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Clonal Analysis of the Glycosylation of Immunoglobulin G Secreted by Murine Hybridomas†

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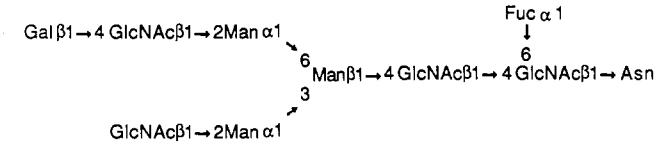
ABSTRACT: A panel of 10 hybridomas was assembled to assess the influence of various genetic and biological factors upon glycosylation of secreted monoclonal IgG. After exhaustive Pronase digestion of IgG, glycopeptides were characterized chromatographically by apparent size, charge, and concanavalin A (Con A)–Sepharose and *Lens culinaris* (LcH)–agarose affinity. Six glycosylation phenotypes were found to be common among all clones studied. Despite this phenotypic heterogeneity in glycosylation of IgG, considerable similarity exists between different clones. In particular, virtually all IgG glycopeptides bear a core fucose residue. Second, the majority of the glycosylation repertoire is comprised of two phenotypes, characterized by glycopeptides which differ in affinity for Con A–Sepharose. Of these, the predominantly expressed phenotype is the same for all clones. The carbohydrate structure derived from this phenotype, elucidated by 500-MHz ^{1}H NMR spectroscopy, is



Significant variability between different hybridomas exists in the relative expression of the two major phenotypes. Other differences between clones may reflect the expression of an additional site which is glycosylated differently. However, there is no apparent correlation of phenotype with either the hybridoma's parentage or the serologically defined polypeptide structure of the IgG which it secretes. In addition to clonal variability, other sources of variability in phenotypic expression were identified. A generational variability is apparent upon continuous culturing of the same hybridoma. Also, differences in culture medium pH or proliferative state of the cells may have a modest influence upon the glycosylation phenotype.

IgG is a glycoprotein secreted by immune splenocytes and malignant myelomas. Although IgG is otherwise well characterized, few detailed, comparative studies exist of the species-specific differences in IgG carbohydrate or the clonal variability in its expression. Similarly, systematic studies are lacking as to whether glycosylation varies with polymorphism in IgG polypeptide structure, which can be defined serologically as isotype, allotype, and idiotype. Structural elucidation of the oligosaccharides expressed has been performed extensively only with human (Parekh et al., 1985; Takahashi et al., 1987; Harada et al., 1987) and rabbit (Taniguchi et al., 1985; Rademacher et al., 1983) IgG, whereas clonal variability in glycosylation has been studied solely with human myeloma IgG (Takahashi et al., 1987; Mizuochi et al., 1982; Savvidou et al., 1984; Grey et al., 1982; Kornfeld et al., 1971).

The carbohydrate structure of human (Kornfeld et al., 1971), rabbit (Taniguchi et al., 1985), murine (Mizuochi et al., 1987), bovine (Tai et al., 1975), and porcine (Rao & Mendicino, 1978) IgG is an Asn-linked oligosaccharide of the biantennary, complex type. However, instead of a single oligosaccharide, an array of structurally related yet distinct oligosaccharides is expressed, a phenomenon referred to as carbohydrate microheterogeneity. Differences exist in the frequency of expression of these various oligosaccharides on human (Parekh et al., 1985; Harada et al., 1987), rabbit (Taniguchi et al., 1985; Rademacher et al., 1983), and murine (Mizuochi et al., 1987) serum IgG. Additionally, clonal differences exist in the carbohydrate microheterogeneity of human myeloma IgG (Takahashi et al., 1987; Mizuochi et al., 1982; Savvidou et al., 1984; Grey et al., 1982; Kornfeld et al., 1971).

The phenomenon of carbohydrate microheterogeneity is a characteristic feature of protein glycosylation. Unlike proteins and nucleic acids, oligosaccharide synthesis is not guided by a template. Rather, it is the structure of the polypeptide undergoing glycosylation, as well as the proteins (enzymes and transporters) comprising the glycosylation apparatus, which is under genetic control (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985; Stanley, 1984; Deutscher & Hirschberg, 1986). The phenotypic expression of the oligosaccharide, however, may be influenced by biological modulation of the cell's environment (pH and substrate availability), proliferative state, or substratum (Warren et al., 1983, 1982; Megaw & Johnson, 1979). Presumably, it is the dynamic interplay of these various genetic and biological influences which accounts

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Table I: Panel of Hybridomas[a]

hybridoma	parental cells		IgG		references
	splenocyte	myeloma	isotype	antigenic specificity	
CSAT	BALB/c	SP2/0	2b	anti-integrin	Neff et al., 1982
34-5-8S	C3H	SP2/0	2a	anti-H-2D[d]	Ozato et al., 1982
H36.15.3	BALB/c	SP2/0	2a	anti-hemagglutinin	Staudt et al., 1983
H36.4.5	BALB/c	SP2/0	2a	anti-hemagglutinin	Staudt et al., 1983
H36.2.6	BALB/c	SP2/0	2a	anti-hemogglutinin	Staudt et al., 1983
H36.15.6	BALB/c	SP2/0	2a	anti-hemagglutinin	Staudt et al., 1983
S-S.1	BALB/c	SP2/0	2a	anti-sheep red blood cell	
B137.17.1	BALC/c	.653	2a	?	Perussia et al., 1987
L5.1	BALB/c	.653	2a	anti-transferrin receptor	Lebman et al., 1982
D4IE4	BALC/c	.653	1	anti-P_0	Miller et al., 1984

[a] The parentage and serologically defined polypeptide structure of the IgG secreted are indicated for each member of the panel of hybridomas employed in the clonal analysis.

for the host-dependent and tissue-dependent variability in glycosylation (Hsieh et al., 1983; Sheares & Robbins, 1986; Parekh et al., 1987).

