Glycosylation at the Fab Portion of Myeloma Immunoglobulin G and Increased Fucosylated Biantennary Sugar Chains: Structural Analysis by High-Performance Liquid Chromatography and Antibody-Lectin Enzyme Immunoassay Using Lens culinaris Agglutinin

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ABSTRACT

An antibody-lectin enzyme immunoassay technique which had been developed for the analysis of sugar chains of α-fetoprotein (N. Kinoshita et al., Clin. Chim. Acta, 179: 143–152, 1989) was used for analysis of sugar chains of myeloma immunoglobulin G (IgG). The IgG sugar chains of four of nine patients with myeloma were found to be highly reactive to Lens culinaris agglutinin as compared with those of six normal controls and 177 patients without myeloma. This reflected a high L. culinaris agglutinin/concanavalin A ratio. The IgGs of these patients were found to have highly siaiylated, fucosylated, and bisected biantennary sugar chains at Fab portions as judged by the lectin-blotting technique as well as by high-performance liquid chromatography analysis. These results indicate that some of the myeloma IgG proteins undergo unusual glycosylation processes.

INTRODUCTION

Human serum IgG has on average 2.8 N-glycoside-type sugar chains per protein molecule. Two of these are invariably located in the conserved N-glycosylation site of asparagine 297, and additional ones are found in the variable regions of the light and heavy chains (1–3). Heterogeneity in the N-linked sugar chains of human IgG has been reported (4–8). In our previous studies we found that the sugar chains of myeloma IgG undergo unusual glycosylation processes.

In this study we found that an antibody-lectin EIA using POD-LCA as a lectin can be used to characterize the sugar chains of myeloma IgG containing bisected biantennary chains with fucose on the Fab portion. The IgG sugar chain structures were confirmed by HPLC analysis of pyridylaminated sugars.

MATERIALS AND METHODS

Materials. The rabbit anti-human IgG antibody, F(ab')2, fraction was obtained from DAKO Japan Co., Ltd., Kyoto, Japan. Five kinds of "POD-conjugated lectins," such as POD-EPHA, POD-Con A, POD-LCA, POD-RCA, and POD-WGA, were kindly supplied by Honen Oil Co., Tokyo, Japan. Neuraminidase (Arthrobacter ureafaciens) was a kind gift from Dr. Y. Ohta. The microtiter plate (Nunc-certified 96-well microtiter plate) was obtained from Intermed. All other reagents used in the study were commercially available and of special grade.

Samples. Serum samples were obtained from 9 patients with IgG myeloma, 177 hospitalized patients without IgG myeloma, and 6 healthy adult volunteers. Sera were stored at −20°C until used. Diagnosis and clinical staging of the patients with IgG myeloma were performed according to the method of Durie (13). The patients consisted of 2 with monoclonal gammopathy of undetermined significance, 2 with Stage I multiple myeloma, one with Stage II multiple myeloma, and 4 with Stage III multiple myeloma as shown in Table 1. Of the above patients, 7 patients were untreated, and 2 patients received chemotherapy with an alkylating agent and prednisolone.

Antibody-Lectin EIA. This was essentially carried out by the method described previously (12), except that the antibody used was anti-human IgG F(ab')2, and the periodate treatment was not carried out. In brief, 100 μl of the anti-human IgG F(ab')2, 0.1 mg/ml with 50 mmol/liter of NaHCO3 (pH 9.6) were added to each well of a microtiter plate. After overnight incubation at room temperature, each well was washed 3 times with PBS containing 0.05% Tween-20. After washing, 100 μl of serum diluted 1000 times with 50 mmol/liter of PBS were added, and then the antigen-antibody reaction was performed at 37°C for 3 h.

Again after washing 3 times, 100 μl of a POD-conjugated lectin that had been diluted 1:1000 with 10 mmol/liter of Tris-HCl buffer (pH 8.0) were added, followed by incubation at 37°C for 3 h, during which time the antibody-antigen complex became sandwiched with the POD-conjugated lectin. Finally, after washing 6 times, 100 μl of 50 mmol/liter of citrate buffer (pH 5.0) containing 0.003 % H2O2 and 0.6 mg/ml of o-phenylenediamine were added, and the enzyme reaction was initiated. When sufficient color was exhibited, the reaction was stopped with 50 μl of 4 N H2SO4. The color intensity was determined by reading the absorbance at 490 nm with a micotiter reader (Intermed).

