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Antibody variable region glycosylation: position effects on antigen binding and carbohydrate structure

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The presence of N-linked carbohydrate at Asn58 in the V_H of the antigen binding site of an antibody specific for $\alpha(1\rightarrow6)$dextran (TKC3.2.2) increases its affinity for dextran 10- to 50-fold. Site-directed mutagenesis has now been used to create novel carbohydrate addition sequences in the CDR2 of a non-glycosylated anti-dextran at Asn54 (TST2) and Asn60 (TSU7). These antibodies are glycosylated and the carbohydrates are accessible for lectin binding. The amino acid change in TSU7 (Lys62 → Thr62) decreases the affinity for antigen; however, glycosylation of TSU7 increased its affinity for antigen 3-fold, less than the >10-fold increase in affinity seen for glycosylated TKC3.2.2. The difference in impact of glycosylation could result either from the position of the carbohydrate or from its structure; unlike the other antibodies, TSU7 attaches a high mannose, rather than complex, carbohydrate in CDR2. In contrast, glycosylation of TST2 at amino acid 54 inhibits dextran binding. Thus slight changes in the position of the N-linked carbohydrate in the CDR2 of this antibody result in substantially different effects on antigen binding. Unlike what was observed for the anti-dextrans, a carbohydrate addition site placed in a similar position in an anti-dansyl is not utilized.

Key words: antibody/anti-dextran/carbohydrate structure/glycosylation

Introduction

The antigen binding sites of antibodies are formed by six complementarity determining regions (CDRs); three from the variable regions of light chain (V_L) and three from heavy chain (V_H). These extend as loops from a conserved framework of β-pleated sheets (Alzari *et al.*, 1988). Variations in the sequences and structures of the binding sites determine the specificities and affinities of the antibodies for antigen (Jones *et al.*, 1986).

A significant number of antibodies also possess carbohydrate addition sequences (i.e. the Asn-X-Ser/Thr tripeptide) in their variable regions. In particular, many members of the murine antibody V_H subgroup IIIB, which are mostly specific for carbohydrate, encode this sequence as Asn58 in the second CDR of V_H (Kabat *et al.*, 1987). In this laboratory it was observed that anti-dextran hybridomas with this potential N-linked glycosylation site attached carbohydrate and that the presence of carbohydrate increased the affinity for antigen. Moreover, this V_H-associated carbohydrate is accessible for lectin binding on the protein surface (Wallick *et al.*, 1988).

Since carbohydrate plays a demonstrable role in their binding behavior, anti-dextrans provide a useful system for studying the role of V-region glycosylation in antibody binding. To investigate whether carbohydrate influences antigen affinity by changing the conformation of the binding site or by direct interaction with the carbohydrate antigen, site-directed mutagenesis and gene transfection techniques have been used to place carbohydrate addition sequences at novel positions in CDR2 of anti-dextrans. We then asked how the attached carbohydrates affected antigen interaction and whether they were accessible to lectin. Further, we placed a carbohydrate addition site in a hapten-specific antibody to investigate whether glycosylation would influence antibody interaction with a non-carbohydrate antigen.

We produced and analyzed these antibodies to gain insight into the 'rules' for positioning of carbohydrate attachment sites, and also to gain understanding of the role played by carbohydrate in the binding of antigen. We found that the new carbohydrate addition sites positioned in the anti-dextran were used and that the attached carbohydrate was accessible to binding by lectin. The effect on binding of the carbohydrate varied depending on its position. At position 54 it blocked antigen binding. While addition of carbohydrate at position 60 increased the affinity of antigen–antibody interaction, it did so less effectively than carbohydrate added at amino acid 58. The structure of the carbohydrate also varied depending on its position in CDR2.

Results

Generation of glycosylation mutants

The anti-dextran series of glycosylation mutants was generated from the V_H gene of 19.22.1, which contains no carbohydrate addition site in CDR2 (Table I). Mutagenic oligomers were designed so that a single amino acid change would result in the generation of sites near position 58 where the Asn residue of the tripeptide acceptor sequence is found in many antibodies. Thus carbohydrate addition sites were placed at position 54 or position 60. These altered V_H region genes were then joined to human IgG4 constant regions in the vector pSV2ΔH-*gpt* and transfected into D3 myeloma cells. Production of functional antibody by the transfectants was evaluated by ELISA. The transfectoma which synthesized antibody glycosylated at Asn54 was designated TST2; that with antibody glycosylated at Asn60, TSU7.

Dansyl-specific antibodies are normally not glycosylated in the V_H region. To assess the potential role of glycosylation in these antibodies, a carbohydrate addition sequence was placed in a position in CDR2 which approximated that of the anti-dextran. To avoid complication of interpretation

