**N-Glycan Structures Derived from Monoclonal Immunoglobulin G Cryoglobulins**

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**Summary**  
N-Glycan structures of Type I cryoglobulin in 3 different samples are reported in detail. The three sample cryoglobulins were purified from sera of monoclonal immunoglobulin G (IgG) 1-κ type cryoglobulinemia: two of them from patients with multiple myeloma (cases 1 and 2) and one from a patient with Sjögren’s syndrome (case 3). Asparagine-linked glycan portions of these cryoglobulins were released by digestion with glycoamidase A (from sweet almond), and the reducing ends of the N-glycans were reductively aminated with 2-aminopyridine. The derivatized N-glycans were separated and structurally identified by a multi-dimensional mapping technique on high performance liquid chromatography (HPLC) columns. The HPLC profiles of N-glycans from these patients were compared with that profile from healthy persons. The N-glycan molecules from the 3 different IgG proteins revealed individually abnormal profiles. A glycoform containing 2 galactose residues predominated in case 1. In contrast, no galactose was found in the predominant glycoforms in cases 2 and 3. Moreover, an unusually high content of bisecting N-acetylgalcosamine characterized one of the predominant glycoforms in case 2. In spite of these striking N-glycan abnormalities, no clear mechanism that could cause cryoglobulinemia was found.

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Cryoglobulins show a so-called thermal reaction behavior. At low temperature (4°C), they form a turbid precipitate or change to a gel state and re-dissolve upon warming (37°C) [1-3]. Cryoglobulins are divided into three types depending on their component proteins. The first type consists solely of monoclonal immunoglobulins. The second is a mixture of monoclonal and polyclonal immunoglobulins [4], and the third is a mixture of two or more polyclonal immunoglobulins. Many studies describing the effect of pH, salt concentration, different buffers, etc. have attempted unsuccessfully to find the cause of cryoglobulinemia [5, 6]. Moreover, the relationship between cryoglobulinemia and complement activation at low temperatures or hepatitis C virus has been pointed out [7, 8]. The mechanism of cryoglobulin formation, however, is still unknown.

Recently, we obtained three different serum samples from patients with cryoglobulinemia. In this paper, we used a high-performance liquid chromatography (HPLC) method that gives a sugar map representing the distribution of N-glycans in each sample immunoglobulin G (IgG) [9, 10]. The profiles obtained by HPLC make it possible to evaluate the amount of each N-glycan that is contained in normal and pathological IgG molecule. We show here how differences in the structure of each of the N-glycans are clearly reflected in the HPLC profile. We also examined the cause-and-effect relationship between N-glycan structures in the IgG protein of cryoglobulins and the characteristic thermal property of cryoglobulin formation.

MATERIALS AND METHODS

Specimens. Cases 1 and 2 were diagnosed as multiple myeloma and case 3, as Sjögren's syndrome. The age of each patient was 70, 78, and 82 years, respectively. In cases 1 and 2, although turbid precipitates were formed in the original sera at 4°C, the precipitates unusually dissolved when they were washed with physiological saline. In case 3, the precipitate usually did not dissolve by when wash with physiological saline.


Purification of cryoglobulins. Since the cryoprecipitates from cases 1 and 2 were dissolved in physiological saline at 4°C, 3% polyethylene glycol/water was used to preserve the precipitates, instead of the physiological saline. As for case 3, the cryoprecipitate was able to be washed with physiological saline under conven-
Fig. 1. Characterization of cryoglobulins. a, Profiles of sera by cellulose acetate membrane electrophoresis. b, Profiles of sera obtained by agar gel immunoelectrophoresis. Arrows in 1a, 2a, 3a, 1b, 2b, and 3b indicate M-protein positions. c, Profiles of purified cryoglobulins obtained by agar gel immunoelectrophoresis. d, Profiles of sera by the Ouchterlony method. e, Profiles of purified cryoglobulins by the Ouchterlony method. Cases 1 and 2, multiple myeloma; case 3, Sjögren's syndrome.
Typing of purified cryoglobulins. The purified cryoglobulins were typed by both the agar gel immunoelectrophoresis and the Ouchterlony double diffusion methods [14]. IgG subclass determination of the purified cryoglobulins was

performed by Ouchterlony double diffusion using anti-IgG 1, 2, 3, and 4 sera. These specific antisera were purchased from Binding Site Products Inc. (Birmingham, England).

