GLYCOSYLATION OF A V_H RESIDUE OF A MONOCLONAL ANTIBODY AGAINST α(1→6) DEXTRAN INCREASES ITS AFFINITY FOR ANTIGEN

By SUSAN C. WALLICK, ELVIN A. KABAT, AND SHERIE L. MORRISON

From the Departments of Microbiology, Genetics and Development, and Neurology, and the Cancer/Institute for Cancer Research, Columbia University College of Physicians and Surgeons, New York, New York 10032

Immunochemical characterization of antibodies against α(1 → 6) dextran has given insights into the size and shape of the antibody-combining site and the nature of the interaction between antibodies and antigen. We are now attempting to correlate the immunochemical properties of the antidextran antibodies with their primary structure. In the course of these studies cDNAs from three monoclonal anti-α(1 → 6) dextran hybridoma cell lines, 14.6b.1, 5.54[1] and 19.22.1 (1, 2), were cloned, and the nucleotide sequences of their V_H and V_L regions were determined (3) (Table I). All synthesize an identical κ light chain with the $V_κ$-OX1 germline gene (4) rearranged to the $J_κ2$ segment; the heavy chains differ by only one or two amino acids in their complementarity-determining regions (CDRs)[2]. When compared with 14.6b.1, 5.54 and 19.22.1 have an identical Thr → Asn amino acid change at position 60 in V_H; 5.54 has an additional change (Ser → Gly) at position 31 in CDR1. The changes in heavy chain sequence result in 5.54 and 19.22.1 having a 10-fold or greater reduction in their binding constants for both polymeric dextran and isomaltoheptaose (IM7) when compared with 14.6b.1 (Table I).

The Thr → Asn change in 5.54 and 19.22.1 leads to the loss of a potential *N*-linked glycosylation site (Asn_{58}—Tyr_{59}—Thr_{60}) present in 14.6b.1. The purpose of this study was to determine whether this potential *N*-linked glycosylation site is used and if so, whether the addition of carbohydrate (CHO) to CDR2 affects the binding constant for dextran. It is difficult to demonstrate glycosylation of V_H in the original hybridoma antibodies since both IgA and IgM isotypes are glycosylated within their C_H1 domains and CHO present in Fd could be linked to either V_H or C_H. Therefore, we have transferred the three V_H regions to the human IgG_4 constant region, which is devoid of CHO in its C_H1 domain. In this report we demonstrate the presence of carbohydrate within the V_H of 14.6b.1. Comparison of the association constants for aglycosylated tunicamycin (Tm)-treated and -untreated antibodies shows

[1] The 5.54 mAb was designated as 5.54.4.24.1 by Newman and Kabat (2).

[2] *Abbreviations used in this paper:* CDR, complementarity-determining region; CHO, carbohydrate; IM7, isomaltoheptaose; Staph A, *Staphylococcus aureus* protein A; Tm, tunicamycin.

TABLE I

Immunochemical Properties of Hybridoma Antibodies Specific for Dextran B512

Hybridoma	Mouse strain	Isotype	Site size	$K_a^{\ddagger}$	K_{ia} (IM7)[†,§]	Heavy Chain amino acid changes vs. 14.6b.1 prototype[‖]			
						CDR1	CDR2	CDR3	J_H
				ml/g	*liter/mole*				
14.6b.1[¶]	BALB/c	IgA,k	6	4.43×10^5	5.76×10^4	—	—	—	3
5.54**	C57BL/6	IgA,k	6	1.78×10^4	3.02×10^3	31 Ser → Gly	60 Thr → Asn	—	3
19.22.1[¶]	BALB/c	IgM,k	7	8.87×10^3	6.46×10^3	—	60 Thr → Asn	—	3

* Maximum number of $\alpha(1 \to 6)$-linked glucose residues that fit the antibody combining site.
‡ Determined by affinity gel electrophoresis according to the method described by Takeo and Kabat (17).
§ Association constants of antidextran combining sites with isomaltoheptaose (IM7).
‖ According to Akolkar et al. (3).
¶ According to Sharon et al. (1).
** According to Newman et al. (2); designated as 5.54.4.24.1 by Newman et al.
These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00809.

that the presence of CHO increases the aK_a of 14.6b.1 for dextran. The effect on binding is unique to the carbohydrate present in V_H, since absence of CHO from C_H2 does not change the aK_a for dextran. Lastly, we have demonstrated that the CHO in V_H is more exposed than in CH_2.