Table I. Antibodies used in this study

Transfectoma	Source of V region	CDR2 sequence[a]																	
		50	51	52	52A	53	54	55	56	57	58	59	60	61	62	63	64	65
Anti-dextran[b]																		
THV8.3	Hybridoma 19.22.1	Glu	Ile	Leu	Pro	Gly	Ser	Gly	Ser	Thr	Asn	Tyr	Asn	Glu	Lys	Phe	Lys	Gly
TKC3.2.2	Hybridoma 14.6b.1	–	–	–	–	–	–	–	–	–	–	–	Thr	–	–	–	–	–
TST2	Site-directed mutagenesis of 19.22.1	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–	–	–	–
TSU7	Site-directed mutagenesis of 19.22.1	–	–	–	–	–	–	–	–	–	–	–	–	–	Thr	–	–	–
Anti-dansyl[c]																		
		50	51	52	52A	52B	52C	53	54	55	56	57	58	59	60	61	62	63	64	65
TPR1.3	Hybridoma 27.44	Glu	Ile	Arg	Asn	Lys	Ala	Asn	Asn	His	Ala	Thr	Tyr	Tyr	Ala	Glu	Ser	Val	Cys	Gly
TSG46.3.1	Site-directed mutagenesis of 27.44	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–	–	–

[a]Dashes denote amino acids identical to those found in THV8.3 (anti-dextran sequences) or TPR1.3 (anti-dansyl sequences).
[b]Anti-dextran transfectomas are formed by murine anti-dextran V region genes joined to human IgG4 constant region genes.
[c]Anti-dansyl V region genes were joined to human IgG1 constant region genes.

of results, it was desirable to produce a carbohydrate addition site using the minimum possible number of amino acid substitutions. Accordingly, a single C → A substitution was used to transform His55 to Asn producing the addition sequence Asn55-Ala56-Thr57. Transfectomas were produced by introducing both heavy and light chain genes into the host cell. All the anti-dansyl transfectoma supernatants identified by binding to anti-IgG antibody also bound dansyl. The transfectoma expanded for further study was designated TSG46.3.1.

To determine whether the transfectoma antibodies were glycosylated in the V_H region, secreted antibodies were biosynthetically labeled in the presence or absence of tunicamycin (Tm) and then digested with papain. Fc fragments were removed by protein A precipitation, and Fab fragments were immunoprecipitated with an anti-Fab antiserum. The Fab and Fc fragments were then analyzed by SDS−PAGE under reducing conditions (Figure 1). When the V_H gene is glycosylated, the Fd migrates more slowly than the kappa light chain (lanes 1, 2 and 3). The differential mobility is abolished when the cells are treated with Tm (lanes 4, 5 and 6). The Fab fragments obtained from all three cell lines show patterns consistent with the presence of Fab-associated carbohydrate. However, the Fds of the three glycosylated antibodies differ notably from each other in mobility. Since their Tm-treated Fds migrate identically, this suggests that the differences in Fd mobility are due to the attached oligosaccharide; further analysis suggests that the mobility differences result at least in part from differences in carbohydrate structure (see below). The glycosylated Fc fragments show no mobility differences (lanes 7, 8 and 9), suggesting that there is no inherent difference in availability of glycosyl transferases among the different cell lines. Wallick *et al.* (1988) have shown previously that the Fc regions of THV8.3 and TKC3.2.2 are glycosylated and migrate similarly on SDS−PAGE gels. In contrast to the anti-dextrans, the anti-dansyl variant TSG46.3.1 exhibits comigration of Fd and light chain identical to that of TPR1.3, indicating that the V_H region of TSG46.3.1 is not glycosylated (Figure 2). In an independently isolated transfectoma, TSG16, V_H also was not glycosylated (data not shown).

To verify that the glycosylation sequence was present in TSG46.3.1, total RNA obtained from TSG46.3.1 and

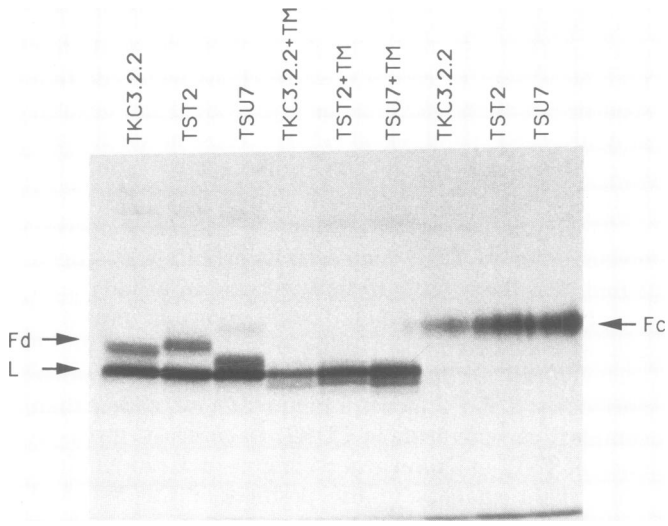

Fig. 1. SDS−PAGE analysis of Fab, aglycosylated Fab and Fc from anti-dextran transfectomas TKC3.2.2, TST2 and TSU7. Immunoglobulins radiolabeled by growing cells in [^{35}S]methionine in the presence or absence of Tm were digested with papain, immunoprecipitated and analyzed by SDS−PAGE. The positions of L chains, Fd and Fc are indicated.

TPR1.3 was hybridized with both the mutant oligomer and, as hybridization control, an oligomer complementary to J_H3, the J gene associated with these heavy chains. Both samples hybridized strongly to the J_H3 probe, while only TSG46.3.1 hybridized with the mutant oligomer (data not shown). These results confirm that the carbohydrate attachment sequence is present in TSG46.3.1 but is not used for reasons that are not clear.

