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Immunochemical Characterization of Antisera Reactivities to *N*-Acetyl-D-glucosamine Oligosaccharides with the β(1-4)-Glycosidic Linkage

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The β(1-4)-linked oligosaccharides of *N*-acetyl-D-glucosamine (GlcNAc) isolated from chitin were used to prepare synthetic immunogens and antigens by reductive amination of $(GlcNAc)_n$ to bovine serum albumin (BSA). The rabbit antisera produced to the $(GlcNAc)_n$-BSA conjugates were characterized using an enzyme-linked immunosorbent assay (ELISA) system under conditions that only the antibodies with carbohydrate specificity were reactive with the solid-phase adsorbed $(GlcNAc)_n$-BSA antigens. Inhibition assays using the $(GlcNAc)_n$-BSA, $(GlcNAc)_n$ oligosaccharides, and the reduced oligosaccharides showed a relative specificity of the antisera for the chain length of the $(GlcNAc)_n$ sequences. For example, the anti-$(GlcNAc)_5$- and anti-$(GlcNAc)_4$-sera were inhibited best by the longer chain $(GlcNAc)_n$ oligosaccharides with the antibody combining sites directed mainly to the cyclic GlcNAc residues of the $(GlcNAc)_n$-BSA conjugates. The antibody combining sites were in part directed to the acyclic moiety of the reducing end of the oligosaccharides as shown by the increased inhibitory activities of the reduced $(GlcNAc)_n$ oligosaccharides particularly with the anti-$(GlcNAc)_2$- and anti-$(GlcNAc)_3$-sera. The best hapten inhibitors for the anti-$(GlcNAc)_2$-BSA and anti-$(GlcNAc)_1$-BSA sera were the *N*-butylamine derivatives of $(GlcNAc)_2$ and $(GlcNAc)_1$, respectively, indicating that the antibodies were also reactive with the secondary amine formed between the reducing end of the oligosaccharides and the ε-amino groups of lysine.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; GlcNAc, *N*-acetyl-D-glucosamine; $(GlcNAc)_n$, oligosaccharides containing GlcNAc in β1-4 linkages; $(GlcNAc)_2$, DGlcNAcβ(1-4)-D-GlcNAc; $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$, the homologous oligosaccharides of $(GlcNAc)_2$: PBS, phosphate buffered saline (0.01 M sodium phosphate, pH 7.3 containing 0.15 %M NaCl); PBSA, PBS containing 1% BSA and 0.1% Tween-20; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

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Neoglycoproteins are produced in the laboratory by covalently linking oligosaccharide sequences to synthetic amino acid polymers or proteins which lack carbohydrate residues. A number of methods have been utilized to couple the oligosaccharides to proteins [1-4], including reductive amination [5, 6]. The neoglycoproteins have been used to study the biologic activities of the carbohydrate portions [1] and to elicit and study the specificities of anti-carbohydrate sequence antibodies [2, 4]. Such antibody and antisera reactivities have been characterized for several glucose disaccharides [3, 7], to isomalto-oligosaccharides [2], and to several blood group oligosaccharides [4, 8]. *N*-Acetyl-D-glucosamine (GlcNAc) residues in β1-4 linkage are found throughout nature as moieties in polysaccharides, glycoproteins and glycolipids, and synthetic conjugates of β(1-4)-linked GlcNAc to poly-L-lysine and BSA have been used to study the binding specificities of lectins and antibodies [6, 9, 10]. In this communication, we describe the antibody reactivities to a series of $(GlcNAc)_n$, the antisera being derived from rabbits immunized with $(GlcNAc)_1$- to $(GlcNAc)_5$-BSA conjugates. The antisera provide a series of reagents with relative specificities to the chain lengths of $(GlcNAc)_n$ oligosaccharides.

Materials and Methods

Mono- and Oligosaccharides

GlcNAc, cellobiose and maltotriose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $(GlcNAc)_n$ oligosaccharides were prepared from a partial acid hydrolysate of chitin as described by Rupley [11], the $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$ being separated by chromatography on charcoal-celite followed by Bio-Gel P-2 (Bio-Rad, Richmond, CA, USA). Cellotetraose was isolated from a hydrolysate of cellulose using the same procedures. $(GlcNAc)_n$ oligosaccharides (10 mg of each) were reduced to their corresponding alditols by addition of 20 mg of sodium borohydride (Ventron Chemical Co., Beverly, MA, USA) in a 1 ml volume for 16 h. The reaction was stopped by addition of excess acetic acid, the solution dried for 48 h over NaOH and P_2O_5, and borate removed by the repeated addition and evaporation of absolute methanol. The precipitate was dissolved in water and the concentrations of reduced $(GlcNAc)_n$ determined by absorbance at 235 nm compared to a GlcNAc standard. $(GlcNAc)_2$- and GlcNAc-*N*-butylamine conjugates were prepared by addition of the oligosaccharides to *N*-butylamine and reduction with $NaBH_4$ as described by Jeffrey *et al.* [12].

