaccharides to type III GBS antiserum. These results were confirmed by two independent immunologic assay methods. In addition, mathematical modeling based on these data suggests that antibody binding to one immunodeterminant site on GBS polysaccharide results in a dramatic increase in the antibody binding affinity of other immunodeterminant sites on the same polysaccharide molecule. This model may explain the high affinity of antigen–antibody interactions for many polysaccharide antigens.

MATERIALS AND METHODS

Type III GBS capsular polysaccharide (5) and derivative oligosaccharides (4, 6) were prepared and radiolabeled (7, 8) as described. Type III GBS antiserum was raised in New Zealand White rabbits as described using type III GBS strain M732 (9). IgG was adsorbed from this serum on a column of protein A-Sepharose and eluted with 0.1 M glycine·HCl, pH 3.0. Radioactive antigen binding assay (RABA) (10) and ELISA inhibition assays (11) have been described.

Statistical Analysis of Saturation Binding Curves. We modeled the relationship between y_s, the amount of antigen bound (in ng) for each oligosaccharide antigen of molecular size s (in ng/pmol), and x, the amount of antigen added (in ng) using a logistic-type regression (12) as follows:

$$\ln[y_s/(c_s - y_s)] = a_s + b_s \ln(x/s) + e \qquad [1]$$

where (x/s) is in pmol and the e values are independent and normally distributed with mean 0 and unknown variance σ^2. The parameter c_s stands for the amount of antigen bound when the antigen-binding sites of the antiserum are saturated (i.e., the amount of antigen bound for an infinite concentration of antigen); the $\exp(a_s)[1 + \exp(a_s)]^{-1} \times 100$ stands for the percentage of c_s that corresponds to the value of y for an amount of antigen added equal to 1 pmol; b_s measures the change of y related to the change of x.

The model in Eq. 1 provides a procedure for investigating the relationship between the amount of antigen bound and the

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Abbreviations: GBS, group B *Streptococcus*; RABA, radioactive antigen-binding assay.

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amount of antigen added. The procedure is free of some of the inherent limitations of conventional plots for analysis of complex binding interactions. Binding-site number and affinity estimates cannot be derived directly from nonlinear Scatchard or Steward–Petty plots, and bias may be introduced by assumptions about the reasons for nonlinearity—i.e., heterogeneity of antibody, antigen multivalency, or cooperativity of binding. As each of these factors could play a role in the interaction of a multivalent antigen with a polyclonal immune serum, we preferred the model equation described above, in which departures of the observed data from the classic binding isotherm are dealt with by letting the data determine the best model in the family of curves described by Eq. 1. The relatively unrestricted nature of the fitting procedure means that the estimates of binding parameters so derived should reflect more accurately the overall phenomenology of antigen–antibody binding in this complex system.

To estimate the unknown parameters a_s, b_s, c_s and σ^2, we used maximum-likelihood methods. In particular, for a given value of c_s, the fitting problem reduces to a standard regression. To find the full solution we proceeded by fitting the model for each value of c in a detailed grid. We refined the grid until we found the values of a, b, c, and σ^2 that maximized the full likelihood of the model in Eq. 1. For hypothesis testing purposes we used the likelihood-ratio test (13). In particular, for the purpose of testing whether the saturation binding values (c values) were different for different molecular sizes, we fit the model in Eq. 1 by assuming a common saturation binding value (i.e., $c_s = c$ for all molecular sizes). Twice the difference between the logarithmic likelihoods of the models with and without the above restriction on the saturation binding values follows an approximate χ^2 distribution with the number of distinct molecular sizes minus one. Similar procedures were done to test whether the a and/or b values were different for different molecular sizes.

RESULTS

Radioactive Antigen-Binding Assay (RABA) Saturation Binding Curves. The results of the saturation-binding experiments are shown in Fig. 1. We measured the amount of antigen bound by a fixed amount of antiserum over a range of antigen concentrations for each of four oligosaccharides of different chain length. Initially we assumed that, in the presence of excess antigen, not more than one antibody molecule would be bound to each antigen molecule (14). If all oligosaccharides were capable of binding to the same population(s) of antibody, we expected all the saturation curves to approach the same asymptote but at different rates depending on relative affinities. In other words, at a theoretically infinite antigen concentration, the number of moles of antigen bound by a fixed amount of antiserum should be the same, regardless of antigen size. Surprisingly, simple inspection of the binding curves shown in Fig. 1 suggests the number of moles of antigen bound at saturation is very different for oligosaccharides of different sizes.