Binding of V_H-associated carbohydrate by Con A

It was previously determined for the TKC3.2.2 anti-dextran antibody that the V_H-associated carbohydrate is accessible to binding by Concanavalin A and thus is exposed on the antibody surface. In contrast, the Fc-associated carbohydrate of THV8.3 was not bound by Con A (Wallick *et al.*, 1988). Con A is a lectin which preferentially binds high mannose sugars but also recognizes complex bi-antennary carbohydrates (Trimble and Maley, 1984). To see if carbo-

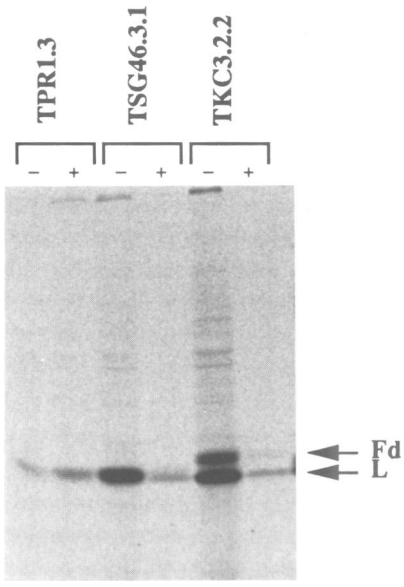

Fig. 2. SDS−PAGE analysis of Fab fragments from anti-dextran and anti-dansyl antibodies. Immunoglobulins radiolabeled by growing transfectomas in [^{35}S]methionine in the presence or absence of Tm were digested with papain, the Fab immunoprecipitated and analyzed by SDS−PAGE. The positions of the Fd and L chain are indicated.

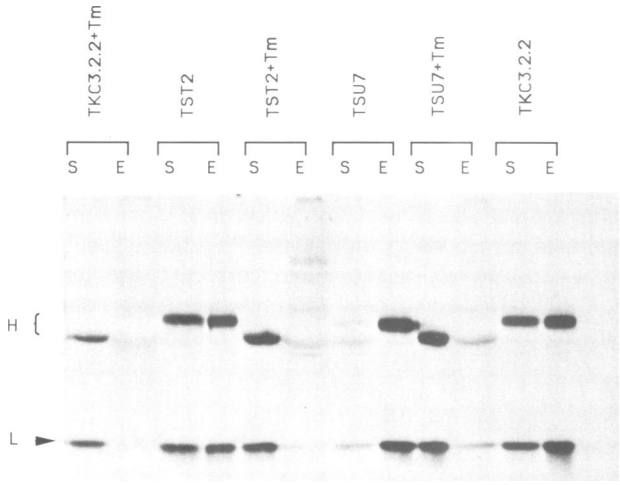

Fig. 3. Con A binding by the transfectoma-produced proteins. Transfectoma immunoglobulins radiolabeled by growth in [^{35}S]methionine in the presence or absence of Tm were incubated with Con A−Sepharose. After washing, bound immunoglobulins were eluted with α-methyl-mannoside. Non-bound and eluted Ig were analyzed by SDS−PAGE following reduction of disulfide bonds by treatment with β-mercaptoethanol. Each pair of lanes shows unbound Ig and Ig eluted from Con A, respectively.

hydrate attached at other sites in CDR2 is also accessible to Con A, radiolabeled antibody was incubated with Con A−Sepharose. After the incubation period, the immunoglobulin was eluted from the Con A with methyl α-D-mannoside; the supernatants and eluates were then analyzed by SDS−PAGE. All of the V_H-glycosylated antibodies bound to Con A. However, it should be noted

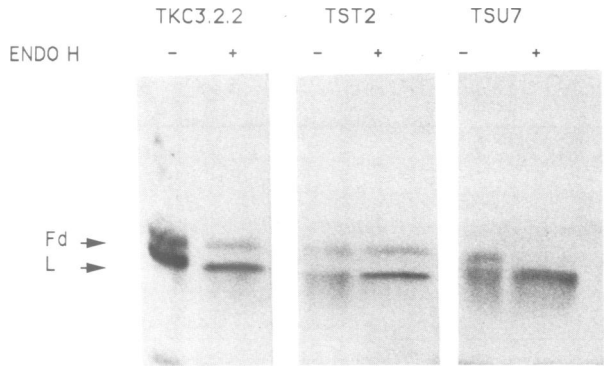

Fig. 4. Endo H treatment of transfectoma proteins. Fab fragments were prepared by papain digestion from biosynthetically labeled immunoglobulins and treated with Endo H.

that TSU7 is more effectively bound to Con A consistent with its high mannose structure (see below). Tm-treated transfectoma proteins were not bound by Con A (Figure 3).

Analysis of carbohydrate structure

The observation of the differential migration of glycosylated Fds led to studies to compare the structures of the V_H-associated oligosaccharides. Antibodies were digested with endoglycosidase H, which cleaves high mannose, but not complex, oligosaccharides. Both secreted and cytoplasmic immunoglobulins were analyzed. In all three transfectomas cytoplasmic IgG was sensitive to Endo H (data not shown). In contrast, when secreted Ig was analyzed, the Fab carbohydrate of TSU7 remained sensitive to Endo H digestion while the Fab carbohydrate of TKC3.2.2 and TST2 was largely or completely resistant to Endo H cleavage (Figure 4). Therefore, the carbohydrate on secreted TSU7 is of the high mannose structure while the carbohydrate on TST2 and TKC3.2.2 is complex. Thus, carbohydrates positioned at nearby residues can be differentially processed. We have yet to define a difference in the structure of the carbohydrate on TST2 although its Fd shows a migration on SDS−PAGE slightly different from that of TKC3.2.2.

