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A Critical Role for Sialylation in Cryoglobulin Activity of Murine IgG3 Monoclonal Antibodies[1]

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Cryoprecipitating IgG3 autoantibodies have been shown to play a significant role in the development of murine lupus-like autoimmune syndrome. However, the structural basis of IgG3 cryoprecipitation still remains to be defined. In view of the implication of positively charged amino acid residues present in variable regions in IgG3 cryoglobulin activity, we explored the role of terminal sialic acids in oligosaccharide side chains for the cryogenic activity of IgG3 mAb. Comparative oligosaccharide structural analysis of different cryogenic and non-cryogenic IgG3 mAb showed an inverse correlation between the extent of sialylation and cryogenic activity. The inhibitory role of sialylation was further confirmed by the demonstration of enrichment of less and more sialylated IgG3 in cryoprecipitated and noncryoprecipitated fractions, respectively, separated from four different cryogenic IgG3 mAb. Significantly, the sialic acid contents of the latter fraction became comparable to those of non-cryogenic IgG3 mAb. Finally, we observed that highly sialylated non-cryogenic IgG3 mAb was more potent in the inhibition of cryoprecipitation of cryogenic IgG3 mAb. Our results thus suggest that the content of negatively charged sialic acids in oligosaccharide side chains is one of the critical factors to determine IgG3 cryoglobulin activity, along with amino acid sequences of the IgG3 variable regions. ***The Journal of Immunology,*** **2005, 175: 1056–1061.**

Cryoglobulins are a heterogeneous group of Ig that precipitates upon cooling and redissolves on warming. Most cryoglobulins are either intact monoclonal Ig or Ig complexes in which one component, usually IgM, has rheumatoid factor activity (1, 2). The presence of cryoglobulins can result in a wide range of vascular, renal, and neurological complications, probably depending on their concentration, their temperature-dependent solubility behavior, and the nature and type of proteins involved (2). In humans and mice, Abs of the IgG3 isotype have a unique physicochemical property which allows them to self-associate via Fc-Fc interactions, independently of their specificities (3–5). This property is necessary to confer cryoglobulin activity, but not sufficient in itself, since not all monoclonal IgG3 proteins in humans (6–8) and mice (5, 9, 10) exhibit cryoglobulin activity. Significantly, a fraction of cryoprecipitating IgG3 monoclonal autoantibodies derived from lupus-prone mice are highly pathogenic, generating glomerular and vascular lesions (10–14).

The molecular basis responsible for cryoglobulin formation by IgG3 molecules is still unclear. Nucleotide sequence analysis of the variable regions of a panel of cryogenic and non-cryogenic IgG3 mAb, in combination with the assessment of mutant Abs, revealed that cryoglobulin activity of IgG3 is associated with the presence of more positively charged amino acid residues at positions 6 and 23 of the H chain variable (V_H) domain (15, 16). The implication of positive charges in IgG3 cryoprecipitation was also supported by the finding that cryoglobulin activity of IgG3 anti-DNP mAb was inhibited or enhanced following the binding of negatively or positively charged DNP-labeled amino acid conjugates, respectively (9).

The CH2 domain of each H chain of IgG bears an asparagine-linked, biantennary complex-type oligosaccharide chain, which is essential for IgG Fc-dependent effector functions, i.e., activation of complement and Fc receptor-bearing effector cells (17, 18). Most of these oligosaccharide side chains end with either two galactose residues, one galactose and one *N*-acetylglucosamine, or two *N*-acetylglucosamines (19, 20). However, a significant, although minor, fraction of galactosylated oligosaccharide chains bear one or two terminal sialic acids and thus become more negatively charged.

Using transgenic mice expressing 6-19 IgG3 heavy chains, we have recently established several 6-19 IgG3 mAb that are identical in the amino acid sequence of their H and L chains, but hardly cryogenic, as compared with the original 6-19 mAb (16). Since these noncryogenic 6-19 IgG3 variants are much more galactosylated, their poor cryogenicity may in part be related to an increased terminal sialylation, which provides more negative charges. In the present study, to explore the role of sialylation in IgG3 cryoprecipitation, we first determined the levels of sialylation and galactosylation of different cryogenic and non-cryogenic IgG3 mAb. Then the contents of sialylated and galactosylated oligosaccharide side chains were compared after separation of cryoprecipitates and supernatants following incubation of three different cryogenic IgG3 mAb at 4°C. Finally, the potential of non-cryogenic IgG3 mAb to inhibit the 6-19 IgG3 cryoprecipitation was assessed in relation to the levels of sialylation and galactosylation of three different non-cryogenic IgG3 mAb. Our results indicated that the content of negatively charged sialic acid, but not of galactose per se, in oligosaccharide side chains is a critical factor in determining the cryoglobulin activity of IgG3 mAb.