To generate an objective estimate of "saturation" binding, we fit a mathematical model of the type described by Eq. 1 separately for each oligosaccharide to estimate the value of the asymptote approached by each curve. Like any mathematical model, the function generated is only an approximation of the true binding interaction(s); it treats as a single function the interaction of a multivalent antigen with, potentially, several populations of antibodies with different binding characteristics. To the extent that such factors influence antigen-binding capacity of the antiserum and overall affinity of binding, they will be reflected, in an unbiased way, in the curve generated by the regression model. When the curves were fit in this manner to the data points shown in Fig. 1, the

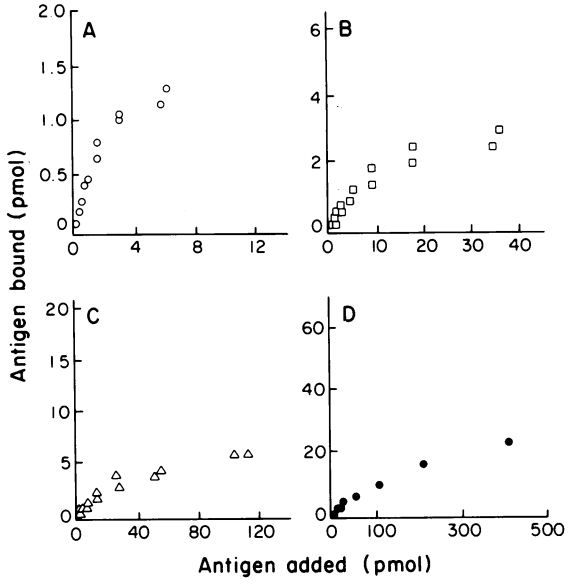

FIG. 1. RABA saturation binding curves for type III GBS antiserum and [^{3}H]oligosaccharides of average molecular size of 92 repeating units (*A*), 29 repeating units (*B*), 9 repeating units (*C*), and 2.6 repeating units (*D*). Data points represent means of duplicate determinations in each of two experiments (one experiment in *D*). Using a fixed concentration of antiserum, RABAs were done with increasing concentrations of antigen, generating a typical saturation binding curve for each oligosaccharide. At highest antigen concentration used, the ratio of free antigen to bound antigen ranged from 5:1 for the largest oligosaccharide (92 repeating units) to 18:1 for the smallest oligosaccharide.

calculated asymptote, c, or the number of moles of antigen bound to antibody at a theoretically infinite antigen concentration, increased from 1.7 pmol for the largest oligosaccharide to 48 pmol for the smallest. An explanation for this result was suggested by the observation that calculating the amount of antigen bound for each oligosaccharide in units of mass, rather than moles, resulted in c values for each oligosaccharide that agreed closely with one another (Table 1). That is, the amount of antigen bound on a weight basis, at saturation, appeared to be essentially independent of oligosaccharide-chain length.

Fig. 2 shows the same data as Fig. 1 with antigen bound expressed in terms of antigen mass, rather than moles, along with the curve calculated by Eq. 1 to give the best fit to the data points for each oligosaccharide. In *A*, the curves have been fit by application of Eq. 1 to the binding data for each oligosaccharide independently. The asymptote (c) values for these curves are those shown in Table 1. In *B*, the curves were fit imposing a single asymptote (c) value for all the curves simultaneously. Casual inspection of the two sets of curves, as well as statistical comparison (likelihood-ratio statistic = 0.16, df = 3, p value = 0.98), shows no difference between the fit of the curves generated by using the individual estimates for c calculated on the binding data for each oligosaccharide independently versus that calculated imposing a common value for c. In other words, when antigen bound is expressed in units of mass, antigen-binding capacity of the antiserum appears the same for each oligosaccharide, regardless of antigen-chain length. Particularly for the smaller oligosaccharides, the actual data points span only a portion of the predicted binding curve, resulting in some uncertainty in the estimate of the asymptote value, as is evident from the width of the confidence intervals in the table. Given this uncertainty, it is remarkable that independent estimates of the asymptote binding value for each of the oligosaccharides should agree so closely in terms of mass of antigen bound.

