Carbohydrate Analysis of Immunoglobulin G Myeloma Proteins by Lectin and High Performance Liquid Chromatography: Role of Glycosyltransferases in the Structures

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ABSTRACT

The carbohydrate structures and the enzymatic basis for glycosylation of IgG by bone marrow plasma cells were determined in 7 patients with monoclonal gammapathy of undetermined significance and 22 patients with IgG MM. Lectin-binding analysis showed that in all cases of monoclonal gammapathy of undetermined significance and normal controls the IgG heavy chains bound to Ricinus communis agglutinin more strongly than to concanavalin A. In contrast, the IgG in 11 of the 17 advanced cases of MM (stages II and III) studied reacted to concanavalin A more strongly. Structural analysis showed that the reduced R. communis agglutinin binding capacity of these MM IgGs was due to hypogalactosylation of IgG. The galactosyltransferase and N-acetylgalcosaminyltransferase activities of the bone marrow myeloma cells from 5 MM cases were found to have a low enzyme activity ratio of galactosyltransferase to N-acetylgalcosaminyltransferase. This indicates that the difference in the carbohydrate moieties observed in myeloma proteins is due to variations in the activities of the two glycosyltransferases.

INTRODUCTION

IgG usually has one asparagine-linked oligosaccharide in the Fc region of each heavy chain (1, 2), and different molecules contain heterogeneous sugar chains characterized by variable terminal galactoses (3). The overall oligosaccharide pattern of IgG is constant in every healthy individual, but variations may be encountered under some pathological conditions (4–6). Asparagine-linked sugar chains have been also found in the variable regions of IgG in diverse clinical entities (7, 8). The glycoform of monoclonal IgG proteins, products of neoplastic plasma cells, also have properties different from those of the polyclonal normal IgG proteins (4, 9). The difference in the MM3 proteins appear to be due to variations in the levels of glycosyltransferase which control the biosynthesis of sugar chains (10).

The present study was undertaken to evaluate detailed structural and enzymological differences in the plasma cells of patients with IgG paraproteinemias, including MGUS and multiple myeloma (MM). To establish the carbohydrate structure of IgG, the separation of the glycosidic moiety by a lectin-binding analysis, using RCA and Con A which recognize galactose and core mannosyl residues, respectively, has been used (11, 12). Following hydrazinolysis of the IgG fraction, the precise sugar structures were determined following their separation by HPLC. It was found that these structures relate to the activities of Gal-T and of GnT III in the bone marrow myeloma cells.

MATERIALS AND METHODS

Patients. The IgG fractions of the sera of 29 patients with IgG paraproteinemias and of 7 healthy control individuals were used. Diagnosis and clinical staging of the patients, according to the report of Durie (13), were as follows: 7 had MGUS; the MM patients included 5 with stage I, 7 with stage II, and 10 with stage III. Of these, 24 patients were untreated (7 with MGUS, 5 with stage I, 5 with stage II, and 7 with stage III). The remaining 5 patients had received chemotherapy with an alkylating agent (melphalan or cyclophosphamide) and prednisolone.