Determination of apparent binding constant (aKa) for anti-dextran antibodies

As it was shown previously that glycosylation at Asn58 enhanced the affinity for antigen of an anti-dextran, we wished to explore further the possible role of V_H-associated carbohydrate in antigen binding. Untreated and Tm-treated preparations of the various anti-dextran antibodies were analyzed by ELISA for binding to dextran-coated plates. Because previous experience had shown that even small amounts of contaminating higher affinity antibody will significantly influence the a*K*a, the Tm-treated antibodies were extensively adsorbed with Con A−Sepharose prior to analysis, to guarantee that aglycosylated antibodies were being analyzed. Dextran binding was initially tested under 'high affinity' conditions, i.e. on plates coated with 5 μg/ml dextran. Only TKC3.2.2 and TSU7 antibodies bound to these plates. Quantitative analyses were therefore carried out under low affinity conditions, on plates coated with 20 μg/ml dextran B512 (Table II). The apparent binding constant of the TKC3.2.2 antibody was consistently 10-fold higher than both its Tm-treated counterpart and the non-glycosylated

Table II. Apparent binding constants for dextran B512

Antibody	Carbohydrate attachment site	Tm treatment	a*K*a (±SD)
TKC3.2.2	Asn58	−	1.10×10^6 (0.15)
		+	1.17×10^5 (0.44)
TST2	Asn54	−	$<1 \times 10^4$
		+	1.15×10^5 (0.53)
TSU7	Asn60	−	1.24×10^5 (0.64)
		+	4.00×10^4 (0.12)
THV8.3	None	−	1.50×10^5 (0.58)

Deglycosylated antibodies were produced by incubating transfectomas in medium containing 8 μg/ml Tm for 24 h. Supernatants were harvested and incubated with Con A−Sepharose to remove trace carbohydrate.

antibody THV8.3. The Tm-treated TSU7 showed a 10-fold lower affinity than aglycosylated TKC3.2.2; glycosylation of TSU7 increases its affinity for antigen 3-fold. Therefore, in this antibody the presence of carbohydrate increases the affinity of the antigen−antibody interaction but the amino acid substitution that produces the carbohydrate addition sequence compromises its affinity for antigen. The glycosylated TST2 antibody did not exhibit detectable binding to dextran under any of the conditions used. When the antibody was aglycosylated, however, binding was restored, with an a*K*a approximately equal to that of aglycosylated TKC3.2.2; therefore, for this antibody the amino acid change does not affect affinity, but the addition of carbohydrate inhibits its ability to bind antigen.

Discussion

Antibody molecules are glycosylated in their Fc regions at characteristic positions according to their isotype (Sutton and Phillips, 1983). All IgG antibody molecules are glycosylated in C_H2 at Asn297; the carbohydrates attached there have been shown to contribute to antibody stability and to several effector functions, notably complement binding and activation and Fc receptor binding (Nose and Wigzell, 1983; Leatherbarrow *et al.*, 1985; Tao and Morrison, 1989). Of antibodies sequenced to date, a significant minority have carbohydrate addition sites in the V_H region. In these, the carbohydrate attachment sites vary in position (Kabat *et al.*, 1987) but among anti-carbohydrate antibodies in particular the carbohydrate addition sequence is positioned in CDR2, at Asn58. Presence of carbohydrate has been proved in only a few cases (Sox and Hood, 1970; Middaugh and Litman, 1987; Wallick *et al.*, 1988); antibodies specific for α(1→6)dextran have been particularly well studied by Wallick *et al.*, who demonstrated that the presence of carbohydrate at this position significantly increases the affinity of the antibody for antigen. They speculated that carbohydrate attached there changes the conformation of the binding site, possibly increasing the accessibility of the amino acid residues which actually contact the antigen.

These findings prompted us to investigate whether carbohydrate attachment sites placed in other positions in CDR2 would be used, and how their utilization would affect antigen binding. Carbohydrate addition sequences were introduced into CDR2 of both dansyl-specific and dextran-specific antibodies. These experiments demonstrated that (i) within CDR2 some carbohydrate addition sequences are used while others are not, (ii) carbohydrate added at closely spaced sites can be processed differently, (iii) some of the amino acid substitutions required to generate carbohydrate addition sequences decrease the affinity for antigen, and (iv) the presence of carbohydrate at different sites has variable effects on antigen binding ranging from being inhibitory to increasing the interactions.