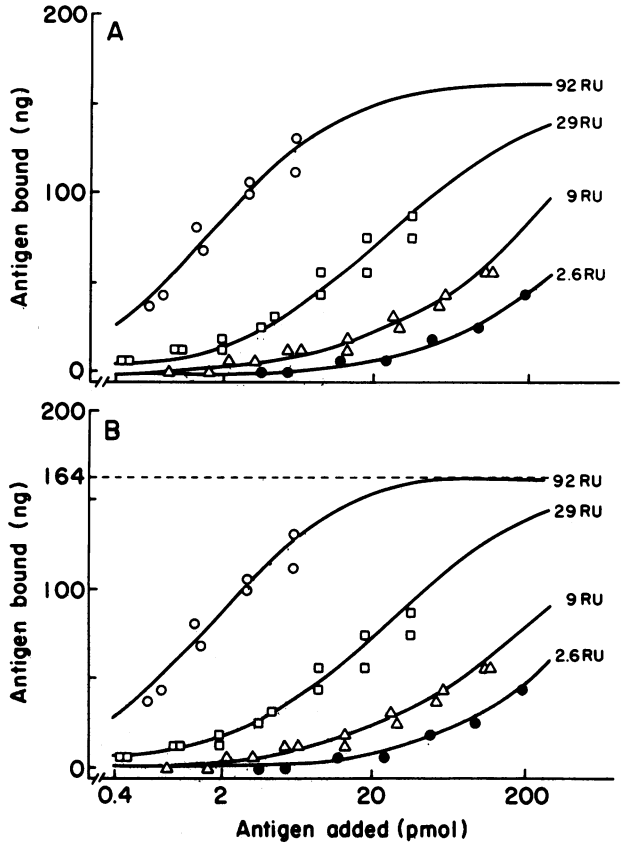

FIG. 2. Analysis of saturation binding curves. Data points are those shown in Fig. 1, here displayed as semilogarithmic plots with antigen binding expressed in ng rather than in pmol on the ordinate and antigen added in pmol on the x axis (logarithmic scale). In *A*, each curve represents the curve of best fit generated by application of Eq. 1 to each set of data points independently. In *B* each curve represents the curve of best fit imposing a single asymptote value for all curves. Binding curves are graphed as semilogarithmic plots so that binding curves of different affinities may be compared in a single plot. RU, repeating units.

Having estimated the antigen-binding capacity of the antiserum for each oligosaccharide, relative "overall affinities" of binding of different antigens can be compared by comparison of concentration of antigen required to achieve half-maximal binding. For a theoretical model system involving a monovalent hapten binding to a homogeneous population of antibody, this antigen concentration represents the dissociation constant (K_d) for the binding interaction (15). Interpretation of this value is complicated by antigen multivalency and by heterogeneous antibody; nonetheless, such "overall K_d" estimates may serve as a useful measure of the relative overall affinity of antigen–antibody interaction for different antigens. These overall K_d values are determined for each oligosaccharide from Fig. 2 as the antigen concentration at 50% saturation. Notwithstanding some uncertainty in the affinity estimates for the small oligosaccharides, there are clearly large differences in relative overall affinities for large oligosaccharides versus small oligosaccharides. Based on the antigen concentrations at half-maximal binding, or overall K_d, this difference in affinity between the largest (92 repeating units, $K_d = 1.6 \times 10^{-7}$ M) and smallest (2.6 repeating units, $K_d = 4.8 \times 10^{-5}$ M) oligosaccharides tested is ≈300-fold.

Table 1. Theoretical saturation binding values calculated by application of Eq. 1 to binding data shown for each oligosaccharide in Fig. 1

Oligosaccharide, ru	M_r	Saturation binding value* (95% confidence limits) pmol	ng
92	98,000	1.7 (1.5–2.3)	165 (148–225)
29	30,500	5.3 (3.8–∞)	161 (117–∞)
9	9,500	25 (7.3–∞)	228 (69.2–∞)
2.6	2,800	48 (32–184)	133 (91.0–515)

ru, repeating units.
*Expressed as pmol or ng bound by a fixed amount of antiserum.