Materials and Methods

Cell Lines.   5.54 is a mouse hybridoma cell line synthesizing a C57BL/6 IgA, κ antibody specific for α(1 → 6) dextran. D3 is a spontaneous heavy chain–loss variant of 5.54 that synthesizes only the κ light chain characteristic of the antidextran hybridomas. The D3 light chain variant cell line was isolated by Dr. P. N. Akolkar (Columbia University, NY). Cell lines were grown in Iscove's Modified Dulbecco's medium (IMDM) (Gibco Laboratories, Grand Island, NY) supplemented with 3–5% FCS (Hyclone Laboratories, Logan, UT).

Gene Transfection.   Gene transfection was by protoplast fusion using the method of Oi et al. (5) and modified as described by Tan et al. (6). Transfectant culture supernatants were tested for antibody production and dextran binding by ELISA (7). Dextran B512 was prepared from *Leuconostoc mesenteroides* strain B512 cultures by Dr. L. Matsuuchi as described (8). Horseradish peroxidase affinity purified goat anti–human IgG antibody was purchased from Sigma Chemical Co. (St. Louis, MO). D3 recipient transfected cells from positive wells were subcloned once in soft agarose (9), and clones that stained heaviest with rabbit anti–human IgG Fc antiserum (Cooper Biomedical, Inc., Malvern, PA) were chosen for further analysis.

Biosynthetic Radiolabeling and Papain Digestion.   Transfectant cells were labeled in the presence of 15 μCi/ml of [^{35}S]Met or 100 μCi/ml D-[^{14}C]glucosamine hydrochloride as described (10).

Secretions from the cells were digested with papain (Sigma Chemical Co.) at 1:100 enzyme/protein ratio for 4 h at 37°C. The reaction was stopped by addition of iodoacetamide to 0.03 M. The Fc fraction and undigested antibody protein were precipitated by incubation with IgG-Sorb (Enzyme Center, Malden, MA). Fab was precipitated from the supernatant using rabbit anti–human Fab (prepared by Letitia A. Wims, Columbia University, NY) or by insolubilized dextran (Sephadex G75). Samples were reduced with 2-ME (0.15 M) and analyzed using 5% SDS-PAGE (5).

Inhibition of Glycosylation.   Tm at a concentration of 8 μg/ml (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to inhibit N-linked glycosylation. Cells were biosynthetically labeled for 3 h with [^{35}S]Met in the presence of Tm as described above. After pretreatment, secreted Ig in the culture supernatant was discarded, the cells were washed twice with IMDM, fresh Tm and [^{35}S]Met added, and treatment continued overnight at 37°C. Removal of CHO from Ig was verified by immunoprecipitation of the secreted antibody and analysis by SDS-PAGE.

Determination of the Antibody Protein Concentration in Culture Supernatants.   Antibodies in culture supernatants diluted into BBS (0.02 M borate-buffered 0.75% saline, pH 8.3) were bound to polystyrene microtiter wells (Corning Glass Works, Corning, NY) for 3 h at 37°C. After blocking any unreacted sites with 1% BSA/PBS/0.05% Tween 20 for 1 h at room temperature, the ELISA plates were washed with PBS/0.05% Tween 20 three times, PBS once, and then bound Ig was quantitated by reaction with horseradish peroxidase-labeled anti–human IgG antibody and compared with a human IgG standard of known concentration. Assay results have been reproduced at least three times. Direct binding of antibody to microtiter plates was a more reproducible method than binding supernatants to plates sensitized with anti–human IgG antiserum, for reasons that are not clear.