In the present study, we describe the clonal repertoire for the glycosylation of IgG secreted by a panel of murine hybridomas, formed by the fusion of immune splenocytes with myelomas. The effect upon glycosylation of the hybridoma's parentage and the serologically defined polypeptide structure of the monoclonal IgG which it secretes are assessed. The contribution of generational differences, resulting from continuous culturing, and differences in culturing conditions are evaluated as further sources of variability in glycosylation.

Materials and Methods

Materials. D-[U-^{14}C]Glucosamine hydrochloride (284 Ci/mol), D-[6(N)-^{3}H]glucosamine hydrochloride (23.4 Ci/mmol), and En^{3}Hance were purchased from New England Nuclear (Boston, MA). Sephadex G-50 (Superfine), Sephadex G-10, Con A–Sepharose,[1] DEAE-Sephadex A-25, and protein A–Sepharose were obtained from Pharmacia (Piscataway, NJ). LcH (E-Y Laboratories, San Mateo, CA) was conjugated to Affi-Gel 10 (Bio-Rad, Richmond, CA) (7 mg/mL of resin) according to an established procedure (Knudsen et al., 1981). Bio-Gel P-2 (200–400 mesh) was also from Bio-Rad. Pronase (grade B) was purchased from Calbiochem (San Diego, CA).

Culturing and Metabolic Labeling of Hybridomas. Hybridomas (Table I) were obtained either from American Type Culture Center (Rockville, MD) or from various laboratories at the Wistar Institute (Philadelphia, PA). The fusion partners were BALB/c-derived, nonsecreting myelomas. For contrast, one hybridoma (34-5-8S) was chosen for its allogeneic C3H background, and the allotypically different IgG which it secretes. The H36 clones were a cluster of related hybridomas derived from a single fusion of one myeloma variant with immune splenocytes from one animal. All H36 clones secreted an IgG2a directed against the same antigen.

Cells were seeded at 5×10^5/mL and cultured in Dulbecco's modified Eagle's medium with low glucose (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum, 10 mM sodium bicarbonate, and 25 mM Hepes (pH 7.2). After a 20-h preincubation, cultures were metabolically labeled with [^{3}H]glucosamine (2 μCi/mL) for an additional 48 h. Parallel cultures of CSAT hybridomas were labeled alternatively with [^{14}C]glucosamine (0.5 μCi/mL).

Isolation of IgG and Preparation of Glycopeptides. IgG in the spent culture medium was affinity purified chromatographically on protein A–Sepharose essentially by the procedure of Ey et al. (1978). The purified IgG was incubated with predigested Pronase for 3 days, according to an established procedure (Blithe et al., 1980). For ^{1}H NMR analysis, IgG also was obtained from the ascites fluid of CSAT hybridomas passaged in pristane-primed BALB/c mice. The IgG was affinity purified and digested with Pronase similar to IgG obtained from tissue culture.

Structural Characterization Studies. To ensure consistency of elution profiles, a common ^{14}C-labeled CSAT IgG Pronase digest was added as an internal standard to all ^{3}H-labeled samples. The resulting doubly labeled Pronase digests were analyzed chromatographically by various techiques. The radioactive distribution in each fraction, determined by liquid scintillation counting, was plotted by computer-aided graphics, which also provided peak area summations.

(1) Gel Filtration Chromatography. Blue dextran and phenol red, added to the sample, served as exclusion and inclusion volume markers, respectively. Samples were analyzed on columns (1 cm × 135 cm) of Sephadex G-50 developed with an alkaline borate buffer composed of 45.5 mM boric acid/4.5 mM sodium tetraborate/2 mM Na_2EDTA/0.02% sodium azide (pH 8.2) (Rothman & Warren, 1988).

For preparative purposes, selected fractions of the ^{3}H-labeled Pronase digests were pooled. Borate was removed by evaporation under a stream of nitrogen after repeated treatment with 1% acetic acid in methanol. The residue in a minimal volume of water was desalted further on a Bio-Gel P-2 column (1 cm × 15 cm) with a 5-cm overlayer of Sephadex G-10 that was developed with water (Blithe et al., 1980).

(2) Anion-Exchange Chromatography. The ^{3}H-labeled IgG Pronase digest was fractionated batchwise on a column of DEAE-Sephadex (0.7 cm × 4 cm). After the column was washed with 10 mM pyridine/17 mM acetic acid (pH 4.5) to remove the neutral glycopeptides, the acidic glycopeptides were eluted with 2 M ammonium acetate/3.5 M acetic acid (pH 4.5). Volatile buffers were removed by lyophilization.

(3) Lectin Affinity Chromatography. After being boiled for 5 min, IgG glycopeptides were analyzed on columns of Con A–Sepharose or LcH–agarose (0.7 cm × 4 cm) according to the procedure of Cummings and Kornfeld (1982). After the glycopeptides lacking lectin affinity were removed by washing with TBS buffer, the column was eluted stepwise. First, glycopeptides with low lectin affinity were eluted with 10 mM methyl α-glucoside in TBS; then, those with high affinity were eluted with 100 mM methyl α-mannoside in TBS, prewarmed to 60 °C.

[1] Abbreviations: Con A, concanavalin A; LcH, *Lens culinaris* agglutinin; GFC, gel filtration chromatography; DEAE, diethylaminoethyl; mAb, monoclonal antibody; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; TBS, 150 mM NaCl/10 mM Tris/1 mM $CaCl_2$/1 mM $MgCl_2$/0.02% NaN_3 (pH 8).