Antigens

$(GlcNAc)_n$ oligosaccharides were conjugated to bovine serum albumin, Miles Laboratories, Elkhart, IN, USA) by reductive amination using sodium cyanogen borohydride ($NaCNBH_3$, Ventron) as described by Schwartz and Gray [5]. Cellobiose, cellotetraose and maltotriose derivatives of BSA were prepared in the same manner. The carbohydrate content of the resulting carbohydrate-protein conjugates analyzed by gas liquid chromatography after methanolysis according to the method of Bhatti *et al.* [13]. The degrees of carbohydrate substitution were determined by the decrease in the number of lysine residues by amino acid analyses.

Immunizations

Rabbits were immunized with $(GlcNAc)_n$-BSA conjugates at approximately three week intervals with multiple subcutaneous, intramuscular and intraperitoneal injections. The first injection consisted of 0.5 mg of the antigen emulsified with complete Freund's adjuvant; subsequent injections were given with incomplete adjuvant. Pre-immune sera were collected and used as controls.

Enzyme-linked Immunosorbent Assay (ELISA)

Dynatech ELISA plates (Cooke, Alexandria, VA, USA) were coated with 200 μl of 0.01-0.1 μg/ml $(GlcNAc)_n$-BSA conjugates in 0.1 M $NaHCO_3$, pH 9.0, for 2 h at 22°C. The wells were then backcoated with a 1% solution of ovalbumin (Sigma) in 0.01 M sodium phosphate, pH 7.3, containing 0.15 M NaCl (phosphate-buffered saline, PBS), for 1 h at 22°C. Plates were washed three times with PBSA [PBS containing 1% BSA and 0.1% Tween-20 (Sigma)] between each step following backcoating. Antisera (200 μl) diluted in PBSA were added to the wells and left for 2 h at 22°C. After washing, 200 μl of β-galactosidase (Sigma) labeled affinity purified goat anti-rabbit IgG (Cappel Labs., West Chester, PA, USA), prepared according to the method of Boraker *et al.* [14] and diluted in PBSA, were added and incubated overnight at 4°C. The plates were washed again, and 200 μl of a 4 mg/ml solution of *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) in 0.1 M sodium phosphate buffer, pH 7.3, containing 0.1 M 2-mercaptoethanol and 5 mM $MgCl_2$ was added. ONPG hydrolysis was monitored by absorbance at 405 nm using a Titertek Multiskan (Flow Laboratories, McClean, VA, USA). All experimental points were done in duplicate and each assay included pre-immune, secondary antibody, substrate, and other controls as mentioned in the text. Inhibition assays were done in the same manner as described above except that the inhibitors were incubated with the diluted antisera for 2 h at 22°C before addition to the ELISA plate wells.

Results

Characterization of $(GlcNAc)_n$-Conjugates and ELISA Conditions

The degree of carbohydrate substitution as determined by the amino acid analyses of the conjugates were 49.6, 26.5, 10.5, 44.8 and 12 for the $(GlcNAc)_1$- through $(GlcNAc)_5$-BSA conjugates, respectively. The molar concentrations in the competitive binding experiments with solution phase $(GlcNAc)_n$-BSA conjugates were calculated on the basis of the oligosaccharide chain substitutions.

The wells of the polystyrene plates were backcoated with ovalbumin and the assays conducted in buffer containing 1% BSA to block binding and measurement of antibodies directed to the BSA carrier proteins. For each of the anti-$(GlcNAc)_n$-BSA sera, no inhibition was obtained using BSA treated with $NaCNBH_3$ as in the conjugation reaction, nor by maltotriose, cellobiose or cellotetraose conjugated to BSA. Also, complete inhibition was obtained with the $(GlcNAc)_n$ oligosaccharides or *N*-butylamine derivatives indicating that the assay conditions did not measure antibodies produced to the BSA carrier or to modified BSA antigenic determinants, and that the reaction detected was specific to antibodies raised against the $(GlcNAc)_n$ carbohydrate portions of the con-