No crystal structures have been solved for the antibodies used in these studies; however, the crystal structure of J539, a galactan-specific antibody homologous to the anti-dextrans, has proved to be a useful cognate structure from which to predict the positions of certain residues. The crystal structure of J539 Fab shows that the carbohydrate attached at position 58 extends out freely from the top of the CDR2 loop (coordinates from E.A.Padlan, personal communication). Recently, based on the quarternary structure of J539, Padlan and Kabat (1988) constructed a model of the Fv region of the anti-α(1→6)dextran antibody 19.1.2, which is identical in sequence to 19.22.1. Space-filling models indicate that the amino acids in CDR2 lie on the antibody surface. Therefore, if alternate carbohydrate addition sites were introduced into this region, they should be accessible for glycosylation and the carbohydrate should be exposed on the outside of the proteins. Consistent with this expectation we found that the new carbohydrate addition sequences in the anti-dextran antibodies were utilized. In contrast, the carbohydrate addition site placed in the CDR2 of the anti-dansyl TSG46.3.1 is not used. The conformation of the heavy chain CDR2s of the anti-dansyl and anti-carbohydrate would be predicted to be slightly different (Chothia *et al.*, 1989); nevertheless, we would expect the site to be exposed.

The dansyl-specific antibodies were expressed in the myeloma cell line P3X63Ag8.653 while the anti-dextrans were produced in D3. We cannot rule out the possibility that V_H-associated glycosylation of TSG46.3.1 was inhibited by some anomalous glycosylation defect in P3, but numerous studies in our laboratory indicate that P3-produced antibodies are assembled and glycosylated correctly, and analysis of TSG46.31 demonstrated the presence of Fc-associated carbohydrate.

The finding that the carbohydrate binding site placed in the CDR2 of the anti-dansyl antibody is not used is not unprecedented. The 'rules' for glycosylation are not well understood, other than the requirement for the Asn-X-Ser/Thr acceptor sequence (Marshall, 1974); so far, the only consistent exception to that rule occurs when the X residue is proline. Numerous examples have been reported of other proteins with 'unused' glycosylation sites; many occur near the carboxy terminus of the protein (Gavel and von Heijne, 1990). Proteins such as RNase A and α-lactalbumin (Pless and Lennarz, 1975) are glycosylated only upon denaturation or, in the case of catalase, protease cleavage (Kronquist and Lennarz, 1978); thus, the most readily demonstrable factor affecting glycosylation is the accessibility of the carbohydrate acceptor sequence to transferases. Folding and surface accessibility would not be expected to vary substantially among IgG molecules. However, in some cases other factors seem to inhibit glycosylation. Studies with synthetic peptides have suggested that the close proximity of amino acids with bulky or charged side groups to the carbohydrate acceptor sequence may inhibit glycosylation (Bause and Lehle, 1979). Recently Machamer and Rose introduced eight carbohydrate addition sites into the G protein of vesicular stomatitis virus in positions presumed to be accessible for glycosylation. Two sites were not glycosylated, although they were near sites

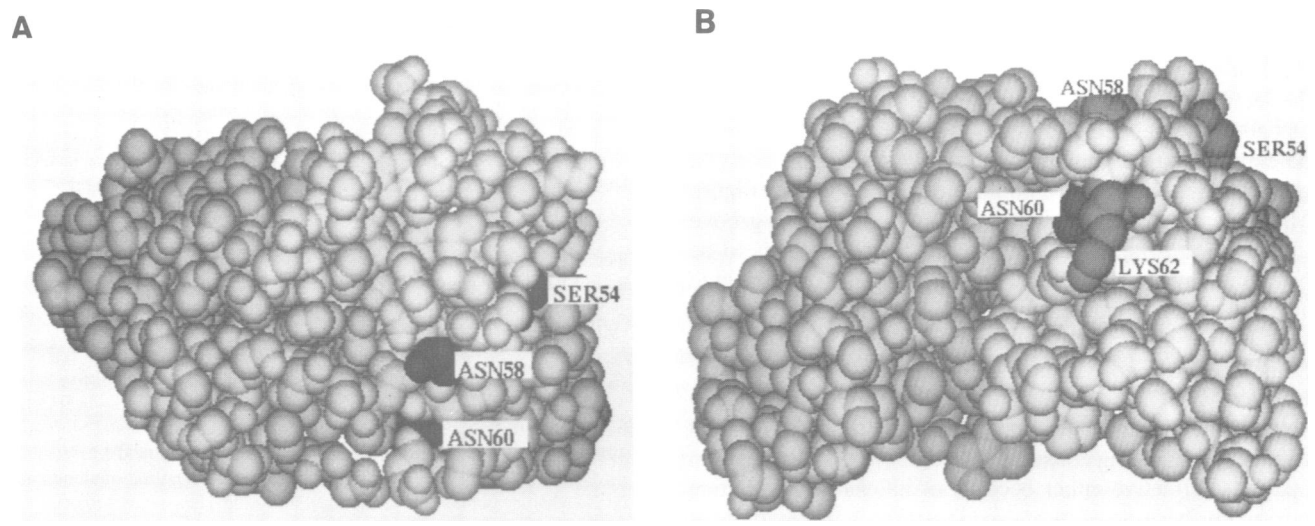

Fig. 5. Space filling model of the variable region of the dextran-specific antibodies. The coordinates used are from the model of Padlan and Kabat (1988) and were visualized using the computer graphics program MacImdad. V_L is to the left (darker), V_H to the right. Amino acids discussed in the text are indicated. **A** is a top view looking onto the antigen combining site (after Padlan and Kabat). Residues 54, 58 and 60 in the V_H portion are identified. (Residue 62 is not visible from this view.) **B**. Space filling model of 19.1.2 Fv, side view. The model is oriented so the proposed antigen contact surface is at the top of the figure. Residues 54, 58 and 62 are identified.