Purification of IgG from Patients. For lectin analysis, serum or plasma collected from patients was diazylated against 0.02 M potassium phosphate pH 8.0, and applied on a DEAE-Affi-Gel blue (Bio-Rad Laboratories, Richmond, CA) column equilibrated with the same buffer (14). In order to obtain purified IgG to be analyzed with HPLC, the IgG fraction from the serum of patients was precipitated by sodium sulfate solution, followed by purification with chromatography on DEAE-cellulose (Whatman DE52; Whatman Biosystems Ltd., United Kingdom) (15). Each IgG isolated showed a single band on sodium dodecyl sulfate-polyacrylamide gel. The concentration of purified protein was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Lectin Analysis of IgG. Four μg of purified IgG was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel in the presence of 2-mercaptoethanol according to the procedure of Laemmli (16) and transferred to nitrocellulose paper by the method of Towbin et al. (17). To avoid nonspecific binding, the nitrocellulose strips were subsequently incubated with 1% bovine serum albumin in phosphate buffered saline containing 0.05% Tween 20 (Sigma Chemical Co, St. Louis, MO) for 1 h at room temperature and then washed three times with phosphate buffered saline containing 0.05% Tween 20. The nitrocellulose strips were incubated either with biotinylated RCA (1 μg/ml, EY Laboratories, Inc., San Mateo, CA) or with biotinylated Con A (1 μg/ml; Vector Laboratories, Inc., Burlingame, CA) for 1 h at room temperature and stained with the avidin-biotin-peroxidase complex method using a Vectastain ABC kit (Vector Laboratories, Inc.) (18). The lectin-binding capacity of each γ chain was expressed as an RCA/Con A ratio of the peak area determined by two dimensional scanning of the gel with a densitometer (CS9000; Shimadzu, Kyoto, Japan) at 600 nm. The heavy and light chains were identified using biotinylated rabbit anti-human IgG (1 μg/ml; Vector Laboratories, Inc.). Occurrence of glycoforms in the Fab papain fragments was determined according to the method of Porter (19). The papain digested IgG was electrophoresed in the absence of 2-mercaptoethanol and electrophoretically transferred to nitrocellulose paper. As control for the specificity of each lectin, we used transferrin (Sigma), which has digalactosylated-biantennary complex sugar chains (20), and ovalbumin (Sigma), which has hybrid type sugar chains (21). In order to determine the proportion of IgG paraprotein to the residual polyclonal IgG, the ε/λ ratio of the purified IgG fraction was obtained after measuring the levels of each light chain by the single radial immunodiffusion assay (22). The ratio of the IgG paraprotein to polyclonal IgG exceeded 90% in all cases except for 4 cases of MGUS (more than 70%).

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MM, multiple myeloma; MGUS, monoclonal gammapathy of undetermined significance; RCA, Ricinus communis agglutinin; Con A, concanavalin A; HPLC, high performance liquid chromatography; Gal-T, galactosyltransferase; GnT III, N-acetylgalcosaminyltransferase III; MNC, mononuclear cells; Gal(0), agalactosylated biantennary sugar chains; RA, rheumatoid arthritis.
CARBOHYDRATE ANALYSIS OF IgG MYELOMA PROTEINS

Fig. 1. Lectin binding patterns of \( \gamma \) chain. Lane A, \( \gamma \) chain of normal control; Lane B, \( \gamma \) chain of MGUS; Lanes C and D, \( \gamma \) chains of multiple myeloma.

<table>
<thead>
<tr>
<th>RCA/Con A</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>A</td>
<td>1.85</td>
</tr>
<tr>
<td>B</td>
<td>2.85</td>
</tr>
<tr>
<td>C</td>
<td>0.74</td>
</tr>
<tr>
<td>D</td>
<td>0.15</td>
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Fig. 2. RCA/Con A ratio of the \( \gamma \) chain among MGUS, multiple myeloma, and control. Dotted area, mean \( \pm 2 \) SD of RCA/Con A ratios from normal controls. For statistical analysis, analysis-variance-techniques of nonparametric ranking were performed among the 5 groups \((P < 0.01)\). The RCA/Con A ratios were significantly lower in advanced MM (stages II and III) than in control \((P < 0.05, \) Dunnett’s test). \( \bigcirc \), treated; \( \Phi \), untreated.

HPLC Analysis. The IgG samples were dialyzed against distilled water and lyophilized. Each purified IgG sample was then subjected to hydrazinolysis as described previously (23). The free oligosaccharides were reductively aminated with the fluorescent reagent, 2-aminopyridine, and the pyridylamino derivatives of each oligosaccharide preparation were separated by gel filtration with Toyopearl HW-40F (Tosoh, Tokyo, Japan) (24). The separated pyridylamino derivatives were then digested with neuraminidase and then separated by HPLC using a Shimadzu LC-3A with a ODS-80TM column (4.6 x 150 mm) (Tosoh). Elution was performed at 55°C with a 0.02 M ammonium acetate buffer, pH 4.0, containing 0.04–1% butanol. The structure of each oligosaccharide resolved was determined with \( ^{1}H \) nuclear magnetic resonance. Details of the nuclear magnetic resonance study were reported by Fuji et al. (25).