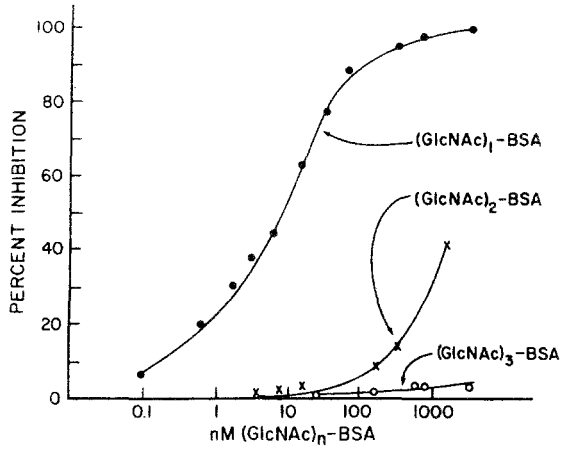

Figure 1. Inhibition of binding of anti-$(GlcNAc)_1$-BSA antibodies to $(GlcNAc)_1$-BSA on the solid phase by $(GlcNAc)_n$-BSA conjugates. The details of the solid-phase ELISA, coating concentration of $(GlcNAc)_1$-BSA and antiserum dilution are described in the text. As described in the text the nM concentrations are calculated on the basis of the $(GlcNAc)_n$ substituted on the $(GlcNAc)_n$-BSA in this Figure and in Figs. 2, 4, 6 and 8. The $(GlcNAc)_1$-BSA was the only effective inhibitor yielding 50% inhibition at a 9 nM concentration (calculated on the basis of the GlcNAc substitution of the conjugate). $(GlcNAc)_3$-, $(GlcNAc)_4$- and $(GlcNAc)_5$-BSA conjugates resulted in no inhibition at the concentrations tested; only the $(GlcNAc)_3$-BSA curve is shown.

jugates. As noted below, a portion of certain antisera reactivities were to the linkage region of carbohydrate to BSA.

For each of the anti-$(GlcNAc)_n$-BSA sera, the assay conditions were initially varied with respect to amounts of coating antigen and dilutions of antisera to yield approximately 1 OD unit at 405 nm in the 30-60 min incubation time with the ONPG substrate. The determined conditions were: $(GlcNAc)_1$-BSA, 0.05 μg/ml coating solution and a 1:30 000 dilution of anti-$(GlcNAc)_1$-BSA serum; $(GlcNAc)_2$-, 0.01 μg/ml and 1:50 000; $(GlcNAc)_3$-, 0.01 μg/ml and 1:60 000; $(GlcNAc)_4$-, 0.1 μg/ml and 1:10 000; and $(GlcNAc)_5$-, 0.1 μg/ml and 1:10 000 dilution. The specificities of the particular antisera described below did not vary with sera collected at different times after the third immunization of the rabbits. Also, the inhibition curves were the same when using either different concentrations of antigens for coating or different antisera dilutions. Thus, the relative inhibitory capacities of the $(GlcNAc)_n$-BSA conjugates or haptens were not dependent on the conditions of the assay system.

Anti-$(GlcNAc)_1$ Sera Specificities

The anti-$(GlcNAc)_1$-BSA antibodies binding to solid-phase $(GlcNAc)_1$-BSA were inhibited effectively only by $(GlcNAc)_1$-BSA in the solution phase (Fig. 1). Fifty per cent inhibition was achieved at approximately 9 nM concentration (calculated on the basis of GlcNAc residue substitution). $(GlcNAc)_2$-BSA resulted in 42% inhibition at the highest concentration used and the other $(GlcNAc)_n$-BSA conjugates gave no inhibition. The *N*-butylamine derivative of $(GlcNAc)_1$ gave 50% inhibition at 1 mM concentration, approximately 10^5 fold greater concentration of the acyclic GlcNAc residue concentration

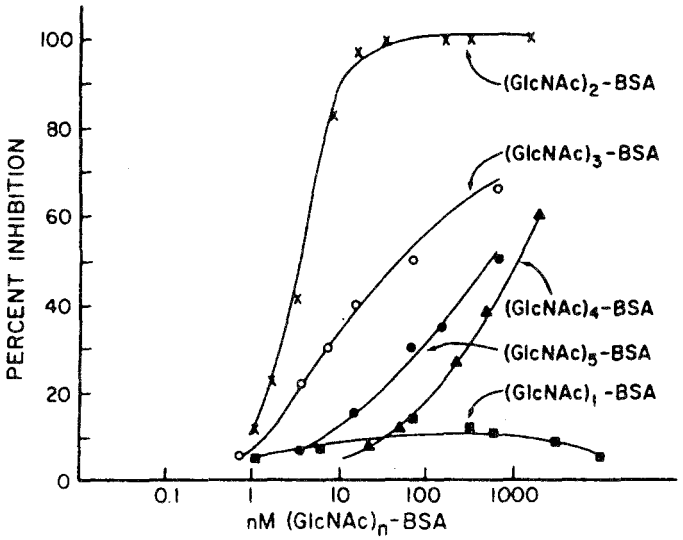

Figure 2. Inhibition of binding of anti-$(GlcNAc)_2$-BSA antibodies to solid-phase $(GlcNAc)_2$-BSA by $(GlcNAc)_n$-BSA conjugates.