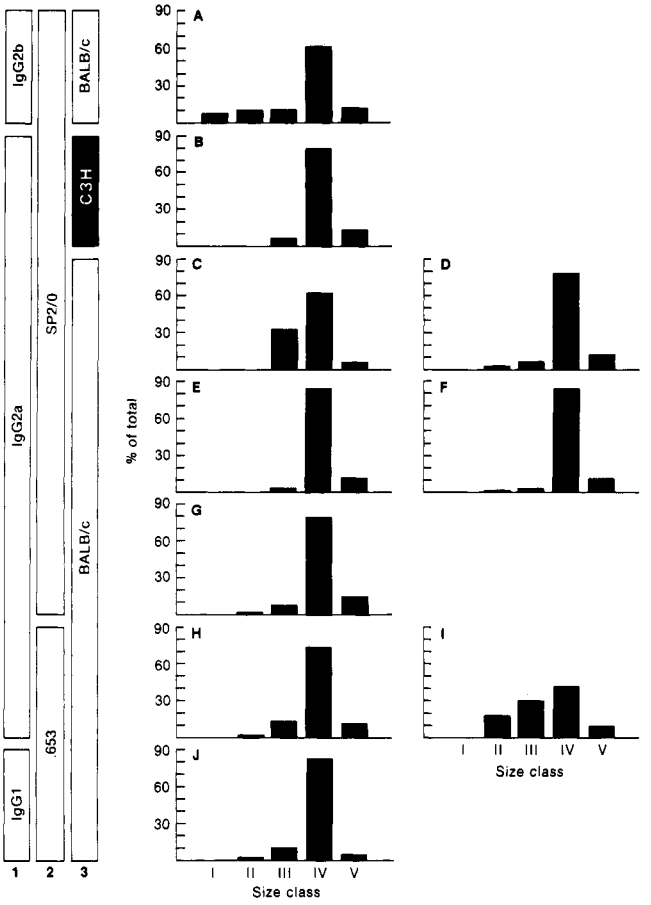

FIGURE 1: Clonal comparison of IgG glycopeptides by alkaline borate GFC. The ^{3}H-labeled IgG Pronase digests were analyzed by alkaline borate GFC. The size class distributions are shown for the IgG glycopeptides of CSAT (A), 34-5-8S (B), H36.15.3 (C), H36.4.5 (D), H36.2.6 (E), H36.15.6 (F), S-S.1 (G), B137.17.1 (H), L5.1 (I), and D4IE4 (J). The panel to the left schematically represents how the hybridomas are related to each other by the isotype (1) of IgG secreted, as well as by their parentage of myeloma (2) variant and splenocyte (3) strain.

For ^{1}H NMR analysis, glycopeptides of IgG isolated from ascites fluid were resolved as described above on a column (1 cm × 8 cm) of Con A–Sepharose.

(4) 500-MHz ^{1}H NMR Spectroscopy. Glycopeptides were repeatedly exchanged in ^2H_2O with intermediate lyophilization and finally dissolved in ^2H_2O which was 99.96 atom % ^{2}H (Aldrich). ^{1}H NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz and a probe temperature of 27 °C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ = 2.225) (Vliegenthart et al., 1983).

Results

Characterization of IgG Glycopeptides by Apparent Size. In an attempt to observe what influence differences in polypeptide structure and parental background might have upon glycosylation, the Pronase digests of IgG secreted by the panel of hybridomas were fractionated according to apparent size by alkaline borate GFC. Clonal analysis revealed that as few as three and as many as five size classes of glycopeptides could be distinguished (Figure 1). Although there is some apparent variability between clones in the relative distribution of these various classes, the predominant size present was always class IV. The molecular weight of this glycopeptide size class is approximately 2000 (Rothman & Warren, 1988).

A second feature common among all the clones was the presence of class III, IV, and V glycopeptides. Of the 10 monoclonal IgGs surveyed, 2 (CSAT and L5.1) were found to have a pattern different by the presence of additional glycopeptides of size classes I and/or II. Although CSAT mAb differs, isotypically, L5.1 mAb is the same as the majority of the panel. Thus, a priori, the absolute number of size classes does not appear to correlate with the isotype.

The clonal differences in the elution profile of CSAT IgG glycopeptides characterized by class I and II glycopeptides have been studied more fully (Rothman & Warren, 1988). Glycopeptide classes I and II were found to differ from the other classes chiefly by sialylation. Their presence reflects the expression of an additional glycosylation site, presumably located within the Fab region, whereas, typically, IgG is glycosylated solely at a single conserved site, located within the Fc portion (Beale & Feinstein, 1976). Furthermore, extensive sialylation appears to be a feature more characteristic of the Fab (and the related Bence Jones proteins) than of the Fc (Taniguchi et al., 1985; Abel et al., 1968; Burton, 1985; Chandrasekaran et al., 1981). Typically, the site of all additional carbohydrate is located within the idiotypically defined variable region of IgG (Spiegelberg et al., 1970; Sox et al., 1970). However, this additional carbohydrate probably does not correlate with the idiotype or the related antigenic specificity. MAbs with either identical specificities (characterized by the H36 series of anti-hemagglutinin mAbs) or different specificities (characterized by the remainder of the panel) generally have similar GFC elution profiles of their glycopeptides, although they differ from that of CSAT mAb (Figure 1). Rather, the expression of an additional site of glycosylation would appear to have been acquired adventitiously in the process of generating antibody diversity.

Characterization of IgG Glycopeptides by Charge. Despite differences in structure and parental background, all hybridomas of the panel yielded IgG glycopeptides which were found to be predominantly neutral by chromatography upon DEAE-Sephadex (Figure 2). Subsequent analysis of the glycopeptides by alkaline borate GFC indicated that the neutral glycopeptides were predominantly of size classes IV and V, whereas classes I and II were composed entirely of acidic glycopeptides. A variable but significant fraction of class III glycopeptides also was acidic. This distribution of acidic glycopeptides by size is an agreement with the sensitivity of CSAT IgG glycopeptides to neuraminidase (Rothman & Warren, 1988).