The lectin activity ratios such as WGA/RCA, RCA/Con A, EPHA/Con A, and LCA/Con A were calculated as described previously (12).

Purification of Serum IgG. Serum IgG was purified by precipitation first with 18% sodium sulfate and second with 14% sodium sulfate, followed by chromatography on DEAE-cellulose (Whatman DE-52). The purity of the IgG was confirmed by SDS-polyacrylamide gel electrophoresis. The IgG samples were dialyzed against distilled water and lyophilized. For HPLC analysis, the purified IgG was then subjected to hydrazinolysis as described previously (9, 14, 15).

For lectin blotting, serum IgG was purified using DEAE-Affigel blue (Bio-Rad) chromatography as described (16).

Fab and Fc fragments of a patient with myeloma were obtained as described (10).
HPLC Analysis of Pyridylaminated Sugar Chains. The released sugar chains were reductively aminated with a fluorescent reagent, 2-amino-pyridine, by use of sodium cyanoborohydride, and the mixture of pyridylamino derivatives of each oligosaccharide was purified by gel filtration on a column of Toyopearl HW-40F (9, 14, 15). The purified pyridylamino derivatives were digested with neuraminidase. Pyridylation of intact (nondesialylated) IgG and of Fc and Fab fragments was carried out as described (17). Separation and identification of the desialylated pyridylamino derivatives were carried out by HPLC using a column (4.6 x 150 mm) packed with ODS-80 TM (Tosoh). Elution was performed at 55°C with 0.02 M ammonium acetate buffer (pH 4.0) containing 0.04% to 1.0% butanol. The structure of each oligosaccharide eluted was determined by 'H NMR. Details of the NMR study were reported previously (9, 15).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Lectin Blotting. In order to determine the localization of the sugar chains on the IgG, we conducted SDS-polyacrylamide gel electrophoresis and the lectin blotting.

Purified IgGs were electrophoresed in the absence of 2-mercaptoethanol after papain digestion according to the method of Porter (18). The digested IgGs were followed by transferring the protein onto nitrocellulose paper (19). The transferred protein was stained by the avidin-biotin complex method using biotinylated RCA lectin as described (10).

RESULTS

Antibody-Lectin EIA. Antibody-lectin EIA was performed to measure the reactivity of the sugar chains of IgGs from 9 patients with myeloma, 6 normal healthy controls, and 177 patients without myeloma. As shown in Table 1, the reactivities to LCA of four myeloma patients (Patients 1, 5, 6, and 7) were markedly different from those of the 6 normal healthy controls and the 177 patients without myeloma, and the reactivities to EPHA and RCA in the same patients were slightly high as compared with those of the 6 normal controls. Furthermore, the reactivities to WGA of the same patients were slightly low as compared with those of other normal patients and normal healthy controls. On the other hand, the reactivity toward Con A was very similar among all the subjects studied. The reactivities of IgG from the other five myeloma patients toward the five lectins were similar to those of normal healthy controls. The high reactivities of the IgG sugar chains of myeloma patients 1, 5, 6, and 7 to LCA lectin were reflected in the LCA/Con A ratio. A decrease in the RCA/Con A ratio, which was previously found by our group to reflect hypogalactosylation in the sugar chains of γ-chain at advanced stages of myeloma (10), was not prominent in this study, because lectin-antibody EIA was carried out using whole IgG sugar chains. However, Patients 2, 3, 4, 8, and 9 gave relatively lower values as compared with those of normal controls, which might reflect hypogalactosylation in the γ-chains of these patients. These results suggest that the IgG from some patients with myeloma has different sugar chains than does IgG from normal subjects.

Typical dilution curves for the IgG antibody-LCA lectin EIA for serum IgG were shown in Fig. 1.