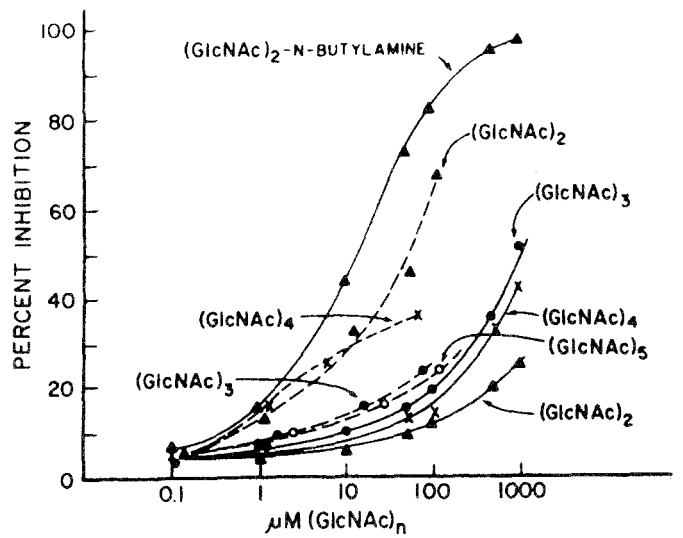

Figure 3. Inhibition of anti-$(GlcNAc)_2$-BSA antibodies binding to solid-phase $(GlcNAc)_2$-BSA by oligosaccharide haptens. The reduced $(GlcNAc)_n$ oligosaccharides are denoted by the broken lines and the non-reduced oligosaccharides by the solid lines. The *N*-butylamine derivative of $(GlcNAc)_2$ was the most effective inhibitor (50% inhibition at 14 μM) followed by reduced $(GlcNAc)_2$, suggesting that the antibody combining sites are directed to one cyclic and one acyclic GlcNAc residue, and in part to the linkage region between the oligosaccharide and the BSA carrier protein.

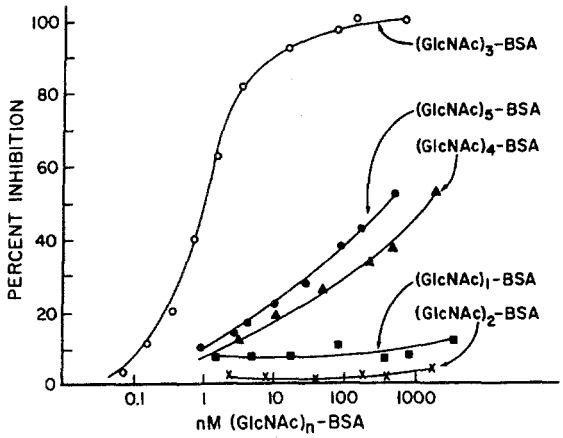

Figure 4. Inhibition of binding of anti-$(GlcNAc)_3$-BSA antibodies to $(GlcNAc)_3$-BSA on the solid phase by $(GlcNAc)_n$-BSA conjugates. The relatively high specificity for the homologous $(GlcNAc)_3$-BSA conjugates is apparent.

compared to $(GlcNAc)_1$-BSA. The other $(GlcNAc)_n$ oligosaccharides and reduced oligosaccharides, including GlcNAc, exhibited little or no inhibitory activity. The GlcNAc alditol resulted in 41% inhibition at the highest concentration tested (5 mM).

Anti-$(GlcNAc)_2$

Fig. 2 shows that the anti-$(GlcNAc)_2$-BSA antibodies were inhibited by the homologous antigen 16, 280 and 160 fold better than with the $(GlcNAc)_3$-, $(GlcNAc)_4$- and $(GlcNAc)_5$-BSA conjugates, respectively. The $(GlcNAc)_1$-BSA exhibited little apparent inhibition. Of the haptens tested, the butylamine derivative of $(GlcNAc)_2$ was most effective resulting in 50% inhibition at a 14 μM concentration (Fig. 3). The reduced oligosaccharides were more effective inhibitors than the non-reduced $(GlcNAc)_n$, reduced $(GlcNAc)_2$ being 4.5 fold less active than the butylamine derivative of $(GlcNAc)_2$.