Characterization of IgG Glycopeptides by Affinity for Con A. Among the clonally common glycopeptide size classes III, IV, and V, the predominant IgG glycosylation phenotype was characterized by glycopeptides of low affinity for Con A–Sepharose (Figure 3A–F). Class IV glycopeptides of low affinity for Con A–Sepharose accounted for 42–62% of the total radioactivity incorporated into IgG. One of the most prominent differences between clones occurred in the relative distribution of class IV glycopeptides of high affinity and those of low affinity for Con A–Sepharose. The ratio between these two phenotypes varied between 1:10 and 2:3. Together, these two phenotypes comprised 50–90% of the total radioactivity incorporated into IgG.

Characterization of IgG Glycopeptides by Affinity for LcH. When analyzed by LcH–agarose, glycopeptides of high affinity for the lectin were characteristic of the predominant phenotype

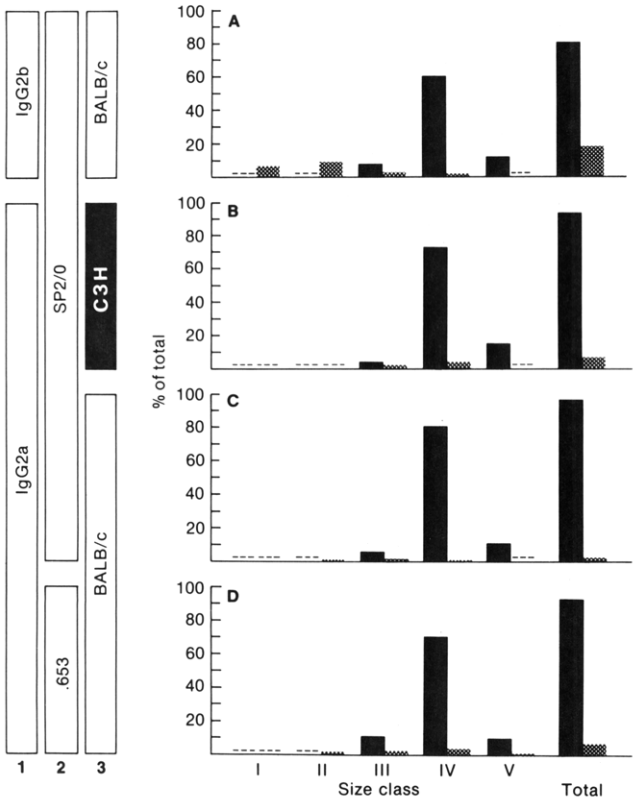

FIGURE 2: Clonal comparison of IgG glycopeptides by DEAE-Sephadex chromatography. The ^{3}H-labeled IgG Pronase digests of CSAT (A), 34-5-8S (B), S-S.1 (C), and B137.17.1 (D) were fractionated batchwise on a column of DEAE-Sephadex into neutral (black bars) and acidic (stiple) glycopeptides. An aliquot of each charge-resolved glycopeptide pool was counted to determine the distribution of acidic and neutral glycopeptides within the total Pronase digest. The balance of each pool was analyzed by alkaline borate GFC to determine the size class distribution. This was weighted for the relative distribution of acidic and neutral glycopeptides within the total digest. The panel to the left is described in Figure 1.

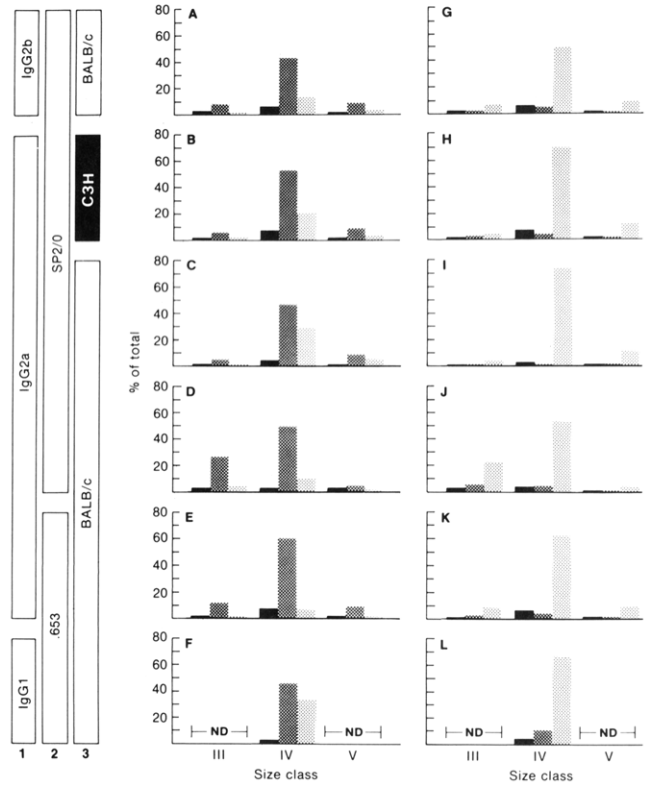

FIGURE 3: Clonal comparison of IgG glycopeptides by lectin affinity chromatography. The ^{3}H-labeled IgG Pronase digests were fractionated by alkaline borate GFC. Next, each of the isolated and desalted glycopeptides was resolved either by Con A (A–F) or LcH (G–L) affinity chromatography into glycopeptides with either no (black bars), low (dark stiple), or high (light stiple) affinity for the particular lectin. The distributions by lectin affinity were then weighted for the relative distribution of the alkaline borate GFC size classes within the total digest (as determined from Figure 2). The lectin affinity distributions are shown for the IgG glycopeptides of CSAT (A and G), 34-5-8S (B and H), S-S.1 (C and I), H36.15.3 (D and J), B137.17.1 (E and K), and D4IE4 (F and L). ND, distribution by lectin affinity not determined. The panel to the left is described in Figure 2.