Saturation Binding Studies with Purified IgG. To exclude the possibility that the preceding results represented an artifact of ammonium sulfate precipitation as a means of quantitating immune complexes, we repeated the saturation binding experiments for three of the oligosaccharides using a different method to measure immune complexes. This method employed formalin-fixed *Staphylococcus aureus* cells (Cowan strain 1) to precipitate immune complexes via the specific interaction of protein A on the surface of the *S. aureus* cells with IgG purified from type III GBS antiserum. Analysis of the binding curves for protein A immunoprecipitation by application of Eq. 1 as described above showed remarkable agreement between the theoretical maximum antigen-binding capacity, in ng, of the antiserum (in this case, purified IgG) for each of the oligosaccharides (Fig. 3). Statistical comparison of theoretical curves generated using a single common value for c (the saturation binding asymptote) for all the curves versus separate values for c generated by the binding data for each oligosaccharide showed no difference in fit (likelihood ratio statistic = 1.29, df = 2, p value = 0.52). Binding capacity (c value) of the purified IgG fraction was 74% of that calculated from the RABA experiments using whole serum, suggesting that the type III antibodies in this serum are predominantly of the IgG class. These data independently confirm the original observation that the amount of antigen bound by antibody approaches the same value on a weight basis rather than a molar basis, with increasing antigen concentration for oligosaccharides of different molecular size.

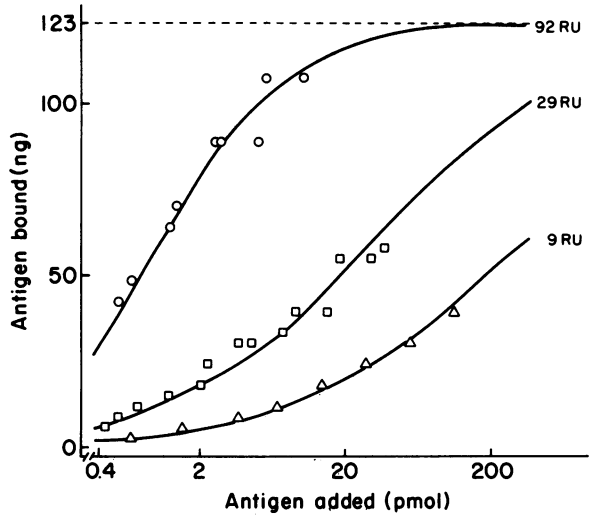

FIG. 3. Saturation binding curves generated by immunoprecipitation of immune complexes with *S. aureus* cells. Data points represent means of duplicate determinations in each of two experiments (one experiment for 9 repeating-unit oligosaccharide) for [^{3}H]oligosaccharides of 92 repeating units (○), 29 repeating units (□), and 9 repeating units (△) plotted as a semilogarithmic plot as in Fig. 2. Each curve represents the line of best fit generated by application of Eq. 1, imposing a single asymptote value (- - -) for all curves. RU, repeating units.

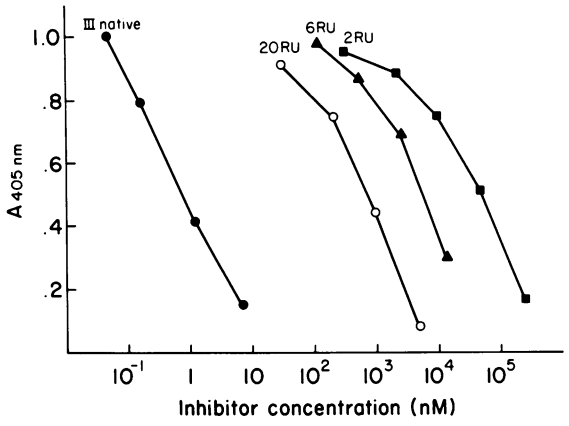

FIG. 4. Type III GBS capsular polysaccharide competitive ELISA. Data points represent A_{405nm} values at indicated inhibitor concentrations for the following inhibitors: type III GBS capsular polysaccharide in its native form (●) and derivative oligosaccharides of average molecular size of 20 repeating units (RU) (○), 6 repeating units (▲), and 2 repeating units (■). Each data point is a mean of duplicate determinations from a representative experiment.