Anti-$(GlcNAc)_3$

Using the $(GlcNAc)_n$-BSA conjugates as inhibitors, the anti-$(GlcNAc)_3$-BSA antibodies showed a relatively high degree of specificity for the $(GlcNAc)_3$-BSA conjugate, the homologous conjugate exhibiting 1800 and 450 fold better inhibitory activity than $(GlcNAc)_4$- and $(GlcNAc)_5$-BSA, respectively (Fig. 4). Little or no inhibition was seen with either $(GlcNAc)_1$- or $(GlcNAc)_2$-BSA conjugates. With the free and reduced oligosaccharides as inhibitors, reduced $(GlcNAc)_3$ resulted in greater inhibition (50% at 2.2 μM concentration) as compared to the corresponding non-reduced oligosaccharide (50% inhibition at 25 μM, Fig. 5). In contrast to the results with the $(GlcNAc)_n$-BSA conjugates, there was not a great difference in the inhibitory activities of the reduced or non-reduced series of $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$ although the reduced oligosaccharides were generally 10 fold better inhibitors than the homologous non-reduced $(GlcNAc)_n$.

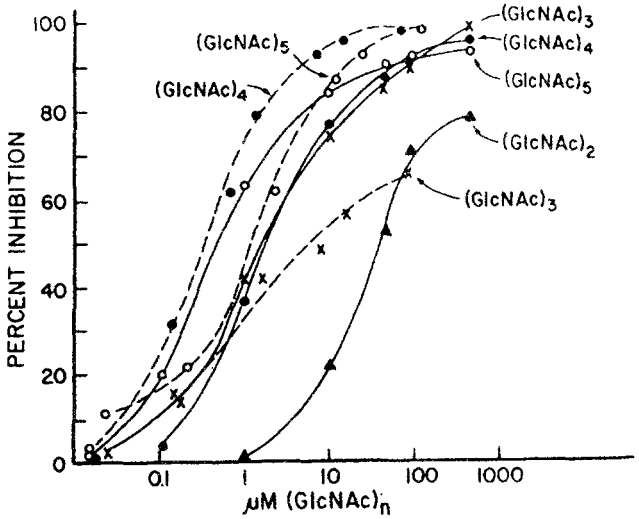

Figure 5. Inhibition of binding of anti-$(GlcNAc)_3$-BSA antibodies to $(GlcNAc)_3$-BSA on the solid phase by reduced (----) and non-reduced (—) $(GlcNAc)_n$ oligosaccharides. In contrast to the specificity exhibited in Fig. 4, the anti-$(GlcNAc)_3$-BSA serum was inhibited approximately equally well by the reduced $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$ oligosaccharides.

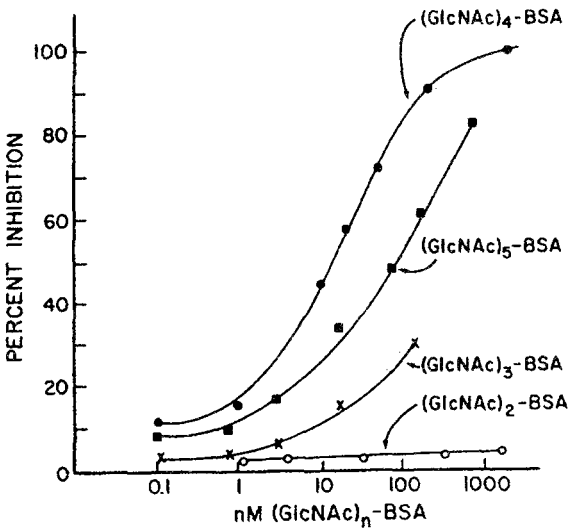

Figure 6. Inhibition of binding of anti-$(GlcNAc)_4$-BSA antibodies to solid-phase $(GlcNAc)_4$-BSA by $(GlcNAc)_n$-BSA conjugates. The antibodies of the antiserum exhibited a relatively high specificity to the homologous antigen suggesting a strong interaction with the three cyclic GlcNAc moieties of the $(GlcNAc)_4$-BSA conjugate.

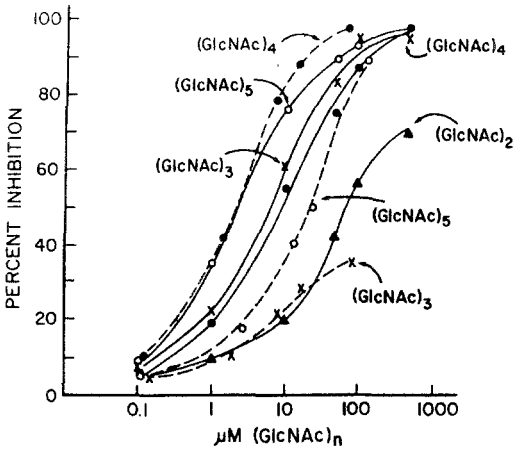

Figure 7. Inhibition of binding of anti-$(GlcNAc)_4$-BSA antibodies to solid-phase $(GlcNAc)_4$-BSA by reduced (----) and non-reduced (—) $(GlcNAc)_n$ oligosaccharides. The greater inhibitory activity of the reduced $(GlcNAc)_4$ suggested that the antibody combining sites were interacting with the three cyclic and one acyclic GlcNAc moieties of the $(GlcNAc)_4$-BSA conjugates. Similar to the results of Figs. 4 and 5, the anti-$(GlcNAc)_4$-BSA antibodies exhibited a greater specificity to the $(GlcNAc)_n$-BSA conjugates than with the oligosaccharide haptens.