(Figure 3G–L). By themselves, class IV glycopeptides of high affinity for LcH–agarose accounted for 50–75% of the total radioactivity incorporated into IgG.

Compared with classes IV and V, however, glycopeptides of size class III were found to be relatively enriched in glycopeptides of either low or no affinity for LcH–agarose. Furthermore, qualitative differences were apparent in the LcH elution profiles of glycopeptides of high affinity. Those derived from glycopeptides of size class III were eluted somewhat more quickly than the analogous ones derived from classes IV and V (data not shown). Similar subtle alterations in the Con A–Sepharose elution profiles of IgG glycopeptides have been reported to result from small differences in oligosaccharide structure (Narasimhan et al., 1979).

Another interesting common feature was that greater than 90% of the IgG glycopeptides of classes III, IV, and V, which are assumed to be derived from the Fc portion, had affinity for both LcH and Con A. Although both lectins share many carbohydrate specificities, core fucosylation is required for binding to LcH, but not to Con A (Kornfeld et al., 1981). Thus, the lectin binding data in the present study would suggest that virtually all murine monoclonal IgG expresses core fucosylation of the Fc with very little, if any, variability in its expression.

Characterization by ^{1}H NMR Spectroscopy of the Carbohydrate Structure Derived from the Predominant Phenotype. To elucidate the carbohydrate structure expressed in the predominant phenotype, the CSAT IgG glycopeptide of size class IV and low affintiy for Con A–Sepharose was analyzed by 500-MHz ^{1}H NMR spectroscopy. Its deduced structure is depicted in Figure 4. Summarized in Table II are the relevant ^{1}H NMR parameters, which are compared with those

Table II: ^{1}H Chemical Shifts of Structural Reporter Groups of the Monosaccharides Present in the Predominant Glycopeptide of CSAT IgG, Together with Those of a Reference Compound

		chemical shift (ppm)	
reporter group	residue[a]	CSAT	GP-II-3 (G Gn)
H-1	1	5.069	5.071
	2	4.681	4.682
	3	n.d.	4.766
	4	5.117	5.115
	4′	4.922	4.926
	5	4.554	4.553
	5′	4.580	4.578
	6′	4.472	4.471
H-2	3	4.250	4.253
	4	4.186	4.188
	4′	4.108	4.114
NAc	1	2.012	2.010
	2	2.093	2.091
	5	2.053	2.051
	5′	2.046	2.045
H-1	Fuc	4.872	4.873
CH_3	Fuc	1.201	1.210

[a] Residue positions refer to Figure 4.

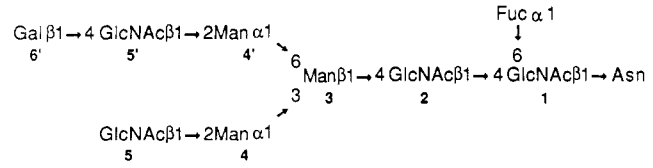

FIGURE 4: Structure of the predominant glycopeptide derived from CSAT IgG. IgG, secreted by CSAT hybridomas grown in vivo, was affinity purified, digested with Pronase, and then fractionated chromatographically. The carbohydrate structure of the size class IV glycopeptide of low affinity for Con A–Sepharose was elucidated by 500-MHz ^{1}H-NMR spectroscopy.

of a reference compound (G Gn) derived from human myeloma IgG (Grey et al., 1982).

The asparagine-bound GlcNAc-1 was characterized by the chemical shift values of its H-1 (δ = 5.069) and *N*-acetyl (δ = 2.012) signals. All GlcNAc-1 residues are α1→6 fucosylated, as revealed by the structural reporter groups for this type of Fuc, namely, H-1 (δ = 4.872) and CH_3 (δ = 1.201). The signals for GlcNAc-2 (H-1, δ = 4.681; NAc, δ = 2.093) also show the presence of the core fucose. No signals for the afuco analogue were detectable.

The biantennary type of branching was seen from the H-2 signals of Man-3, Man-4, and Man-4′, occurring at δ = 4.250, δ = 4.186, and δ = 4.108, respectively. This was consistent with the corresponding H-1 signals for Man-4 and Man-4′ at δ = 5.117 and δ = 4.922. The doublet at δ = 4.580 is assigned to GlcNAc-5′, and the doublet at δ = 4.554 to GlcNAc-5. Therefore, the *N*-acetyl signal at δ = 2.046 is assigned to GlcNAc-5′, and the signal at δ = 2.053 to GlcNAc-5.

Only one Gal seemed to be present and was localized in the Man α1→6 arm by comparison with compounds G Gn and Gn G described by Grey et al. (1982). G Gn was a biantennary glycopeptide terminating with Gal in the Man α1→6 arm and GlcNAc in the Man α1→3 arm. In glycopeptide Gn G, the terminal Gal was located in the Man α1→3 arm instead. The chemical shift of H-1 of Gal in the CSAT IgG glycopeptide was assigned at δ = 4.472. The placement of the Gal in the Man α1→6 arm is based on the chemical shift values of the NAc resonances of GlcNAc-5 and GlcNAc-5′ as well as the value of Man-4′ H-1 (see above). These values are in closer agreement with the structure of G Gn than of Gn G.

Evaluation of Variability in Expression of Glycosylation Phenotypes. Typically, parallel cultures of the same clone, grown under identical conditions, yielded very similar IgG glycopeptide elution profiles (Figure 5). However, comparison of the glycopeptide profiles of IgG secreted by different generations of the same clone revealed an interexperimental (generational) variability (Figure 5). Although the number of size classes remained constant, the relative distribution of all classes varied in a random fashion. One of the most dramatic changes was in the distribution of class III glycopeptides. The percentage of the total radioactivity incorporated into this size class varied nearly 3-fold between the different generations. This generational variability characterized by class III glycopeptides might account for the apparent clonal difference observed in the glycopeptide elution profile of H36.15.3 IgG (Figure 1C).