Determination of the Apparent Association Constants of Aglycosylated Con A-adsorbed or -untreated Transfectoma Antibody Against Dextran B512.   Apparent binding constants were determined using the method of Nieto et al. (11). In brief, the association constant for an antibody is defined as the reciprocal free ligand concentration necessary for occupying one half of the antibody-combining sites. If a fixed amount of antibody is reacted with an increasing amount of free ligand on a plate coated with antigen, the reciprocal of the free ligand concentration that causes 50% inhibition of binding to the plate is considered to be a function of the intrinsic K_a and is designated as the apparent affinity constant (aK_a). The aK_a is calculated from the

amount of ligand necessary for 50% inhibition of binding. The following experimental conditions were used to measure the aK_a values: Corning microtiter plates were coated with 0.5 µg/ml or 20 µg/ml dextran B512 (high-affinity and low-affinity assay conditions, respectively). Bound Ig was quantitated using anti–human IgG labeled with horseradish peroxidase.

Results

The expressed V_H regions from the three hybridoma antibodies against $\alpha(1 \rightarrow 6)$ dextran were joined to the human IgG_4 constant region gene (Fig. 1), and after transfection of D3, a cell line producing only the hybridoma-specifc light chain (5, 6) directed the expression of an H chain that assembled with the endogenous light chain and was secreted (results not shown). Nomenclature for the mAbs and transfectoma antibodies used in this study are presented in Table II.

To determine if the 14.6b.1 chimeric antibody contained CHO in V_H, we fractionated the molecule into Fab and Fc by papain cleavage, reduced the molecules with 2-ME, and analyzed them on 5% SDS-PAGE gels. Proteins were labeled with [35S]Met, and the Fab was precipitated using specific anti-Fab antiserum (Fig. 2 A). Transfectoma antibodies with V_H derived from 5.54 and 19.22.1 cDNA clones (T5.54 and T19.22, respectively) show comigration of their Fd and κ light chains. Precipitation of Fab with insolubilized dextran results in the same pattern, verifying that both κ and Fc are present (data not shown). In contrast, in transfectoma antibodies with the H chain variable region of 14.6b.1 (T14.6b), the Fd portion migrates more slowly than the L chain. The reduced mobility of the T14.6b Fd fragment is consistent with glycosylation of its V_H.

To confirm the presence of CHO in the V_H of T14.6b, we labeled secreted Ig with [14C]glucosamine, prepared Fab and Fc fractions, and analyzed the products by SDS-PAGE (Fig. 2 B). As anticipated, the κ light chains do not contain CHO and bands are absent from the position indicated by the [35S]Met-labeled κ light chain. We find [14C]glucosamine labeling of the human IgG Fc fragment that contains N-linked CHO within its C_H2 domain (12). However, the Fab from only T14.6b, with its Fd containing the 14.6b.1 V_H, shows glucosamine labeling. The reduced intensities of the Fd bands relative to the Fc is probably due to poor recovery of the Fab fragment rather than incomplete glycosylation (13). In SDS-PAGE gels in which we can resolve H chains containing no, one, or two CHO moieties (Fig. 3 B) we find only one heavy chain band for T14.6b.

We have used the glycohydrolase Endo H to investigate the structure of the V_H oligosaccharide. The di-N-acetylchitobiose linkage of high-mannose core oligosaccharides found on newly synthesized IgG H chains is susceptible to Endo H cleavage (14), while processed complex CHO are resistant to Endo H cleavage. H chains obtained from cell cytoplasms were hydrolyzed by Endo H (data not shown). In contrast, heavy chains from the secretions of both T19.22 and T14.6b were unaltered by Endo H treatment. Thus the N-linked CHO present in V_H does not appear to differ from that present in the constant region.