ELISA Inhibition Studies. Further confirmation of the results of RABA saturation binding experiments came from ELISA inhibition studies. Native type III polysaccharide and oligosaccharides of different chain lengths were used to compete for binding of type III GBS antiserum to type III polysaccharide linked to microtiter wells via poly(L-lysine) (11, 16). Over a range of inhibitor concentrations, curves were generated for the native polysaccharide and each of the oligosaccharides (Fig. 4). These inhibition profiles revealed a similar relationship to molecular size of the inhibiting antigen as that observed in the RABA saturation binding studies. The magnitude of the relative affinity differences was even more dramatic in the ELISA inhibition experiments than in the RABA saturation binding studies. These results provided further support for the affinity differences seen in the RABA experiments. Moreover, the ability of the oligosaccharides to compete for antibody binding with the higher-M_r form of the antigen indicates that the smaller oligosaccharides bind to the same population of antibodies as does the native polysaccharide but bind with lower affinity.

Molecular Size of Immune Complexes. The model we propose (see below) of high-affinity binding of antibody to a polysaccharide antigen predicts the formation of high-M_r immune complexes consisting of multiple antibody molecules bound to a single antigen molecule, even when antigen–antibody interaction occurs in antigen excess. We confirmed the formation of high-M_r complexes experimentally by Sepharose-CL6B chromatography of immune complexes formed by the reaction of ^{3}H-labeled type III polysaccharide (at a 6-fold molar excess) with IgG purified from type III GBS antiserum. The elution profile of ammonium sulfate-precipitable radioactivity (representing [^{3}H]polysaccharide bound to antibody) is shown in Fig. 5. When run separately, the [^{3}H]polysaccharide and the IgG preparation elute in single peaks corresponding to M_r of 110,000 and 150,000, respectively. Immune complexes of a single IgG molecule combined with a molecule of polysaccharide would be predicted to yield a peak corresponding to M_r 200,000–300,000, as in peak 2 (M_r 220,000). In addition, however, a peak is seen in V_o of the CL-6B column (peak 1), indicating immune complexes of M_r > 1,000,000.

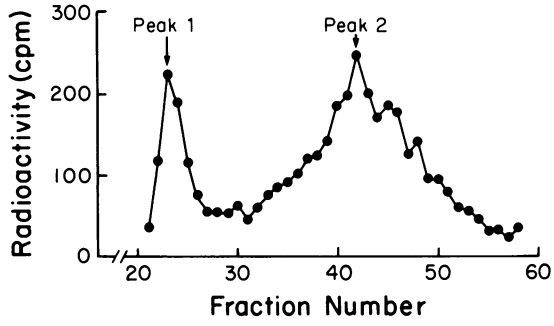

FIG. 5. Sepharose-CL-6B elution profile of immune complexes of ^{3}H-labeled type III GBS capsular polysaccharide and type III GBS antiserum, formed in antigen excess. Immune complexes were detected by precipitation from aliquots of column fractions in 50% saturated ammonium sulfate and subsequent counting in a liquid scintillation counter. Data points are means of duplicate determinations from a representative experiment.

DISCUSSION

Utilizing derivative oligosaccharides of varying size we have developed several independent lines of experimental evidence demonstrating that the affinity of antibody binding to the type III GBS capsular polysaccharide is determined by antigen-chain length. This relationship between affinity of antibody binding and antigen chain length was seen in three different immunologic assays: RABA, protein A immunoprecipitation, and ELISA inhibition. In addition, analysis of oligosaccharide saturation binding curves by mathematical modeling indicated that a given amount of antibody bound the same mass (rather than molar amount) of antigen in extreme antigen excess, regardless of antigen-chain length. We propose the following model of antigen–antibody interaction to explain these findings: the affinity of binding of antibody to the polysaccharide is relatively low; however, binding of one immunoglobulin molecule to a site on the polysaccharide leads to a marked increase in the affinity of interaction between adjacent immunodeterminant sites and other immunoglobulin molecules. Subsequent binding of antibody will tend to be on molecules that have already bound antibody, whereas the interaction with "naive" polysaccharide molecules will be infrequent because of the lower affinity of their immunodeterminant sites. Thus, a chain reaction might be initiated on a large polysaccharide bearing multiple immunodeterminant sites, such that antibody would preferentially bind to all available sites on a given molecule before binding to the lower-affinity sites on a naive molecule.