Assay for Gal-T and GnT III of Myeloma Cells. The MNC of heparinized bone marrow enriched with myeloma cells was separated from heparinized bone marrow by centrifugation on a Ficoll-Hypaque gradient (26). The proportion of myeloma cells in the mononuclear cells was determined both morphologically by May-Grünwald staining and immunocytochemically by the indirect immunoperoxidase method (27). Only samples that contained more than 60% myeloma cells were subjected to enzyme assay. The contaminating cells in the samples consisted mainly of lymphocytes. The samples were stored in liquid nitrogen in the presence of 10% dimethyl sulfoxide (28). Prior to enzyme assay, the MNC were thawed, washed three times with Tris-buffered saline (0.9% NaCl-0.05 M Tris-HCl, pH 7.4), and sonicated. The supernatants were subjected to the Gal-T and GnT III assays as

Symbols: G, galactose; M, mannose; F, fucose; GN, N-acetylglucosamine
RESULTS

Lectin Binding Analysis. Control studies showed that RCA bound transferrin but not ovalbumin, while Con A bound both proteins. The binding of transferrin by RCA was not enhanced by treatment with neuraminidase. Thus, RCA recognized galactose at the nonreducing end of the biantennary-complex oligosaccharides irrespective of whether it was sialylated. Con A detected core mannosyl residues in the biantennary complex as saccharides irrespective of whether it was sialylated. Con A bound to the core mannosyl residues in the biantennary-complex oligosaccharides, while RCA bound to the nonreducing end of the biantennary-complex oligosaccharides.

The heavy and light chains of IgG were detected with specific antisera. Electrophoretically resolved heavy and light chain were separated into about 55 kDa and 25 kDa, respectively. RCA and Con A bound to the heavy chains of all immunoglobulins isolated including normal IgG but reacted with light chains only in three cases (one of MGUS and two of MM); in these instances, the light chains reacted strongly with RCA and weakly with Con A. In normal controls, the staining intensity to IgG heavy chains was similar for RCA and Con A. However, in 29 paraproteins, the staining patterns of the heavy chains varied as revealed by the data of Fig. 1. The RCA/Con A binding ratio was used to compare the lectin affinity for the heavy chain of individual isolates. The RCA/Con A ratios are presented in Fig. 2, together with clinical data. The RCA/Con A ratios were: 1.40 ± 0.26 in normal controls (SD); 1.47 ± 0.69 in MGUS; 1.74 ± 0.66 in stage I; 0.60 ± 0.27 in stage II; and 1.07 ± 0.70 in stage III. RCA bound to the γ chains more strongly than Con A in all cases of MGUS and stage I myeloma. In contrast, Con A bound the γ chains of advanced MM cases (stages II and III) more strongly in 6 of 7 cases in stage II and 5 of 10 cases in stage III. Reduced RCA binding affinity for the heavy chain, evidenced by a RCA/Con A below normal range, was encountered only in cases with advanced myeloma (stages II and III). The lectin binding patterns had no apparent relationship with other clinical parameters, such as the quantity of IgG paraprotein, the age of patients, and whether or not the patients had received chemotherapy.

HPLC Analysis. The IgGs from cases with various RCA/Con A ratios were subjected to HPLC analysis in order to elucidate whether the diverse lectin binding patterns reflected the degree of galactosylation.

The sugar chains of normal IgG were divided into three groups (Fig. 3): afucosylated, nonbisected biantennary complexes (Peaks A, B, C, and D); fucosylated, nonbisected biantennary complexes (Peaks E, F, G, and H); and fucosylated, bisected biantennary complexes (Peaks I, J, K, and L). The afucosylated, nonbisected complexes were the major fraction. Within this group, the digalactosylated sugar chain was the major component. Other studies have shown almost the same distribution in normal individuals (9).