To examine the role of CHO in Ag binding we determined the association constants for Tm-treated aglycosylated and untreated native antidextran transfectoma antibodies. Although Tm is a potent inhibitor of N-linked glycosylation (15), it is difficult to produce proteins completely free of glycosylated species. From reconstruction experiments it was apparent that even a trace contamination of high-affinity
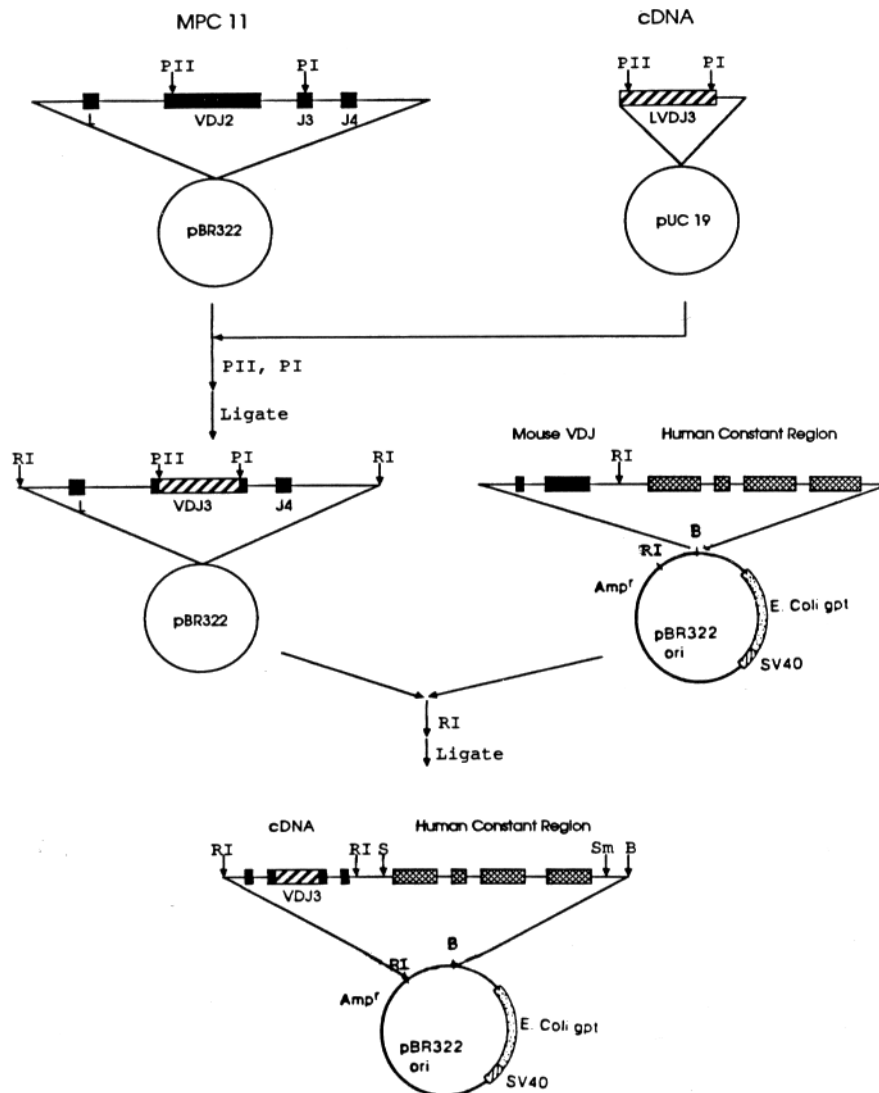
FIGURE 1.   Substitution of the genomic V_H region with V_H cDNA and isotype switch. A genomic Eco RI fragment containing the MPC11 H chain promoter, leader sequence, rearranged V region, and Ig enhancer (24) was cloned into the Eco RI site of a pBR322 derivative from which the sequences lying between the Hind III site (nucleotide 29) and the Pvu II site (nucleotide 2,066) had been deleted. Using cDNA produced from the anti-$\alpha(1 \rightarrow 6)$dextran hybridomas (3), the V region of the MPC11 was replaced by the antidextran V region by inserting the Pvu II-Pst I cDNA fragment into Pvu II-Pst I-cleaved MPC11. The first four V_H amino acids are derived from MPC11, but are identical to those found in the three cDNAs (24). The Eco RI fragment containing the dextran V_H was joined to a human IgG$_4$ constant region within the pSV2-gpt expression vector (25, 5). The coding sequences of the MPC11 and cDNA genes are shown as solid and hatched lines, respectively. The crosshatched boxes represent the coding sequences of the human IgG$_4$ constant region. The maps are not drawn to scale. Restriction enzymes have been abbreviated as follows: Eco RI, RI; Pvu II, PII; Pst I, PI; Bam HI, B; Sal I, S; and Sma I, SM.