that were (Machamer and Rose, 1988). Both sites contained hydrophobic X residues, as does the addition site in the anti-dansyl (Asn-Ala-Thr). Other proteins such as miraculin (Theerasilp *et al.*, 1989) and α-lactalbumin (Pless and Lennarz, 1975) also contain unused glycosylation sites with hydrophobic X residues. However, such sites are utilized in other proteins. Thus, while certain characteristics of primary amino acid sequence influence glycosylation in some proteins, they are not sufficient in themselves always to inhibit glycosylation. Defining additional structural features of the carbohydrate addition site determining its utilization will be useful in predicting whether glycosylation sites will be used, whether native or engineered.

A surprising observation has been that the carbohydrate added to position 60 remains in the high mannose form while carbohydrate added to the nearby residues 58 or 54 is processed to the complex form. In oligosaccharide biosynthesis, a lipid-linked high mannose precursor is synthesized and attached to protein in the rough endoplasmic reticulum. When the protein is then transferred to the Golgi apparatus, resident glycosidases and transferases can process the precursor to a complex form (Kornfeld and Kornfeld, 1985). Unprocessed high mannose oligosaccharides are sensitive to cleavage by Endo H while complex, processed carbohydrates are resistant. The carbohydrate added to Asn60 in TSU7 appears completely sensitive to Endo H cleavage and so is of the high mannose form. This proposed structure is supported by its favored binding by Con A and by the more rapid migration of its Fab on SDS−PAGE. The factors determining this difference in processing are unclear. It does not seem to be a difference in the available glycosyl-transferases in the cells because we do not detect any differences in mobility of the glycosylated Fc in TSU7. Instead it is possible that the TSU7 carbohydrate is positioned so that further processing is inhibited. Precedent exists for proteins with both high mannose and complex carbohydrates. The degree of oligosaccharide processing appears to be site-specific (Hsieh *et al.*, 1983; Trimble *et al.*, 1983) and has been ascribed to constraints of protein folding which render the carbohydrate at 'high mannose' sites inaccessible to processing enzymes. Among the immunoglobulins, both IgM and IgD possess high mannose and complex carbohydrate on their heavy chains. For IgM, complex structures are found toward the NH_2-terminal region while high mannose oligosaccharides are at the carboxy terminus with the degree of processing increasing with the distance from the carboxy terminus (Anderson and Grimes, 1982). In contrast, for IgD, the high mannose carbohydrate is on C_H2 while the two C_H3 carbohydrates are complex (Mellis and Baenziger, 1983).

A decrease in affinity of dextran binding results in TSU7 as a consequence of the Lys → Thr substitution at amino acid 62. Aglycosylated TSU7 has an a*K*a for dextran of 4×10^4 compared with the a*K*a of 1.11×10^5 observed for aglycosylated TKC3.2.2. Why this amino acid change from Lys to Thr decreases binding is not clear; perhaps the substitution of an aliphatic for a charged residue has a deleterious effect on antigen−antibody contact.

In the anti-dextran variants the new carbohydrate addition sites were used with substantially different effects. The glycosylated TSU7 antibody with carbohydrate at position 60 bound antigen with an a*K*a of 1.23×10^5, an increase of ~3-fold over its aglycosylated form. Therefore, carbohydrate at this position increased the affinity of the interaction with antigen, but less effectively than the carbohydrate placed at Asn58. This could be a consequence either of its position or its structure since the carbohydrate at Asn58 is complex while the carbohydrate at Asn60 is high mannose. In marked contrast, the carbohydrate positioned at Asn54 actually prevents antigen binding. Deglycosylated TST2 binds dextran approximately as well as deglycosylated TKC3.2.2, therefore the substitution at amino acid 54 does not affect the strength of the antigen−antibody interaction. However, when the carbohydrate is present at Asn54, it is impossible to detect binding of dextran.

The model of the Fv of the anti-dextran antibody 19.1.2, identical to 19.22.1, can be used to evaluate the effects we observed of carbohydrate added at different positions

(Figure 5). The proposed combining site of 19.1.2 (and by extrapolation, 14.6b.1, 19.22.1 and its derivatives) is a shallow groove in a basically flat surface. Consistent with the lectin binding data, a top view of the model indicated that all three sites (i.e. Ser/Asn54, Asn58 and Asn60) would be exposed on the antibody surface. Asn58 appears to be at or near the top of the molecule, while Asn60 (Figure 5B) appears to be positioned slightly inside the groove tipped toward the V_L half of the Fv. Ser54 appears to be positioned away from the V_L chain. Whereas carbohydrate attached at Asn58 may, as proposed, enhance antigen contact with key amino acid residues, an alternative and possibly more likely explanation is that carbohydrate at position 58 interacts with the carbohydrate antigens and this interaction between two hydrophilic structures leads to an increase in the apparent binding constant. The carbohydrate at position 60 is less effective either because of its change in relative position or because of its altered structure. Carbohydrate at residue 54 may block access of the large antigen dextran to the combining site. It should be remembered that complex carbohydrate groups are large, bulky structures (Deisenhofer, 1981).