As culturing conditions are known to influence phenotypic heterogeneity in glycosylation (Buck et al., 1971; van Beek et al., 1975; Megaw & Johnson, 1979), several typical cultural differences were evaluated as a possible source of the interexperimental variability observed in this study. One such difference might be the pH of the culturing medium. Culturing hybridomas at either a lower or higher pH than normal

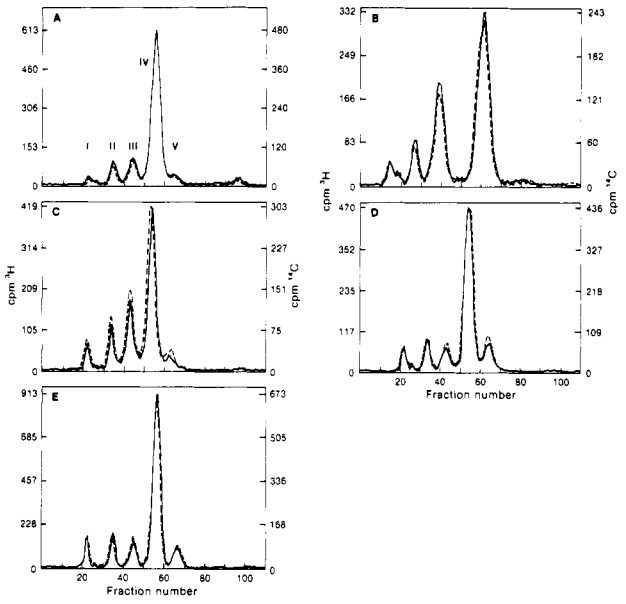

FIGURE 5: Generational variability in the glycosylation of IgG. CSAT hybridomas were cultured continuously in vitro for 16.5 months. At various times, parallel cultures were metabolically labeled with either [^{3}H]- or [^{14}C]glucosamine. The secreted IgG was affinity purified and digested with Pronase. The ^{14}C-labeled (—) and ^{3}H-labeled (--) Pronase digests obtained from parallel cultures of the same generation of CSAT were cochromatographed by alkaline borate GFC. The initial profile (A) and the profiles after culturing for an additional 2 (B), 10 (C), 13.5 (D), and 16.5 (E) months are presented.

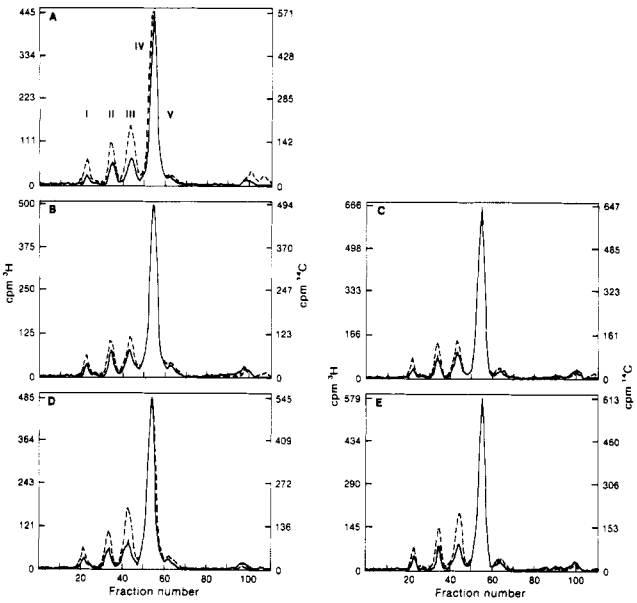

FIGURE 6: Effect of culture medium pH upon IgG glycosylation. Parallel cultures of CSAT, buffered with 10 mM $NaHCO_3$ and either 25 mM Pipes, pH 6.7 (A), 25 mM Hepes, pH 7.2 (B), 25 mM Tes, pH 7.2 (C), 25 mM Hepes, pH 8.0 (D), or 25 mM Hepps, pH 8.0 (E), were metabolically labeled with [^{3}H]glucosamine. The ^{3}H-labeled IgG Pronase digests (--) were analyzed by alkaline borate GFC. A ^{14}C-labeled IgG Pronase digest (—) derived from an earlier generation of CSAT served as a common internal standard.

did enhance the distribution of glycopeptides of size classes I, II, and III (Figure 6). However, the variability in the relative distribution of class III glycopeptides was not as extensive as the observed generational variability depicted in Figure 5.

A second possible cultural difference could be the proliferative state of the hybridomas. To assess its effect upon the IgG glycopeptide elution profile, different inhibitors were