TABLE II

Nomenclature for Transfectoma* and Hybridoma Antibodies

Laboratory name of hybridoma/ transfectoma antibodies	Name used in this study[‡]
14.6b.1	—
19.22.1	—
5.54.4.24.1	5.54
TKC3.2	T14.6b
THV8.3	T19.22
TJC8.5	T5.54

* An immortalized antibody-synthesizing cell line produced by gene transfection and expression techniques.

[‡] The names of the transfectoma cell lines have been shortened for ease and clarity in reading the text. The letter T denotes transfectoma; the number following lists the origin of V_H segment: 14.6b is derived from mAb 14.6b.1; 19.22 from 19.22.1; 5.54 from 5.54.4.24.1. A dash indicates that the name has not been changed.

antibody could dramatically increase the apparent binding constant for dextran of the low-affinity antibody (data not shown). To avoid this we used Con A, which binds high-mannose and biantennary complex oligosaccharides (16), to separate unglycosylated from glycosylated Ig. Adsorption experiments showed that the CHO in T19.22 antibody was not accessible by binding to Con A–Sepharose (Fig. 3 *A*, lanes *3* and *5*). In contrast, the T14.6b antibody was adsorbed to Con A–Sepharose (Fig. 3 *A*, lanes *2* and *4*), thus the additional CHO present in V_H, unlike the CHO buried between CH_2, must be accessible to binding by Con A. The residual T14.6b antibody seen in the Con A supernatant (Fig. 3 *A*, lane *2*) may reflect our inability to separate the Con A slurry completely from the culture fluid.

TM treatment of both T14.6b and T19.22 antibodies resulted in an electrophoretic mobility change consistent with the loss of CHO from the H chain (Fig. 3 *B*, lanes *1-4*). H chains that contain two, one, and zero *N*-linked CHO moieties (Fig. 3 *B*,
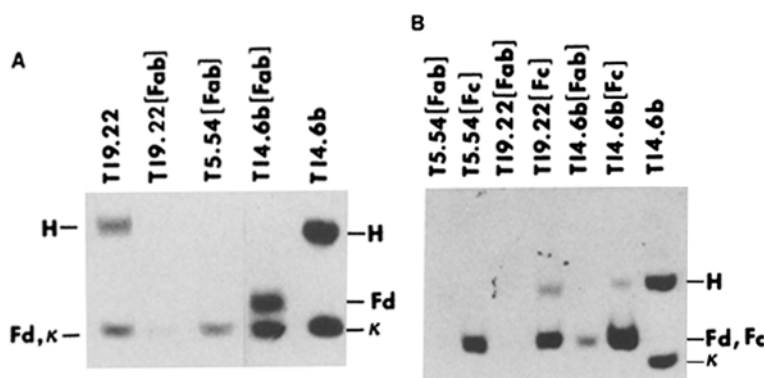