Anti-$(GlcNAc)_4$

Fig. 6 shows that anti-$(GlcNAc)_4$-BSA antibodies were inhibited best by $(GlcNAc)_n$-BSA conjugates containing the longer chain oligosaccharides, 50% inhibition being obtained at 14 nM $(GlcNAc)_4$-BSA and 89 nM $(GlcNAc)_5$-BSA. Much less inhibition was detected with $(GlcNAc)_3$-BSA and no inhibition with the $(GlcNAc)_2$-BSA conjugate. No great difference in the extent of inhibition was seen when using non-reduced $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$ oligosaccharides (50% inhibitions at 6.3 μM, 8.9 μM and 2.2 μM for the trimer, tetramer and pentamer, respectively, Fig. 7). The reduced $(GlcNAc)_4$ was the most effective hapten inhibitor; reduction of $(GlcNAc)_3$ or $(GlcNAc)_5$ resulted in substantially less effective hapten inhibitors compared to their non-reduced forms.

Anti-$(GlcNAc)_5$

Anti-$(GlcNAc)_5$-BSA antibodies were inhibited to approximately the same degree with $(GlcNAc)_4$- and $(GlcNAc)_5$-BSA conjugates, whereas $(GlcNAc)_3$- and $(GlcNAc)_2$-BSA conjugates were much less effective (Fig. 8). Using the non-reduced oligosaccharides as inhibitors (Fig. 9), $(GlcNAc)_5$ was about four times more effective than either $(GlcNAc)_3$ or $(GlcNAc)_4$. The reduced $(GlcNAc)_4$ oligosaccharide was elquivalent in inhibitory activity to non-reduced $(GlcNAc)_5$ and exhibited greater inhibition than the reduced $(GlcNAc)_5$. Both of the reduced $(GlcNAc)_4$ and $(GlcNAc)_5$ oligosaccharides were more effective inhibitors than the non-reduced homologous oligosaccharides at the higher concentrations used.

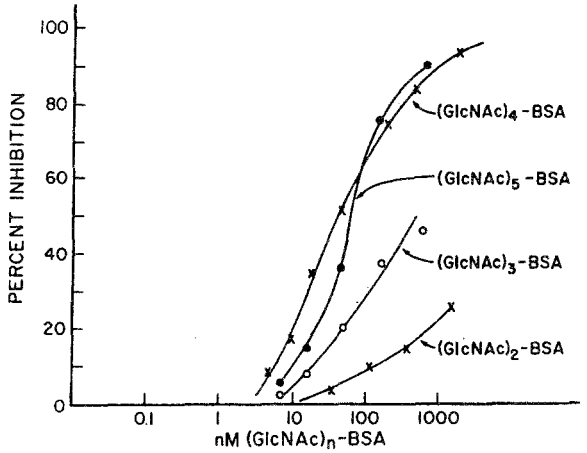

Figure 8. Inhibition of binding of anti-$(GlcNAc)_5$-BSA antibodies to $(GlcNAc)_5$-BSA on the solid phase by $(GlcNAc)_n$-BSA conjugates.

Discussion

A solid-phase ELISA was used for these studies. As noted in the Methods and Results sections, the assay construction was such so as to measure binding of antibodies reactive with $(GlcNAc)_n$ moieties and not with the BSA carrier molecule. No inhibition of any of the antisera, under the conditions of the assay, was obtained with BSA treated with $NaCNBH_3$ or with maltotriose, cellobiose or cellotetraose conjugates of BSA. The latter two conjugates, containing glucose in β1-4 linkages and therefore similar to the $(GlcNAc)_n$-BSA conjugates, also showed the specificity of the antibodies for the $(GlcNAc)_n$ oligosaccharide sequences.

It is interesting to note that a general feature of most of the anti-$(GlcNAc)_n$ sera is the relatively high specificity in reactivity to the $(GlcNAc)_n$-BSA conjugates and the much lower specificities exhibited in the $(GlcNAc)_n$ oligosaccharide inhibition experiments. The relative ratio of inhibitory activities for anti-$(GlcNAc)_2$, anti-$(GlcNAc)_3$ and anti-$(GlcNAc)_4$ with the homologous antigen versus its next best $(GlcNAc)_n$-BSA inhibitor ranged between 10 and 120 fold, whereas the reduced or non-reduced $(GlcNAc)_n$ oligosaccharide inhibitors showed generally less differences in the molar inhibitory activities. Thus, the functional affinity consideration of the two antibody combining sites binding to two haptenic groups on the $(GlcNAc)_n$-BSA antigen may be important in determining relative specificities of these antisera.

