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

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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

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[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.

Published September 1, 1988

TABLE I

Immunochemical Properties of Hybridoma Antibodies Specific for Dextran B512

Hybridoma	Mouse strain	Isotype	Site size	K_a‡	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 α(1 → 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

Published September 1, 1988

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 α(1 → 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 [^{35}S]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 [^{14}C]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 [^{35}S]Met-labeled κ light chain. We find [^{14}C]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

Published September 1, 1988

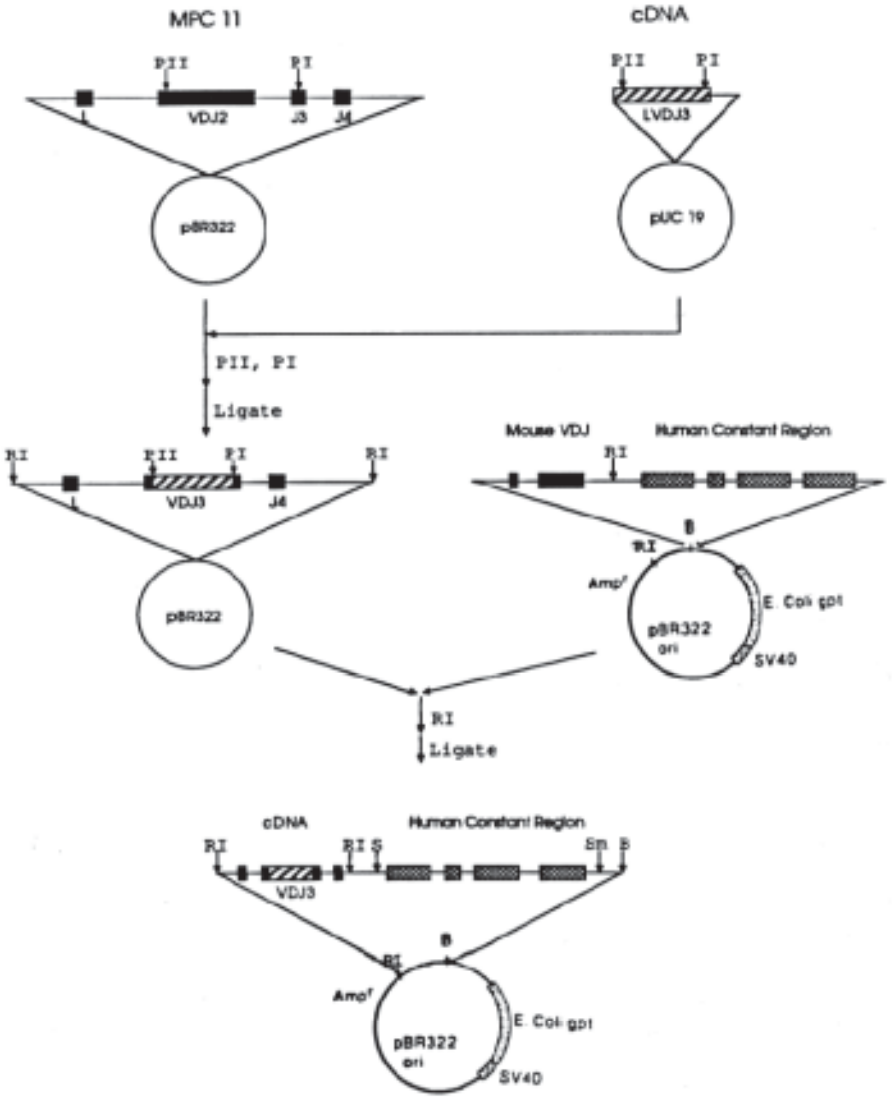

FIGURE 1. Substitution of the genomic V_H region with V_H cDNA and isotype switch. A genomic Eco RI fragment containing the MPC11 H chain promoter, leader sequence, rearranged V region, and Ig enhancer (24) was cloned into the Eco RI site of a pBR322 derivative from which the sequences lying between the Hind III site (nucleotide 29) and the Pvu II site (nucleotide 2,066) had been deleted. Using cDNA produced from the anti-α(1 → 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.

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