**Molecular mass determination.** Purified cryoglobulins were treated with an equal amount of 2-mercaptoethanol at 37°C for 1 h and subsequently subjected to a 12% polyacrylamide gel electrophoresis (PAGE; size, 10 X 10 X 0.1 cm³). The molecular mass of the γ-chains was determined by immunoblotting [15]. A HMW SDS Calibration Kit was purchased from Pharmacia Co. (Lund, Sweden).

**N-Glycan analyses of IgG.** Neutral and sialyl N-glycan analyses of IgG were performed by HPLC using the multi-dimensional sugar mapping technique [9, 10, 16]. Briefly, the N-glycan moieties were released from purified sample IgG by enzymatic cleavage with glycoamidase A (EC 3.5.1.52), (Seikagaku Kogyo, Tokyo, Japan). The reducing ends of the N-glycans were then reductively aminated with 2-aminopyridine by use of sodium cyanoborohydride [17]. The mixture of

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**Fig. 2.** HPLC profiles of PA-N-glycans of IgG from healthy and cryoglobulinemia individuals. N-Glycan fractions released by glycoamidase A digestion of IgG were pyridylaminated and subjected to HPLC analysis as described in the text. 1, 2, and 3 correspond to cases 1, 2 and 3. Symbols: G, galactose; M, mannose; F, fucose; GN, N-acetylglucosamine. Peak P, for example, represents.

Neutral N-glycans

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Mono-sialyl N-glycans

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pyridylamino (PA) derivatives of the \( N \)-glycans was separated by HPLC on a TSK gel DEAE-5PW column (Tosoh, Tokyo, Japan) according to its sialic acid content. Then, neutral and mono-sialyl \( N \)-glycans were individually separated and analyzed sequentially on a Shim-pack HRC-octadecyl silica (ODS) column (Shimadzu, Kyoto, Japan) and a TSK-gel Amide-80 column (Tosoh).

**RESULTS AND DISCUSSION**

*Characterization of serum proteins*

The total protein concentration in case 1 serum was 7.0 g/dl, in which the ratios of the serum protein fractions separated by cellulose acetate membrane electrophoresis were as follows: albumin, 54.4%; \( \alpha-1 \), 3.6%; \( \alpha-2 \), 15.0%; \( \beta \), 7.3%; and \( \gamma \), 19.7% (Fig. 1-1a). The corresponding values for case 2 serum were 13.8 g/dl, 19.5%, 1.3%, 4.6%, 3.8%, and 66.1% (Fig. 1-2a), and those for case 3 serum 8.6 g/dl, 39.9%, 2.0%, 10.9%, 9.6%, and 37.6% (Fig. 1-3a). The immunoglobulin concentrations determined by the nephelometric immunoassay method in case 1 serum were IgG, 1,490; IgA, 93; and IgM, 56 mg/dl. In case 2 serum these concentrations were IgG, 11,300; IgA, 134; and IgM, 85 mg/dl, and in case 3 serum, IgG, 3,190; IgA, 333; and IgM, 108 mg/dl. Concentrations of IgA and IgM in cases 1 and 2 were lower, and the concentration of IgG paraprotein was higher than those of healthy age-matched sera (IgG, 800–1,200; IgA 150–250; and IgM 90–150 mg/dl). Such a profile is characteristic of multiple myeloma.