This model is analogous to the concept of positive cooperativity in ligand–receptor binding. Evidence compatible with such a mechanism has recently been reported by Greenspan *et al.* (17) in studies of antibody binding to the group A streptococcal carbohydrate. Using a series of monoclonal antibodies in a solid-phase radioimmunoassay, these authors found that binding of certain antibodies in low concentrations greatly increased the affinity of binding of a second antibody to the same antigen. The molecular basis for such an increase in affinity of antibody binding is unknown. Initial antibody-binding interaction might induce a conformational change in the polysaccharide, resulting in increased affinity of adjacent immunodeterminant sites for antibody. Alternatively, the bound antibody itself may interact in some way with other immunoglobulin molecules subsequently bound to adjacent sites on the polysaccharide.

Our model predicts the formation of high-M_r immune complexes consisting of multiple antibody molecules per molecule of polysaccharide even in the presence of antigen excess. A population of such high-M_r complexes was demonstrated by gel filtration chromatography of immune complexes formed in excess type III GBS polysaccharide, lending further support to our hypothesis. If the high-M_r complexes represented cross-linking of antigen molecules by bivalent antibodies, one would predict the molecular size distribution

of the complexes to be a single broad peak with a maximum at the molecular size representing the most prevalent form of complexes (15). That distinct populations of complexes are seen, differing in molecular size by at least 800,000 Da, suggests that cross-linking of antigen molecules by antibody is not the mechanism of formation of high-M_r complexes.

Several linear transformations of the classic binding isotherm have been described for estimation of equilibrium binding constants (K_a and K_d) and estimation of number of binding sites, such as the Steward–Petty plot and the Scatchard plot (14, 18). As these methods assume a homogeneous population of immunodeterminant binding sites, the results of their application to more complex systems may not be interpretable. Note, however, that Scatchard analysis of these binding curves results in a plot with a concave upward shape (data not shown). This pattern is expected for a multivalent antigen in high antibody concentrations; that is, under conditions permitting formation of complexes consisting of multiple antibody molecules bound per molecule of antigen (15). That Scatchard plots show a marked departure from linearity in our system, even in antigen excess, is compatible with the hypothesis that multiple antibody molecules are bound per antigen molecule, despite apparent antigen excess. Heterogeneity of antibody affinities could also explain, at least in part, a Scatchard plot with a concave upward shape.

That this relationship between antibody-binding affinity and antigen-chain length is unique to the type III GBS polysaccharide is unlikely. Kabat and coworkers have clearly demonstrated that maximum antigenicity in the antidextran system is attained by oligosaccharides of six or seven sugar residues corresponding to a molecular size predicted to completely occupy the Fab binding region (19, 20). However, Goodman *et al.* (21) found that a mixture of peptides derived from the poly(D-glutamic acid) capsule of *Bacillus anthracis* with an average polymer size of nine amino acids was 4-fold more efficient than the homologous hexapeptide in inhibition of the poly(D-glutamic acid) quantitative precipitin reaction. Similarly, Jennings *et al.* (7) found oligomers of α-(2→8)-linked sialic acid oligosaccharides increasingly antigenic to chain length of at least 17 residues in inhibiting the quantitative precipitin reaction between group B meningococcal antiserum and colominic acid.

The mechanism for increased affinity of antibody binding to high-M_r forms of these antigens is unknown. Some increase in avidity of binding is anticipated as antigen-chain length increases sufficiently to permit simultaneous interaction of both Fab regions of IgG molecules with separate immunodeterminant sites on the antigen. However, we saw further graded increases in affinity of binding as antigen-chain length increased beyond that expected to accommodate bivalent antibody binding (i.e., beyond a backbone-chain length of 10–20 sugar residues, or 3–6 repeating units). The high-M_r form of the type III GBS capsular polysaccharide thus seems to be associated with a conformation of the repeating immunodeterminant that binds antibody with high affinity. One implication of these findings is that antibodies with a binding-site specificity for a small oligosaccharide may functionally discriminate between simple haptens consisting of that oligosaccharide and a polysaccharide of multiple repeating units of the same oligosaccharide. Binding interaction with an oligosaccharide on a host glycoprotein would be of very low affinity, insufficient to result in activation of potentially damaging immune effector mechanisms. Antibody interaction with the same oligosaccharide structure as part of a bacterial capsular polysaccharide would lead to high-affinity binding of antibody molecules to other immunodeterminant sites on the polysaccharide, with subsequent activation of immune effector mechanisms.

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