Localization of IgG Sugar Chains by SDS-Polyacrylamide Gel Electrophoresis and RCA Lectin Binding. The IgGs of 4 of 9 myeloma patients (Patients 1, 5, 6, and 7 in Table 1) were found to stain at a molecular weight of approximately 45,000, which is equivalent to a dimer of the IgG Fab portion, in addition to a band corresponding to the Fc portion (M, approximately 50,000) as shown in Fig. 2. On the other hand, the IgGs of 5 of 9 myeloma patients (Patients 2, 3, 4, 8, and 9) and normal healthy controls were found to stain at only the Fc portion. This indicates that the sugar chains of the above 4 IgGs react to RCA strongly and are located on the Fab portion in addition to the Fc portion.

Characterization of Sugar Chains of Normal and Myeloma IgGs by HPLC of Pyridylaminated Sugar Derivatives. In order to characterize the differences in IgG sugar chains between normal and myeloma IgGs, we carried out HPLC analysis of the pyridylamino derivatives of the IgG sugar chains from the nine patients with myeloma and the 6 normal healthy controls.

Table 1 Lectin reactivities of IgGs from myeloma patients, normal control, and patients without myeloma

<table>
<thead>
<tr>
<th>Serum samples, diagnosis, treatment</th>
<th>Lectin-POD activities</th>
<th>Lectin reactivity ratios</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EPHA</td>
<td>WGA</td>
</tr>
<tr>
<td>Myeloma patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1° MGUS (†)</td>
<td>0.046</td>
<td>0.066</td>
</tr>
<tr>
<td>2 Stage III (+)</td>
<td>0.021</td>
<td>0.175</td>
</tr>
<tr>
<td>3 Stage III (+)</td>
<td>0.025</td>
<td>0.183</td>
</tr>
<tr>
<td>4 Stage III (+)</td>
<td>0.024</td>
<td>0.157</td>
</tr>
<tr>
<td>5° Stage I (†)</td>
<td>0.058</td>
<td>0.058</td>
</tr>
<tr>
<td>6° Stage II (†)</td>
<td>0.046</td>
<td>0.030</td>
</tr>
<tr>
<td>7° Stage I (†)</td>
<td>0.065</td>
<td>0.097</td>
</tr>
<tr>
<td>8 Stage I (†)</td>
<td>0.025</td>
<td>0.125</td>
</tr>
<tr>
<td>9 MGUS (†)</td>
<td>0.024</td>
<td>0.156</td>
</tr>
<tr>
<td>Normal controls (n = 6)</td>
<td>0.038 ± 0.005</td>
<td>0.124 ± 0.025</td>
</tr>
<tr>
<td>Patients without myeloma (n = 177)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Patients whose IgG sugar chains are located in their Fab portion, as judged by lectin blotting as shown in Fig. 3.
* MGUS, monoclonal gammopathy of undetermined significance; ND, not determined; NC, not calculated.
* Mean ± SD.
SUGAR CHAINS OF MYELOMA IgG AND ANTIBODY-LECTIN EIA

the same as those of the whole nondesialylated IgG from the normal control (Fig. 4, A and B). On the other hand, sugar chains of the Fab portion of a patient with myeloma were markedly different from those of the control IgG (Fig. 4C). All major sugar chains were found to be fucosylated. This indicates that the high LCA/Con A ratio observed in this study was reflected by the high content of the fucosylated, biantennary sugar chains at the Fab portion in the myeloma patient.

DISCUSSION

It is known that roughly 30% of IgG myeloma proteins is glycosylated at Fab portions (20, 21).

In this study, we found that some of the IgG myeloma proteins are highly reactive to LCA as judged by antibody-lectin EIA. This finding has been supported by the HPLC data indicating that the major sugar chain components of the IgG are fucosylated, bisected and fucosylated, nonbisected, biantennary sugar chains. Kornfeld et al. (2) reported that the presence of an α-fucosyl residue attached to position C-6 of an asparagine-linked N-acetylglucosamine residue (innermost asparagine-linked N-acetylglucosamine residue) and two α-mannosyl residues either free or substituted only at position C-2 are essential for sugar chains to be strongly bound to LCA. Therefore, the high reactivity of myeloma IgG to LCA observed in normal controls and myeloma patients, pyridylaminated sugar chains from the IgGs were analyzed by HPLC. As shown in Fig. 3, in healthy controls, the sugar chains are classified into three groups: afucosylated, nonbisected, biantennary complexes (Components A to D); fucosylated, nonbisected, biantennary complexes (Components E to H); and fucosylated, bisected, biantennary complexes (Components I to L). Expected reactivities of lectins were shown in the lower panel of Fig. 3.