Our findings demonstrate that carbohydrate attachment sites may be deliberately placed so that they are accessible to lectin binding without denaturation of the protein. This is a potentially useful technique for antibody purification. Affinity purification techniques tend to be chemically harsh, time consuming and/or untenable due to the unavailability of quantities of purified antigen. Lectin purification is comparatively gentle and quick and could be applied to large numbers of antibodies. Clearly, the position of the glycosylation site must be chosen with care, but given the close homologies of most V_H sequences, the strategy may be very useful in the future.

The importance of glycosylation in various general processes of glycoprotein configuration, cell–cell recognition, intracellular targeting and others is becoming increasingly appreciated. The complexity of glycosylation patterns is illustrated by reports of multiple oligosaccharide structures within a single protein (Parekh *et al.*, 1987). It will therefore be useful to understand better the rules governing glycosylation: position, structure and other features of the microenvironment of the carbohydrate. This system demonstrates that carbohydrate at a particular position exerts a quantifiable effect on antigen–antibody interaction and that seemingly slight changes in the position of carbohydrate can effect changes of substantial magnitude.

Materials and methods

Cell lines

The mouse transfectoma cell lines THV8.3 and TKC3.2.2 were previously produced in this laboratory. Both synthesize chimeric antibodies consisting of a human IgG4 constant region joined to variable (V_H) region genes from mouse hybridomas specific for $\alpha(1\rightarrow6)$dextran. The V_H gene in TKC3.2.2 was derived from the mouse hybridoma 14.6b.1 and is glycosylated at Asn58 (Table I). The V_H gene in THV8.3 was obtained from the mouse hybridoma line 19.22.1, and differs from 14.6b.1 by a single amino acid change (Thr60 → Asn60) which removes the carbohydrate addition site (Alkolkar *et al.*, 1987). Their light chains are identical, as both cell lines were produced by transfection of the cell line D3, which is a heavy-chain-loss variant of an anti-dextran hybridoma (Wallick *et al.*, 1988). The variant V_H genes produced for this study were also expressed in D3. Dansyl-specific antibodies were produced in P3X63Ag8.563, an Ig non-producing mouse myeloma cell line (see below).

Site-directed mutagenesis and vector preparation

The *Bam*HI–*Pst*I restriction fragment containing the anti-dansyl variable region gene 27.44 (Dangl *et al.*, 1982) was subcloned into M13mp18; the anti-dextran variable region gene was similarly subcloned into the *Eco*RI site in M13mp19. In all cases, site-directed mutagenesis was carried out by the two-primer method of Zoller and Smith (1983) using mutagenic oligomers prepared by Dr Thomas Sutherland (Molecular Biology Institute, UCLA). Mutations were verified by dideoxy sequencing (Sanger *et al.*, 1977), using materials and procedures supplied with a Pharmacia T7 sequencing kit (Pharmacia, Piscataway, NJ). The variant V_H genes were then cloned into the appropriate heavy chain expression vectors: anti-dansyl into pSV2ΔH-*gpt* with human IgG1 constant region genes; anti-dextran V_H genes were joined to human IgG4 constant region genes in pSV2ΔH-*gpt* (Morrison *et al.*, 1984).

Gene transfection

To produce dextran-specific antibodies the light-chain-producing hybridoma line D3 was transfected by protoplast fusion as described (Oi and Morrison, 1986). Transfectants were selected with mycophenolic acid (in selection medium containing 0.5 μg/ml hypoxanthine, 8.3 μg/ml xanthine and 0.02 μg/ml mycophenolic acid) and surviving cells were screened for antibody production by ELISA. Microtiter plates (Corning, Arcadia, CA) were coated with anti-mouse kappa antibody (Sigma, St Louis, MO), diluted to 5 μg/ml in PBS. Detecting antibody was goat anti-human IgG coupled to alkaline phosphatase (Sigma). Positive clones were expanded, and those which retained activity were maintained in Iscove's modified Dulbecco's medium (IMDM) with 5% bovine calf serum (Hyclone, Logan, UT) for further analysis.

Dansyl-specific antibodies were expressed in the myeloma line P3X63Ag8.653. The heavy chain genes were cloned into the pSV2ΔH-*gpt* as described; the light chain genes were encoded in a separate vector pSV184ΔH-*neo*, which confers resistance to the drug G418. The vectors were linearized by digestion with the restriction enzymes *Pvu*I and *Bam*HI, respectively, and introduced into the myeloma cells by electroporation (Shin and Morrison, 1989). Briefly, $\sim 3 \times 10^6$ myeloma cells were washed and resuspended in 0.8 ml cold PBS. Approximately 5 μg each of heavy and light chain linearized DNA, which has been ethanol precipitated, dried and resuspended in PBS, were simultaneously added to the cells and incubated briefly on ice in a chilled electroporation cuvette (0.4 cm gap; Bio-Rad, Richmond, CA). The cells were then pulsed with a Bio-Rad Gene Pulser electroporation apparatus with settings at 200 V, 960 μF capacitance. Afterwards, the cuvette was immediately returned to ice for 10 min, and the cells were then diluted in plating medium (IMDM + 10% serum + 1% gentamicin + 1% Nystatin), pelleted, resuspended to a final concentration of $\sim 1 \times 10^5$ cells/ml, and dispensed into microtiter plates. Selection medium containing 1.0 mg/ml G418 was added 2 days later and, after ~2 weeks, surviving clones were screened by ELISA on microtiter plates coated with dansyl–BSA, and on plates coated with goat anti-human IgG. Positive clones were subcloned and expanded as described.