HPLC analysis was performed on the IgGs of 4 cases of MGUS (2 of IgG1 and 2 of IgG2) and of 12 cases of MM (9 of IgG1, 2 of IgG2, and 1 of IgG4). Lectin analysis of the IgGs and of papain digestion had shown that 10 of these 16 cases had sugar chains in only the Fc region ("Fc" case) and that the other 6 cases possessed sugar chains in both the Fc and Fab regions ("Fab" case). Lectin blotting patterns of "Fab" and "Fc" cases were shown in Fig. 4. Examples of the HPLC profiles of the IgGs with various RCA/Con A ratios are presented in Fig. 5. All IgG isolates had the major 3 groups of oligosaccharides as observed in normal IgG, but the patterns differed. In the "Fc" cases with RCA/Con A ratios of 0.15 and 0.74, agalactosylated sugar chains were the most prominent component (Fig. 5, A and B). For "Fc" cases proteins with RCA/Con A ratios of 1.14 and 1.06, the HPLC profiles were similar to normal IgG (Fig. 5, C and D). All "Fab" case proteins (2 cases of MGUS and 4 cases of MM) had an increased amount of digalactosylated bisected biantennary complex (Fig. 5, E and F).

The level of Gal(0) sugar chains in each "Fc" case isolate was determined and correlated with different RCA/Con A ratios (Fig. 6). The RCA/Con A ratio showed an inverse correlation to the Gal(0) contents ($r = -0.87$, $P < 0.01$). There was no relationship between these contents and the IgG subclass.

Gal-T and GnT III Activities in Myeloma Cells. The enzymatic activities of myeloma cells and the oligosaccharide contents of the IgG of five patients glycosylated only in Fc region are shown in Table 1. The IgG of case 1 had the highest content of digalactosylated complex sugar chains. Cases 2, 3, and 4 had hypogalactosylated IgG [Gal(0)]. In case 5, in which IgG was also hypogalactosylated, the content of bisected oligosaccharides was highest. In case 1, nevertheless, the Gal-T activity was only 2.39 nmol/mg/h. In case 2, the Gal-T activity was the highest. Thus there appeared to be no simple correlation between Gal-T activity and the galactose content of oligosaccha-
Fig. 5. Examples of HPLC profiles of protein isolates with various RCA/Con A ratios. Cases A, B, C, and D were "Fc" cases isolates and cases E and F were "Fab" case samples (see text for details). In cases A and B (RCA/Con A = 0.15 and 0.74), the major component of oligosaccharides was the agalactosylated biantennary sugar chains (Peak E). In cases C and D (RCA/Con A = 1.14 and 1.06), the HPLC profiles were similar to the pattern of normal IgG. In cases E and F (the Fab fragments were also glycosylated; "Fab" case), the digalactosylated bisecting-type oligosaccharide was markedly increased (Peak L).

Fig. 6. Correlation between RCA/Con A ratio and the content of agalactosylated biantennary sugar chains [Gal(0)].

The IgG of many of the MM patients studied, particularly those from advanced clinical stages, had a reduced affinity for RCA lectin, compared with normal polyclonal IgG. On the other hand, the monoclonal IgG in the MGUS patients indi-
The alterations in the carbohydrate moiety of monoclonal IgG in MM have been interpreted as coming from an expansion of a clone equipped with a definitive set of glycosyltransferase (34). Based on this, the difference in the carbohydrate moiety of IgG between MGUS and advanced MM in our study may imply that MM clones have a glycosylation pattern profile different from that of MGUS or that the clone with hypogalactosylated IgG is liable to evolve into advanced MM. Alternatively, a reduction in Gal-T/Gnt III activity during the course of clinical progression might occur. The last possibility is as yet to be examined, because we were unable to analyze the enzyme activities in cases with a rapid progression from stage I to stage III. It is of interest that Ishibashi et al. (35) and Narasimhan et al. (36) reported activation of Gnt III during the clinical progression from liver cirrhosis to hepatocellular carcinoma. Our findings may reflect some intrinsic cytogenic difference between the plasma cells in MGUS and myeloma cells.

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