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[3] Abbreviation used in this paper: ABEE, *p*-aminobenzoic acid ethyl ester.

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Materials and Methods

Monoclonal Abs

6-19, 2-6D, and 9-106 IgG3 mAb, derived from autoimmune-prone MRL-*Fas*^{lpr} mice, were described previously (21, 22). A6C, P11C, and Y12C IgG3 mAb bearing H and L chains identical to those of the 6-19 mAb were established from mice expressing a transgene encoding the 6-19 IgG3 H chain (16). C11M, CB1, and J606 IgG3 mAb and a hybrid IgG3 mAb with 6-19 H chains and 9A6 L chains (6-19H/9A6L) were described previously (4, 9, 23). All hybridoma cells were grown in conventional stationary culture flasks, and supernatants were collected from confluent cultures. IgG3 mAb were purified from culture supernatants by protein A affinity column chromatography. The purity was >95% as documented by SDS-PAGE.

Analysis of oligosaccharide structures

The analysis of the structure of carbohydrate side chains present in the IgG3 samples was conducted as previously described (24). The samples were subjected to gas-phase hydrazinolysis for 3 h at 90°C using Hydraclub S204 (Honen) followed by *N*-acetylation to quantitatively liberate *N*-linked oligosaccharides (25). The oligosaccharides liberated from the IgG3 samples were purified by cellulose column chromatography (26) and then labeled with *p*-aminobenzoic acid ethyl ester (ABEE; Nacalai Tesque) by reductive amination (27). The ABEE-labeled oligosaccharides were subjected to HPLC using a COSMOGEL DEAE column (Nacalai Tesque) and separated into neutral (N), acidic monosialylated (A1), and acidic disialylated (A2) oligosaccharide fractions. Neutral oligosaccharide mixtures obtained by treatment of ABEE-oligosaccharide fraction with *Arthrobacter ureafaciens* sialidase (Nacalai Tesque) were applied to HPLC using a Wakosil 5C18-200 octadecylsilane column (Wako Pure Chemical). Desialylated ABEE-oligosaccharides were separated into eight oligosaccharide fractions which were eluted as a series of authentic biantennary complex-type oligosaccharides with or without fucosylation. Representative elution profiles by HPLC of total oligosaccharides and desialylated neutral oligosaccharides from 6-19 IgG3 mAb are shown in Fig. 1.

Cryoglobulin activity

One milliliter of different concentrations of purified IgG3 mAb in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4; ionic strength, 0.2, pH 7.4) was placed in conical glass tubes at 4°C for 24 h. The samples were centrifuged at 3000 rpm at 4°C for 30 min. The amounts of cryoprecipitates were estimated by measuring A_{280} of supernatants, and results are expressed as percentages of cryoprecipitation. In some experiments, 0.1 ml of 2 mg/ml 6-19 mAb was incubated with 0.1 ml of various concentrations of non-cryogenic IgG3 mAb or PBS at 4°C for 24 h. The precipitates were washed five times with cold PBS and resolubilized in 4 M urea. The concentrations of IgG3 in cryoprecipitates were determined by ELISA as described elsewhere (12). Results are expressed as percent inhibition of the 6-19 cryoprecipitation.

IgG3 self-association assay

The ability of different non-cryogenic IgG3 mAb to form IgG3-IgG3 complexes with 6-19 mAb was determined by a radioimmunoassay as described previously (10). Briefly, various amounts of mAb in PBS were incubated with ^{125}I-labeled 6-19 mAb (10 ng) in PBS at 4°C overnight in the presence of 10 μl of normal mouse serum. After 1 h of incubation at 4°C with polyethylene glycol (M_r 6000; Hänseler) at a final concentration of 7.5%, IgG3 complexes formed with radiolabeled 6-19 mAb were precipitated by centrifugation at 3000 rpm at 4°C for 30 min. The precipitates were washed once with 7.5% polyethylene glycol. Results are expressed as the percentage of labeled 6-19 mAb precipitated specifically after correction for the nonspecific precipitation (<10%) obtained in the presence of normal mouse serum alone.