Among the three groups of sugar chains from normal IgG, fucosylated, nonbisected complexes (E + F + G + H) were the main component. Within this group, the digalactosylated sugar chain (Component H) was the major component.

As described above, a markedly different pattern was seen for IgGs from the four myeloma patients whose sugar chains were found to be located in the Fc and Fab portions as judged by the lectin-blotting technique (Fig. 2). In a separate experiment, we found that all 4 myeloma IgGs have sugar chains on their light chains (data not shown). The major components of the IgGs from these patients were found to be fucosylated, bisected (I + J + K + L) and fucosylated, nonbisected biantennary complexes (E + F + G + H) as shown in Table 2. Among them the major component was found to be a fucosylated, bisected, biantennary chain, Component L. These structural studies are consistent with the finding that the IgGs from these myeloma patients are highly reactive to LCA in the antibody-lectin EIA.

Characterization of Nondesialylated Intact Sugar Chains of Fab, Fc, and Whole IgG in Normal Control and a Patient with Myeloma. Fig. 4 indicates HPLC patterns of nondesialylated sugar chains of whole IgG and Fc and Fab of a patient with myeloma, whose LCA/Con A ratio was extremely high. Most of the sugar chains in the Fc portion of the patient were almost the same as those of the whole nondesialylated IgG from the normal control (Fig. 4, A and B). On the other hand, sugar chains of the Fab portion of a patient with myeloma were markedly different from those of the control IgG (Fig. 4C). All major sugar chains were found to be fucosylated. This indicates that the high LCA/Con A ratio observed in this study was reflected by the high content of the fucosylated, biantennary sugar chains at the Fab portion in the myeloma patient.

Fig. 2. Typical sugar chain localizations for serum IgG from a normal control (Lane a), a myeloma patient whose IgG sugar chains are located on the Fab portion (Patient 1 in Table 1 and Lane b), and a myeloma patient whose IgG chains are not located on the Fab portion (Patient 2, Lane c).

Fig. 3. A typical sugar chain structure of serum IgG from a normal control. The sugar chains of normal IgG are classified into 3 major groups. The sugar chains in each group are further separated into 4 subgroups according to the extent of galactosylation. Agalactosylated, biantennary sugar chains (Components A + E + J); biantennary sugar chains monogalactosylated at the Mano1-6 branch (Components B + F + J); biantennary sugar chains monogalactosylated at the Mano1-4 branch (Components C + G + K); digalactosylated, biantennary sugar chains (Components D + H + L). Expected reactivities to each lectin are shown at the bottom.

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the present study could be well explained by the presence of fucosylated, bisected and fucosylated, nonbisected sugar chains in the Fab portion of the IgG.

In this study we also found that serum IgG of myeloma patients whose IgG sugar chains are located on the Fab portion also strongly reacted to RCA. RCA binds fucosylated or nonfucosylated, biantennary sugar chains which have galactose at the nonreducing end. This again is consistent with the data that, in the myeloma IgG, a high amount of fucosylated, biantennary sugar chains which have galactose at the nonreducing end appears (Components J, K, and L in Fig. 3).

In order to characterize sugar chains of Fab in patients with myeloma whose sugars were a high LCA/Con A ratio, nondesialylated sugar chains of these IgGs were separated into Fab and Fc portions. The data suggested that the Fab portions containing most of the sugar chains were fucosylated. In addition, a complete biantennary sugar chain was found (see Fig. 4, Component p).

The N-linked sugar chains of the Fab moiety of IgG tend to influence solubility/aggregation phenomena (22). The cryoglobulin and cold agglutinin properties of certain monoclonal IgG and IgM molecules have been shown to arise from sialylated...
**REFERENCES**

Glycosylation at the Fab Portion of Myeloma Immunoglobulin G and Increased Fucosylated Biantennary Sugar Chains: Structural Analysis by High-Performance Liquid Chromatography and Antibody-Lectin Enzyme Immunoassay Using \textit{Lens culinaris} Agglutinin

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