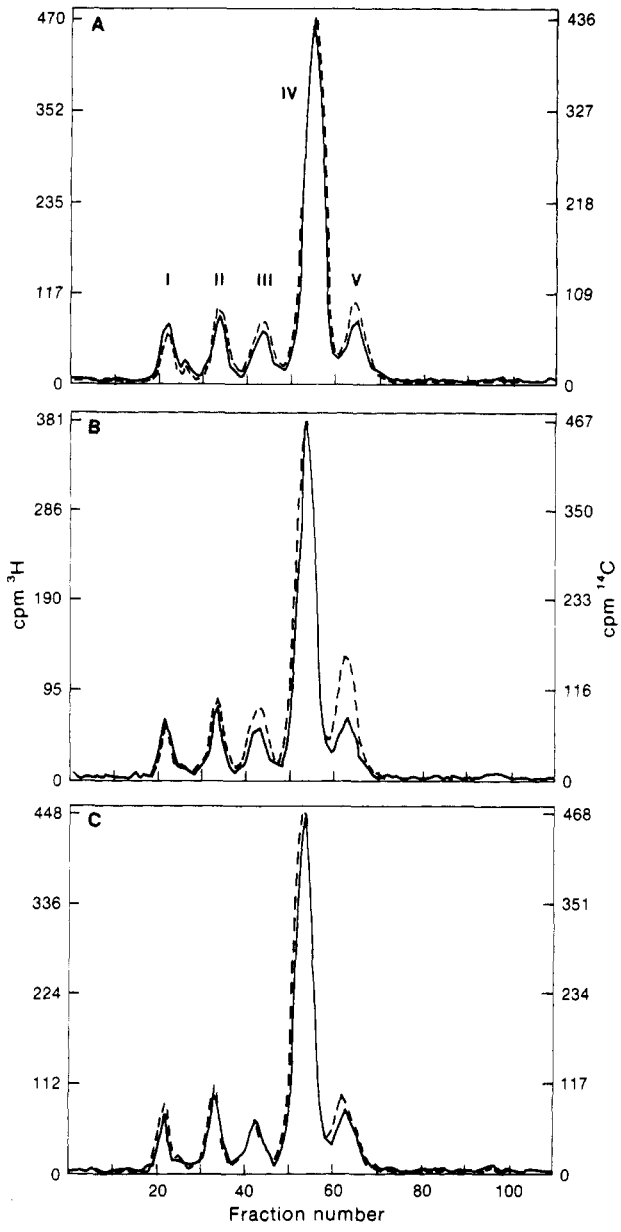

FIGURE 7: Effect of growth inhibitors upon IgG glycosylation. Parallel cultures of CSAT were metabolically labeled with [^{3}H]glucosamine in the absence (A) or presence of either 0.1 μg/mL vinblastine sulfate (B) or 10 mM thymidine (C) by the protocol described in under Materials and Methods. An additional culture of CSAT was metabolically labeled with [^{14}C]glucosamine in the absence of inhibitors. The secreted IgG was affinity purified and digested with Pronase. Aliquots of the ^{14}C-labeled digest (—), added as an internal standard, were cochromatographed by alkaline borate GFC with the ^{3}H-labeled digest (--). Treatment with either inhibitor resulted in a 60% reduction in cell number.

chosen to synchronize the growth of the hybridomas at various stages of the cell cycle. Whereas thymidine (inhibition at G_1/S interphases) had no apparent influence upon glycosylation, vinblastine (inhibition in M phase) had a modest effect. This effect was particularly noticeable in the enhanced percentage of class V glycopeptides (Figure 7).

Discussion

Previously, we have shown that the CSAT hybridoma repertoire for glycosylation of IgG comprises a minimum of eight distinct phenotypes (Rothman & Warren, 1988). In the present study, six of these eight phenotypes were found to be commonly expressed by all ten clones analyzed and presumably reflect glycosylation of the same site located within the Fc portion of IgG. These clonally common phenotypes were characterized by glycopeptides of either low affinity, high affinity, or no affinity for Con A–Sepharose and were distributed differently by size between classes III and IV (plus V). Class IV and V glycopeptides differ from each other only by the size of the peptide remaining after exhaustive Pronase digestion (Rothman & Warren, 1988). The inability of Pronase to completely remove all amino acid residues surrounding the glycosylation site is characteristic of most glycoproteins and appears to be dependent upon the nature of the oligosaccharide (Kobata, 1984).

Despite the phenotypic heterogeneity in glycosylation of IgG, there is considerable similarity between different clones. In particular, virtually all Fc-derived oligosaccharides are believed to express a core fucose residue, as, typically, greater than 90% of IgG glycopeptides have affinity for LcH–agarose. Moreover, the majority of the IgG glycosylation by all hybridomas is of only two phenotypes. Of these, the predominantly expressed phenotype is the same for all clones. Upon digestion of IgG by Pronase, this phenotype is characterized chromatographically by neutral glycopeptides of size class IV (M_r ~2000) which are of high affinity for LCH–agarose but of low affinity for Con A–Sepharose. The second most prevalent phenotype is characterized by neutral glycopeptides also of size class IV, but which are of high affinity for Con A–Sepharose.

For CSAT IgG, the carbohydrate structure derived from the predominant phenotype has been identified by ^{1}H NMR. Similar structures are also the predominant ones expressed on rabbit (Taniguchi et al., 1985) and bovine (Tai et al., 1975) serum IgG, as well as on human myeloma IgG (Kornfeld & Kornfeld, 1980; Kornfeld et al., 1971). Chromatographic similarity of glycopeptides by four criteria (apparent size, charge, and affinity for two lectins) suggests that a similar oligosaccharide also is the predominant one expressed on all IgG secreted by our panel of murine hybridomas. Due to its high affinity for Con A–Sepharose, the second most prevalent phenotype is consistent with the expression of an oligosaccharide similar to the predominant one, but lacking the single galactosyl residue, such that both branches terminate with *N*-acetylglucosamine (Narasimhan et al., 1979).

Very recently, Mizuochi et al. (1987) have elucidated the structure of the oligosaccharides expressed on IgG from pooled murine serum. The major structure is identical with the predominant one identified for CSAT IgG in the present study. Furthermore, they have reported that 94% of the oligosaccharides are core fucosylated and that 80% bear either a single galactose residue or none, which is consistent with our analysis by lectin affinity.

Interestingly, though, we find that a lesser percentage of sialylated structures are expressed on IgG secreted by murine hybridomas than Mizuochi et al. (1987) reported for murine serum IgG. Whereas a comparable percentage of murine, rabbit, and human serum IgG is sialylated, there is considerable variability in the (Fc) sialylation of human myeloma IgG (Mizuochi et al., 1987, 1982; Taniguchi et al., 1985). Thus, the reduction in sialylation we observe may be a feature of murine hybridomas, perhaps as a consequence of the somatic hybridization of two cell types.