Statistical analysis

Statistical analysis was performed with the Wilcoxon two-sample test. Probability values >5% were considered to be insignificant.

Results

Correlation of the cryoglobulin activity of murine IgG3 mAb with levels of sialylation, but not galactosylation

Our recent studies showed that IgG3 6-19 variants obtained from 6-19 H chain transgenic mice displayed barely detectable cryoglobulin activities associated with increased levels of galactosylation in the oligosaccharide side chains, as compared with highly

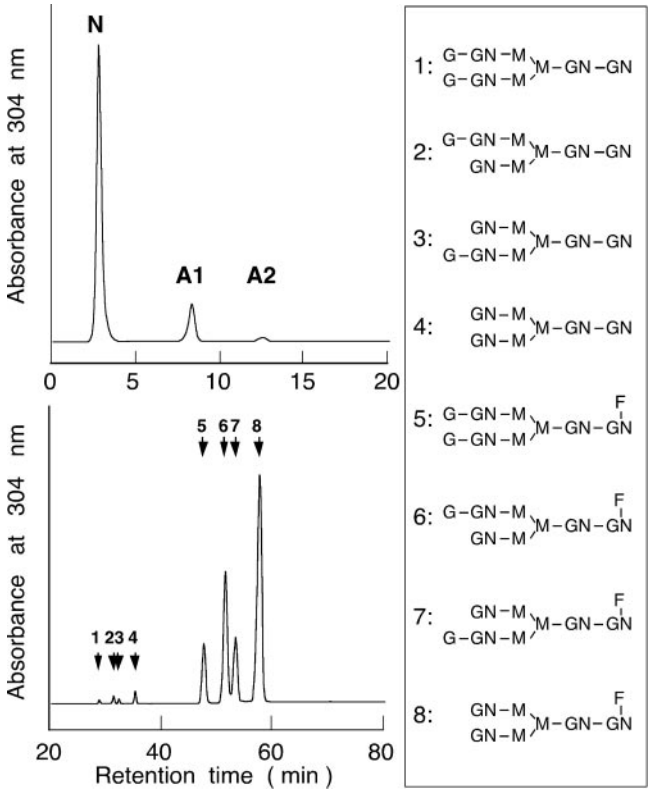

FIGURE 1. Representative elution profiles by HPLC of ABEE-oligosaccharides from 6-19 IgG3 mAb. ABEE-oligosaccharides obtained from 6-19 IgG3 mAb were separated by DEAE-HPLC into nuetral (N), acidic monosialylated (A1), and acidic disialylated (A2) fractions (*upper panel* of the *left column*). Desialylated ABEE-oligosaccharides of 6-19 IgG3 mAb were separated by octadecylsilane-HPLC into eight oligosaccharide fractions (*lower panel* of the *left column*). The structures of these authentic biantennary complex-type oligosaccharide structures (positions 1–8) of ±Galβ1–4GlcNAcβ1–2Manα1–6(±Galβ1–4GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4(±Fucα1–6)GlcNAc are shown in the *right column*. The peaks 1 and 5 correspond to digalactosylated (G2) oligosaccharide chains without or with a fucose residue, the peaks 2, 3, 6, and 7 to monogalactosylated (G1) oligosaccharide chains without or with a fucose residue, and the peaks 4 and 8 to nongalactosylated (G0) oligosaccharide chains without or with a fucose residue. G, Galactose; GN and GlcNAc, *N*-acetylglucosamine; M mannose; F and Fuc, fucose.