For each anti-$(GlcNAc)_n$ serum, the $(GlcNAc)_n$-BSA conjugates were much better inhibitors than the reduced or non-reduced oligosaccharides, 50% inhibition being obtained at nM concentration ranges for the $(GlcNAc)_n$-BSA conjugates and in the μM concentration range for the oligosaccharides. It can be estimated that the functional affinity (avidity) factor of the multiple $(GlcNAc)_n$ substituted BSA antigens is in the range of 10 to 10^3. For anti-$(GlcNAc)_2$ and anti-$(GlcNAc)_3$ antibodies, the effective inhibitory activities of the $(GlcNAc)_n$-BSA conjugates showed 10^3 more relative inhibition than the

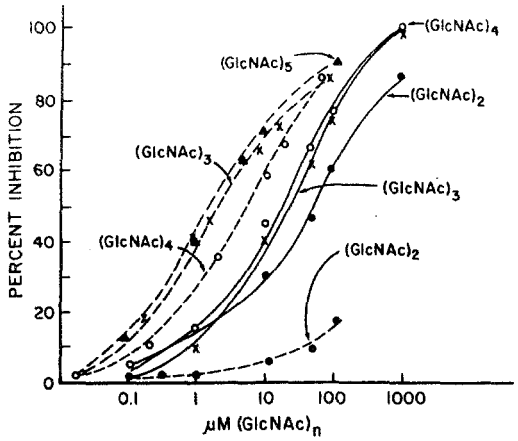

Figure 9. Inhibition of binding of anti-$(GlcNAc)_5$-BSA antibodies to $(GlcNAc)_5$-BSA on the solid phase by reduced (----) and non-reduced (—) $(GlcNAc)_n$ oligosaccharides. Several of the $(GlcNAc)_n$ reduced and non-reduced oligosaccharides exhibited similar inhibitory activities indicating the antibodies were interacting predominantly with four GlcNAc residues.

best $(GlcNAc)_n$ hapten; for anti-$(GlcNAc)_4$ and anti-$(GlcNAc)_5$ antibodies the ratios were approximately 100 and 10, respectively.

The $(GlcNAc)_n$ oligosaccharide versus the $(GlcNAc)_n$-BSA inhibition results clearly showed that the anti-$(GlcNAc)_n$ antibodies were reacting with the linkage region between oligosaccharide and protein, and with a portion of the BSA carrier molecule for the anti-$(GlcNAc)_1$, anti-$(GlcNAc)_2$ and perhaps for the anti-$(GlcNAc)_3$ sera. The differences in the molar inhibitory activites of the corresponding reduced oligosaccharides increased 16 to 40 fold for anti-$(GlcNAc)_2$ compared to anti-$(GlcNAc)_4$ or -$(GlcNAc)_5$. Also, the greater inhibitory activities observed with the *N*-butylamine derivatives of GlcNAc and $(GlcNAc)_2$ indicated that those antibody combining sites were in part interacting with the acyclic GlcNAc moiety and to the secondary amine formed with the side chains of lysine residues of the BSA carrier molecule. The anti-$(GlcNAc)_2$ sera exhibited the relative inhibitory activities of: $(GlcNAc)_2$-*N*-butylamine > reduced $(GlcNAc)_2$ > > $(GlcNAc)_2$. These results are comparable to those of Kamicker *et al.* [7] who produced and characterized rabbit antisera to the cellobiose derivative of BSA made by the reductive amination conjugation method. Using inhibition of precipitation between the antibodies and cellobiose-BSA with a series of haptens the order of relative inhibitory activities were: α-*N*-acetyl-ε-*N*(1-deoxycellobiitol)-lysine was slightly better than 1-deoxyaminocellobiitol > cellobiitol > > cellobiose. Thus, the anti-cellobiosyl-BSA recognized one cyclic ring, the acyclic ring and a portion of the linkage region similar to the anti-$(GlcNAc)_2$-BSA reactivities.