Variability in Expression of Glycosylation Phenotypes. As anticipated, there is some variability in the relative expression of the various phenotypes. In part, some of this variability is clonal in origin. For example, significant variability between different hybridomas exists in the relative expression of the two major phenotypes characterized by glycopeptides which

bind to Con A–Sepharose but which differ in their affinity. In some instances, other differences between clones may reflect the expression of an additional site which is glycosylated differently. This is exemplified by class I and II IgG glycopeptides derived from CSAT, which are sialylated and lack affinity for Con A (Rothman & Warren, 1988). However, there is no phenotype which appears to be uniquely characteristic of either the hybridoma's parentage or the serologically defined polypeptide structure of the IgG which it secretes.

Instead, there appears to be a continuum of shared phenotypes. Although the predominantly expressed one is always the same, these phenotypes otherwise are variably expressed in an apparently random fashion by the different clones. To some extent, this may be a consequence of the variability in glycosylation evident between different generations of the same hybridoma upon continuous culturing. This generational variability does not influence the number of phenotypes expressed. Rather, it results in a random drift in the relative expression of these phenotypes, particularly the one characterized by class III glycopeptides.

Anderson et al. (1985) also have reported what appears to be a generational variability in glycosylation of mouse myeloma IgM. By contrast, Sweidler et al. (1985) have reported stability in glycosylation of major histocompatibility complex (MHC) antigens by lymphomas. Whether this discrepancy reflects intrinsic differences between cell surface and secreted glycoproteins is uncertain. Alternatively, generational variability may be an intrinsic feature of immunoglobulins.

Lastly, some variability in phenotypic expression of glycosylation may be a consequence of differences in culturing conditions of the hybridomas. However, typical culturing differences which would be anticipated, such as culture medium pH and proliferation status, have only a modest influence upon the IgG glycopeptide elution profile. In fact, a survey of environmental challenges, including a comparison of the effect of growth of hybridomas in vitro with that in vivo, revealed only occasional differences in the relative expression of the various phenotypes, whereas the nature of the predominant phenotype remained conserved (unpublished data).

The relative clonal similarity in IgG glycosylation poses an intriguing question. If a single, predominant phenotype is sufficiently important enough to have been conserved, why is there phenotypic heterogeneity? One possibility might be that glycosylation defines functional subsets of mAb. Thus, heterogeneity in effector function might be a consequence of structural heterogeneity in glycosylation. Studies to determine the effect of metabolically induced structural alterations in glycosylation upon IgG effector function should help to answer this question.

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Effect of Homo Poly(L-amino acids) on Fibrin Assembly: Role of Charge and Molecular Weight

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ABSTRACT: Positively charged molecules such as protamine, leukocyte cationic protein, and the carboxyl terminus of platelet factor 4 have been shown to increase fibrin fiber thickness. Synthetic homo poly(L-amino acids) were used to explore the role of charge and molecular weight of cationic molecules on fibrin assembly. The effects of poly(L-lysine) (PLL), poly(L-glutamic acid) (PLG), poly(L-aspartic acid) (PLA), poly(L-histidine) (PLH), and poly(L-arginine) (PLArg) on the assembly and structure of fibrin gels were studied by using light-scattering techniques. At a PLG (M_r 60 000) concentration of 80 μg/mL and a PLA (M_r 20 000) concentration of 64 μg/mL, neither of these negatively charged polymers produced a detectable change in either fibrin assembly kinetics or final structure. Positively charged PLArg (16 μg/mL) caused a 30% increase in fibrin fiber mass/length ratio without calcium. In contrast, PLH (16 μg/mL), also positively charged, had no effect in the absence of $CaCl_2$ but produced a 40% increase in fiber mass/length ratio with 5 mM $CaCl_2$. At concentrations as low as 1 μg/mL, positively charged PLL increased the initial fibrin assembly kinetics and led to larger fiber mass/length ratio. The impact on fibrin mass/length ratio was equivalent for three different molecular weight preparations of PLL (M_r 25 000, 90 000, and 240 000). The lack of a molecular weight effect on fiber thickness and the low polymer concentrations required to produce the perturbation argue against an excluded volume effect as the mechanism by which lateral fiber growth is augmented. Mechanisms by which poly(L-amino acids) may perturb fibrin assembly are discussed.

Fibrinogen normally circulates as a soluble asymmetric protein, M_r 340 000, until the amino-terminal peptide, fibrinopeptide A, is enzymatically removed by thrombin. The subsequent fibrin polymerization kinetics and resulting fibrin structure are exquisitely sensitive to and modified by the microenvironment in which fibrin assembly occurs (Jones & Gabriel, 1988). Small shifts in pH, ionic strength, or calcium concentration during the assembly process result in dramatic changes in the material properties of fibrin (Ferry, 1947; Shulman et al., 1953a,b; Shen et al., 1975; Latallo et al., 1962; Carr et al., 1986a,b). Agents with known positive charge, including protamine (Stewart et al., 1969), dextran (Carr & Gabriel, 1980), and hydroxyethyl starch (Carr, 1986), have been shown to modify the final clot structure. Recently, native plasma proteins such as immunoglobulins (Gabriel et al., 1983), histidine-rich protein (Leung, 1986), leukocyte cationic protein (Carr et al., 1986a,b), thrombospondin (Bale et al., 1986), platelet factor 4 (Carr et al., 1987), and actin (Janmey et al., 1985) have been reported to alter fibrin assembly kinetics and fibrin material properties. The roles of molecular charge and size of nonpolymerizing perturbing molecules in determining fibrin assembly kinetics and structure remain incompletely examined. This study reports the effects of charged synthetic homo poly(L-amino acids) of varying size and charge

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