FIGURE 2.   SDS-PAGE analysis of immunoprecipitates obtained after papain digestion of (*A*) [³⁵S]Met or (*B*) [¹⁴C]glucosamine-labeled secreted Ig. [³⁵S]Met-labeled and reduced Igs were used as markers. In *A* the T14.6b (Fab) and T14.6b samples were analyzed on a separate SDS-PAGE gel.
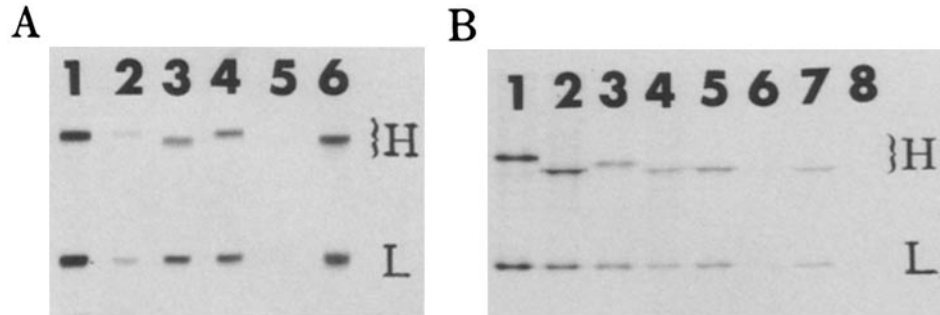
A

1 2 3 4 5 6

-    — —   — }H

- — — —   — L

B

1 2 3 4 5 6 7 8

—   }H

— — — — —   — L

FIGURE 3.   12.5% Tris-glycine SDS-PAGE analysis of [^{35}S]Met-labeled transfectoma culture supernatants, immunoprecipitated with rabbit anti-human Ig Fc antiserum after Con A adsorption (as indicated) and/or tunicamycin treatment. Samples were reduced with 0.15 M 2-ME before SDS-PAGE. The positions of the H and L chains are indicated. (*A*) Con A-Sepharose adsorption of secreted transfectoma Ig. (Lanes *1* and *6*) Untreated T14.6b and T19.22 secreted Ig, respectively. (Lanes *2* and *4*) T14.6b unbound, and T14.6b bound and eluted from Con A-Sepharose. (Lanes *3* and *5*) T19.22 unbound, and bound and eluted from Con A-Sepharose. (*B*) Tunicamycin-treated cell supernatants without or with Con A-Sepharose adsorption. (Lanes *1* and *2*) T14.6b before and after tunicamycin treatment; (lanes *3* and *4*) T19.22 before and after tunicamycin treatment; (lanes *5* and *6*) tunicamycin-treated T14.6b Con A supernatant and eluate, respectively; (lanes *7* and *8*), tunicamycin-treated T19.22 Con A supernatant and eluate, respectively.

lanes *1*, *3*, and *2*, or *4*, respectively) can be resolved. The H chain bands of the untreated samples (Fig. 3 *B*, *1* and *3*) appear homogeneous, suggesting that all H chains are uniformly glycosylated. From the lack of visible glycosylated H chain bands in lanes *2* and *4*, we estimate that TM treatment results in >97% deglycosylation of the Ig. Lanes *5–8* show the results obtained from Con A adsorption of Tm-treated Ig. Both the T14.6b and T19.22 aglycosylated antibodies were not bound by Con A (Fig. 3 *B*, *5* and *7*). The faint bands that represent CHO$^-$ H chains in lane *6* probably reflect nonspecific trapping in the Con A-sepharose slurry.

Having established that Con A adsorption could remove glycosylated contaminants from T14.6b Tm-treated preparations, we used Con A-adsorbed material for dextran binding studies. The results from one typical experiment are graphically illustrated in Fig. 4. For the native T14.6b antibody 50% inhibition of binding to ELISA plates coated with 0.5 or 20 µg/ml dextran was obtained when 1.2 µg/ml of dextran
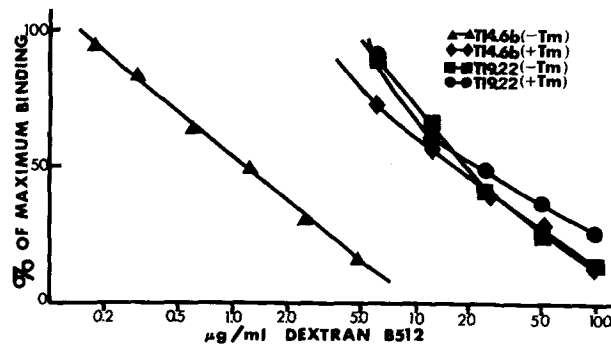