The inhibition results allowed an approximation of the antibody combining site sizes for the oligosaccharides with GlcNAc residues. The anti-$(GlcNAc)_5$ serum was inhibited equally well by $(GlcNAc)_5$- and $(GlcNAc)_4$-BSA conjugates and approximately equal inhibitory activities were seen with the reduced or non-reduced $(GlcNAc)_4$ and $(GlcNAc)_5$ oligosaccharides. The anti-$(GlcNAc)_4$ serum was inhibited slightly better by $(GlcNAc)_4$- than $(GlcNAc)_5$-BSA; the antiserum was also inhibited to about the same extent with reduced $(GlcNAc)_4$ and non-reduced $(GlcNAc)_5$, both being slightly better inhibitors than $(GlcNAc)_4$ and $(GlcNAc)_3$. The results indicate that the antibody combining sites

may be predominantly interacting with four GlcNAc cyclic ring structures of $(GlcNAc)_5$-BSA and three cyclic and one acyclic GlcNAc residues of $(GlcNAc)_4$-BSA. The anti-$(GlcNAc)_3$ antibodies appeared to be interacting with the two cyclic GlcNAc residues and the acyclic GlcNAc residue as shown by the relatively high specificity for the $(GlcNAc)_3$-BSA conjugate, the greater inhibitory activities of the reduced versus the non-reduced $(GlcNAc)_n$ oligosaccharides, and the inefficient inhibition obtained with reduced $(GlcNAc)_2$ versus non-reduced $(GlcNAc)_2$. Thus, the combining site regions of the polyclonal antibodies in the antisera may interact with three to five GlcNAc residues (including the acyclic GlcNAc at the linkage region) and predominantly with four GlcNAc residues. Danielson and Gray (personal communication) obtained similar results using rabbit antisera to β(1-4)-linked glucose oligosaccharides (isolated from cellulose) reductively aminated to carrier protein. In their studies the antibody combining sites exhibited an upper limit of interacting with four glucose residues, and the immunodominance of the linkage region diminished with increasing size of the oligosaccharide hapten.

Kabat [15, 16] and Outschoorn *et al.* [2] defined anti-dextran antibody combining sites as having an upper limit of six α(1-6)-linked glucose units which measured 34 × 12 × 7 Å in the isomaltohexaose's most extended form. The lesser number of carbohydrate residues interacting with the anti-$(GlcNAc)_n$ antibodies could be due to the presence of the *N*-acetyl groups which increases the width of the GlcNAc residue to 15 Å compared to the 12 Å of the glucose molecule. Thus, the volumes occupied by isomaltohexaose and $(GlcNAc)_4$ are similar, being 2856 and 2520 $Å^3$, respectively. An additional consideration is that the anti-dextran combining sites may interact with forms other than the most extended one for the isomaltohexaose molecule, the relatively free rotation of the glucose moieties around carbon-6 rendering the molecule capable of assuming more compact structures. Space-filling models of the β(1-4)-linked GlcNAc oligosaccharides suggest that the glycosidic linkage and the bulky *N*-acetamido groups restrict rotation around the glycosidic bond yielding an extended structure with maximization of volume.

The lower limit of the antibody combining site region as discussed by Kabat [16] was between one and two monosaccharide units. The results presented here suggest that the predominant antibody combining site regions for the $(GlcNAc)_n$-BSA conjugates were greater than one or two GlcNAc moieties (including the acyclic GlcNAc moiety of the linkage region). The anti-$(GlcNAc)_1$-BSA serum was inhibited poorly by the reduced GlcNAc monosaccharide and 50% inhibition was obtained by the GlcNAc-*N*-butylamine derivative at the relatively high concentration of 1 mM. Also, the anti-$(GlcNAc)_2$-BSA serum was inhibited best by the *N*-butylamine derivative of $(GlcNAc)_2$ indicating an average combining site region larger than two GlcNAc residues. It is also of interest to note that the anti-$(GlcNAc)_1$-BSA serum was reactive with the homologous antigen but not by the control BSA treated with $NaCNBH_3$. This latter result suggests a great dependence of reactivity for the substitution of BSA by the immunodominant acyclic GlcNAc residue, and/or that the acyclic GlcNAc substitution alters the conformation at the local site of covalent binding to the BSA molecule.

Carbohydrate sequences of the type $(GlcNAc\beta1\text{-}4)_n$ are found in chitin and other glycoconjugates and have been implicated in several biologic systems. Some examples are: rabbit polyclonal antisera to di-*N*-acetylchitobiose have been shown to be a possi-

ble probe for the disaccharide structure of human and mouse peripheral lymphocytes and plasmacytoma cells [9]; the shiga cytotoxin binding to HeLa and rat liver cell membranes may involve the $(GlcNAc)_n$ structures and is inhibited by $(GlcNAc)_3$ and $(GlcNAc)_4$ and blocked by the wheat germ lectin [17]; the *N*-acetylchito-oligosaccharides have been shown to have anti-tumor effects [18] and to attract peritoneal exudate cells in mice [19]. In addition, biosynthesis of the $(GlcNAc)_n$ polymer chitin in fungi and insects has been studied using the wheat germ lectin [20-22]. The relative specificities to the different chain lengths of $(GlcNAc)_n$ described here may allow the anti-$(GlcNAc)_n$ sera to be used in various carbohydrate receptor, cell surface carbohydrate sequence expression, and biosynthetic studies.

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