In cases 1, 2, and 3, M-protein (myeloma protein) at the \( \gamma \)-position was observed by agar gel immunoelectrophoresis (Fig. 1-1b, -2b, and -3b). Purified cryoglobulins were also shown by using the same agar gel immunoelectrophoresis (Fig. 1-1c, -2c, and -3c). In the serum protein fractionation of cases 1 and 2, the migration positions of IgG M-proteins and the purified cryoglobulins on the cellulose acetate membrane did not conflict with those on the agar gel electrophoresis. In case 3, although the M-protein was found at the slow \( \gamma \)-position on the cellulose acetate membrane (Fig. 1-3a), and the M-protein was identified as IgG-\( \kappa \) (Fig. 1-3d), both the M-protein and the purified cryoglobulin stayed at the origin and lacked any protein at the \( \gamma \)-position on the agar gel immunoelectrophoresis. These results suggest that the IgG molecule in case 3 interacted with the agar gel and made a sediment (Fig. 1-3b and -3c) [18].

In cases 1, 2, and 3, the purified cryoglobulins were shown to be single Type I protein, each one consisting solely of a single IgG-\( \kappa \) molecule (Fig. 1-1d, -2d, and -3d). These three cryoglobulins were determined to be of the monoclonal IgG-1 subclass type by using the Ouchterlony method (Fig. 1-1e, -2e, and -3e). Each \( \gamma \)-chain was estimated to have a molecular mass of 50,000 Da by immunoblotting (normal range 50,000–60,000 Da).

*Structural analysis of \( N \)-glycans of purified cryoglobulins*

Figure 2 shows HPLC profiles of the \( N \)-glycans obtained from polyclonal IgG

of healthy persons (in Fig. 2, control) and from the cryoglobulins of cases 1, 2, and 3 (in Fig. 2, 1, 2, and 3, respectively). The neutral N-glycan profile of control IgG (top, left) showed 4 main peaks (E, F, G, and H), whereas in case 1, only peak H (a glycoform with two galactose residues) was found to be predominant. Peaks e and f (the second row, right) were the main components in mono-sialyl N-glycans of case 1 and were also introduced from glycoform H. In contrast, in case 2, the content of glycoform H showed a remarkable decrease, and 2 other glycoforms (E and M, both having no galactose) showed a marked increase. Furthermore, the abnormally predominant peak M also had a bisecting N-acetylglucosamine residue. In case 3, only glycoforms E and Q (bottom, left) showed a large increase. In contrast, glycoforms H and e (mono-sialyl H) in case 3 were negligible. Glycoform Q was a truncated mono-antennary N-glycan without any galactose residue and was almost absent in healthy polyclonal IgG. The characteristic molar ratio of N-glycans in the 3 cases is summarized in Table 1.

Since patients with the type of cryoglobulins chosen for the present experiments (Type I consisting solely of monoclonal IgG) are rare, only 3 samples could be obtained. Changes in the profiles of pathological IgG N-glycans have been discussed so far mostly on the basis of the decrease of the galactose contents.

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Especially in rheumatoid arthritis, IgG N-glycans without galactose residue (peak E) were found to increase [19–21]. In Sjögren’s syndrome (case 3) which is an autoimmune disease like rheumatoid arthritis, N-glycans without galactose residue (peaks E and Q) were also predominant. In contrast, N-glycan structures of IgG from multiple myeloma patients have been reported not to show a similar tendency, while structural changes appear to be different in each case [9, 10]. An abnormal increase of peak H (two-galactose containing N-glycan) in case 1, or peak M (bisecting N-acetylglucosamine containing N-glycan) in case 2 were observed also in cryoglobulins accompanying multiple myeloma. The abnormal increase of galactose-rich N-glycans in pathological IgG proteins has very rarely been reported [10].

In this study, each of the 3 IgG N-glycans in cryoglobulinemia showed a uniquely abnormal profile, and there was no common tendency among their structural changes. The abnormality of N-glycan structures may reflect the nature of the original diseases accompanied by cryoglobulinemia. These results suggest that there is no direct relationship between cryoglobulinemia and the M-protein N-glycan structures.

REFERENCES