FIGURE 4.   Inhibition by soluble dextran of antibody binding to dextran-coated ELISA plates. Percentage of antibody binding (ordinate) is plotted against dextran inhibitor concentration (abscissa). Plates were coated with 20 µg/ml dextran. Native antibodies and antibodies aglycosylated by tunicamycin treatment were used; trace quantities of glycosylated Ig present in tunicamycin treated T14.6b were removed by adsorption to Con A-Sepharose.

inhibitor was added. CHO-depleted T14.6b antibody could not bind to 0.5 µg/ml dextran-coated plates (data not shown). Using low-affinity binding conditions (microtiter wells coated with 20 µg/ml dextran) the aglycosylated T14.6b and T19.22 antibodies and native T19.22 antibody showed half-maximal binding when 18–24 µg/ml dextran B512 inhibitor was added.

The apparent association constants for Tm-treated aglycosylated and untreated native antidextran antibodies are summarized in Table III. The binding constant of the CHO-depleted T14.6b was 14–15-fold lower than the native antibody. In contrast, carbohydrate removed from the Fc of T19.22 did not affect that antibody's ability to bind antigen. All experiments except those noted were performed using an antibody concentration of 1 µg/ml; we observed a slight affect of antibody concentration on apparent aK_a values. The aK_a values determined using the inhibition ELISA were, in general, slightly higher than those obtained previously using affinity gel electrophoresis. For a discussion of affinity gel electrophoresis see Takeo and Kabat (17). However, the differences in binding strength between antibodies were similar using the two assays. We found a 32-fold difference in binding affinity between the T14.6b and T19.22 antibodies, versus the 50-fold difference between the parental mAbs 14.6b.1 and 19.22.1 reported (1). In summary, it is clear that the presence of CHO within the antidextran V$_H$ region significantly affects its affinity for antigen, however, we cannot rule out an additional contribution of the altered amino acids to the differences in binding.

Discussion

Antibodies are glycoproteins with all heavy chains containing at least one and frequently several N-linked carbohydrate residues (18). The role postulated for carbohydrate found on the heavy chain constant regions includes solubilization of the H chain, facilitation of subcellular transport and secretion, promotion of assembly,

TABLE III

Apparent Binding Constants for Dextran B512

Hybridoma or transfectoma antibody	aK_a (tabulated from Fig. 4)*	aK_a (calculated from several experiments)[‡]	Number of experiments
		ml/g	
14.6b.1	ND	2.30 ± 0.1 × 10^6	4
19.22.1	ND	ND	
T14.6b (without Tm)	1.7 × 10^6	1.68 ± 0.6 × 10^{6}[§]	8
		(2.10 ± 0.3 × 10^6)[¶]	5
T14.6b (with Tm) Con A-adsorbed	1.1 × 10^5	1.18 ± 0.04 × 10^5	5
T19.22 (without Tm)	1.0 × 10^5	8.22 ± 3.6 × 10^{4}[‖]	10
		(6.5 ± 0.3 × 10^4)[¶]	6
T19.22 (with Tm) Con A-adsorbed	8.3 × 10^4	1.09 ± 0.4 × 10^5	4

* Calculated from the reciprocal concentration of dextran B512 necessary to inhibit 50% of the maximal binding of antibody to dextran-coated plates. 1/[Dex]$_{I50}$ has been doubled to give the final aK_a value because dextran inhibitor and antibody were added to microtiter wells at a 1:1 molar ratio.

[‡] The aK_a value represents an average obtained from the experiments indicated. The error for the sum total of all the values is represented by the first standard deviation.