Biosynthetic labeling and papain digestion

Secreted antibodies were obtained from [^{35}S]methionine labeled transfectants. Approximately $3-5 \times 10^6$ cells were washed in methionine-free Dulbecco's modified Eagles's medium (DME; Irvine Scientific, Santa Ana, CA) and then resuspended in the same medium supplemented with 15 μCi/ml [^{35}S]methionine (Amersham, Arlington Heights, IL) and 1% fetal calf serum (FCS; Hyclone) and incubated at 37°C for 3 days. The supernatants were then harvested and the antibody immunoprecipitated with rabbit anti-human IgG Fc antiserum (prepared in this laboratory by Letitia A.Wims) followed by *Staphylococcus aureus* protein A precipitation and washing (IgG Sorb, The Enzyme Center, Boston, MA). The antibodies were resuspended in sample buffer (25 mM Tris, pH 6.7, 2% SDS, 10% glycerol, 0.008% bromophenol blue) and eluted from the protein A by boiling. The samples were analyzed by SDS–PAGE and autoradiography.

For papain digestion cells were labeled and supernatants collected as described and dialyzed against PBS overnight, sodium phosphate (pH 8.0) was added to a final concentration of 0.1 M, EDTA to 0.002 M and cysteine to 0.01 M, and then incubated with papain (Sigma) at a 1:100 enzyme:protein ratio for 4 h at 37°C (Parham, 1986). The reaction was stopped by adding iodoacetamide (Sigma) to 0.03 M and allowing the mixture to stand briefly at room temperature. The Fc fraction and undigested antibody were removed by precipitation with protein A. After incubation on ice for 15 min the protein A–Ig complexes were pelleted and the procedure was repeated. The supernatants were then incubated overnight at 4°C with a rabbit anti-human Fab antiserum and immune complexes precipitated with protein A. Samples were recovered by boiling the protein A–Ig complexes with sample buffer,

reduced with 0.15 M 2-mercaptoethanol (Kodak, Rochester, NY) and analyzed by SDS–PAGE.

Inhibition of glycosylation by Tm

Cells were grown in Tm, an inhibitor of N-linked glycosylation, to produce antibodies deficient in carbohydrate. Cells were biosynthetically labeled for 3 h in medium containing [^{35}S]methionine in the presence of 8 μg/ml Tm (Boehringer Mannheim, Indianapolis, IN) (Wallick *et al.*, 1988). The cells were then pelleted, washed and incubated overnight in medium freshly supplemented with Tm and [^{35}S]methionine. The supernatants were subjected to immunoprecipitation as described and analyzed by SDS–PAGE to verify that the carbohydrate was removed.

Lectin binding

Immunoglobulin labeled with [^{35}S]methionine was incubated overnight on a Labquake rotator with Con A coupled to Sepharose (Sigma). The Con A–Sepharose had been previously washed and resuspended in Tris-buffered saline. The Con A–Sepharose–Ig complexes were then pelleted, washed several times in buffer, and the Ig eluted with 0.5 M methyl-α-D-mannoside in the same buffer. The elution step was carried out at room temperature on a rotator. The supernatants were collected and immunoprecipitated as before.

Determination of the apparent association constants (aKa) of transfectoma antibodies against dextran B512

Apparent binding constants were determined using the method of Nieto *et al.* (1984). The association constant for an antibody is defined as the reciprocal free ligand concentration required to occupy one-half of the antibody combining sites. If a plate is coated with antigen and a fixed amount of antibody is reacted with it in the presence of an increasing amount of free ligand, the reciprocal of the amount of free ligand required to cause 50% inhibition of antibody binding to fixed ligand is considered the apparent affinity constant. In the assay, Immulon 1 microtiter plates (Dynatech, Chantilly, VA) were coated with 20 μg/ml dextran B512 in BBS and allowed to dry overnight at 37°C. The plates were washed and blocked with 3% BSA for 1 h at room temperature, then washed again.

The test supernatants were added along with increasing amounts of dextran B512 and allowed to incubate at room temperature for at least 4 h. After washing, the plates were incubated overnight at 4°C with anti-human IgG conjugated with alkaline phosphatase (Sigma) diluted 1:1000 in BBS/1% BSA. The plates were washed again, substrate was added (*p*-nitrophenyl phosphate disodium, purchased from Sigma), and plates were read at 410 nm on a Dynatech MR700 plate reader.

Endoglycosidase H hydrolysis

Biosynthetically labeled Ig was precipitated from supernatants with protein A as previously described. The protein A–Ig complexes were pelleted, washed and resuspended in 50 ml of 0.1 M sodium citrate, pH 5.5. The samples were incubated with 5 mU Endo H (Boehringer Mannheim) for 24 h at 37°C. An equal volume of sample buffer was then added and the samples were boiled for 2 min. The protein A was pelleted and the samples were analyzed by SDS–PAGE.

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