[§] Antibody concentration was 0.8 µg/ml.

[‖] Antibody concentration was 0.3 µg/ml.

[¶] Culture supernatants were not from tunicamycin experiments. Antibody concentration was 1 µg/ml.

and maintenance of Ig conformational features that contribute to effector functions (19). Carbohydrate can also be found within the V region of an antibody molecule. 15% of human myeloma light chains have carbohydrate within their variable regions (20). In a study of 76 human IgG myeloma proteins, ~25% were shown to contain a carbohydrate moiety on the Fab fragment (21). The carbohydrate was linked to either the light chains or the Fd fragments, and in a few cases to both.

In an earlier study, Matsuuchi et al. (22) isolated and characterized a spontaneously arising mutant of the myeloma J558 [IgA, λ, anti-α(1 → 3) α(1 → 6)dextran] with decreased reactivity with polymeric dextran. The mutant differed from the wild-type in that it had increased amounts of sialic acid on the carbohydrate in its Fab region. Since the variable region of J558 does not contain the canonical carbohydrate addition sequence, the altered carbohydrate probably resides within the CH_1 domain. The change in carbohydrate content was the consequence of the altered availability of cellular enzymes involved in glycosylation.

It has been well documented how variation in amino acid sequence of the variable region contributes to antibody diversity, and the many genetic mechanisms that can generate different amino acid sequences have been defined. In this report we demonstrate directly that the presence of carbohydrate in CDR2 of V_H is critical for the high-affinity binding of a monoclonal antibody specific for polymeric α(1 → 6)dextran, and we infer that the carbohydrate also contributes to the increased affinity for IM7. Thus not only the specific amino acid sequence of the variable region, but also its carbohydrate moieties can determine the specificity and magnitude of the antigen-antibody interaction. Variable expression of glycosyltransferases could be used to modulate antibody binding.

Of great interest is the mechanism by which presence of an oligosaccharide attached to amino acids in the combining site of the antibody 14.6b.1 leads to increased K_a for both polymeric dextran and IM7. X-ray crystallographic studies of unrelated antibodies predict that the residues to which the carbohydrate is attached in V_H should be exposed on the hypervariable loops. Our Con A binding experiments also suggest that, in contrast to the carbohydrate in CH_2, the V_H oligosaccharide is relatively exposed, and is positioned at the surface of the Ig. Thus it is possible that the V_H carbohydrate directly interacts with the antigen; however, it is difficult to see how direct interactions could occur both with polymeric dextran and a site-filling oligosaccharide, IM7.

A more likely explanation for the effect of glycosylation is that the carbohydrate linked to amino acid 58 alters the conformation of the combining site. Such alterations might increase the accessibility of the Thr residue at position 60 in the 14.6b.1 V_H region so that it may contact the antigen more closely. Indeed, Feldman and coworkers have predicted from the hypothetical space-filling model of the V region of the galactan-binding myeloma Ig J539 that H chain Thr residue 56 may contact galactan (23). The X-ray crystallographic structure of the 14.6b.1 Fab would aid in our understanding of how the presence of carbohydrate affects the topology of the combining site.

Summary

We have observed that antidextran hybridomas with potential N-linked glycosylation sites in V_H have higher affinity for polymeric dextran and for isomaltoheptaose

than those lacking potential glycosylation sites. In these studies we have used gene transfection and expression techniques to verify that the carbohydrate addition sites in V_H were used. The carbohydrate of the V_H region was accessible for binding by the lectin Con A. By ELISA analysis it was demonstrated that the aK_a of the antibody for dextran was influenced by the presence of carbohydrate in V_H, with the aglycosylated antibody having an aK_a 15-fold lower than its untreated counterpart. The aK_a for antigen of antibodies that contain carbohydrate only in their constant region was unaffected by lack of carbohydrate. Thus, not only the amino acid sequence of the variable region but also its carbohydrate moieties can determine the magnitude of the antigen-antibody interaction.

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