cryogenic 6-19 mAb established from MRL-*Fas*^{lpr} mice (16). To determine whether decreased galactosylation is indeed responsible for cryoglobulin activity of IgG3 mAb, we compared the levels of galactosylation of three other cryogenic IgG3 mAb, C11M, CB1, and hybrid 6-19H/9A6L, and three non-cryogenic IgG3 mAb, 2-6D, J606, and 9-106. Octadecylsilane-HPLC analysis of the neutral oligosaccharides obtained after sialidase treatment of total oligosaccharides showed that the contents of nongalactosylated (G0; no galactose, terminating in *N*-acetylglucosamine), monogalactosylated (G1; one terminal galactose residue), and digalactosylated (G2; two terminal galactose residues) oligosaccharides isolated from C11M, CB1, and 6-19H/9A6L cryogenic IgG3 mAb were comparable to those of the cryogenic 6-19 mAb (Table I). However, even the two non-cryogenic 2-6D and J606 IgG3 mAb bore oligosaccharide chains in which the galactose contents were similar, but not higher than those of the cryogenic 6-19 mAb, while the content of G0 oligosaccharides in non-cryogenic 9-106 IgG3 mAb was comparable to those of the three noncryoprecipitating 6-19 variants, A6C, P11C, and Y12C.

Table I. *Structural analysis of N-linked oligosaccharide chains purified from cryogenic and non-cryogenic IgG3 mAb*[a]

IgG	Cryoglobulin	Sialylated Glycoforms				Galactosylated Glycoforms			
		N	A1	A2	A1 + 2A2	G0	G1	G2	G1 + 2G2
6-19	+	90.0	9.0	1.0	11.0	50.8	38.2	11.0	60.2
C11M	+	94.4	3.7	1.9	7.5	50.9	39.7	9.4	58.5
CB1	+	92.9	4.8	2.3	9.4	55.4	35.0	9.6	54.2
6-19H/9A6L	+	89.2	9.4	1.4	12.2	49.0	44.2	6.8	57.8
A6C	−	85.1	12.8	2.1	17.0	31.6	44.5	23.9	92.3
P11C	−	84.3	13.7	2.0	17.7	32.5	45.9	21.6	89.1
Y12C	−	87.4	10.8	1.8	14.4	33.6	45.3	21.1	87.5
2-6D	−	84.1	15.0	0.9	16.8	57.5	35.2	7.3	49.8
J606	−	83.1	11.2	5.7	22.6	60.8	30.3	8.9	48.1
9-106	−	51.6	25.0	23.4	71.8	30.5	27.6	41.9	111.4

[a] Percent molar ratios of neutral (N) and two acidic sialylated (A1 and A2) oligosaccharides among total oligosaccharides, and of nongalactosylated (G0) and two galactosylated (G1 and G2) glycoforms among desialylated neutral oligosaccharides were calculated on the basis of their UV absorbance at 304 nm. Representative results obtained in two or three independent experiments are shown. The analysis was carried out in duplicate, and results of mean values are shown.

In contrast, analysis by DEAE-HPLC of total oligosaccharides showed significant, although small, differences in molar ratios of neutral asialo-oligosaccharides and acidic monosialylated oligosaccharides (A1, one terminal sialic acid) between cryogenic and non-cryogenic IgG3 mAb ($p < 0.02$), independently of the extent of galactosylation (Table I). When the content of sialic acids was expressed as [percentage of A1 + 2 × (percentage of A2)], because of the presence of two terminal sialic acid residues in the A2 glycoform, non-cryogenic 2-6D, J606, and the three 6-19 variants had approximately two times higher values than the cryogenic 6-19, C11M, CB1, and 6-19H/9A6L mAb ($p < 0.02$). Notably, the 9-106 mAb displayed an aberrant glycosylation pattern in which the contents of G2 oligosaccharides and sialylated (A1 and A2) oligosaccharides were markedly increased, as compared with other non-cryogenic IgG3 mAb (Table I).

Loss of cryoglobulin activity of IgG3 supernatant fractions obtained after separation from initial cryoprecipitates

Cryoglobulin formation of IgG3 mAb is known to be a concentration-dependent phenomenon (16). When the cryogenic activities of 6-19, C11M, CB1, and 6-19H/9A6L mAb were assessed as a function of their concentrations following an incubation at 4°C for 24 h, the amounts of cryoprecipitates generated were dependent on the initial concentrations of IgG3 for each mAb, although the extent of cryoprecipitation was variable among these three mAb (Table II). However, once cryoprecipitates formed during the first 24-h incubation were removed, the IgG3 mAb remaining in supernatants were no longer cryoprecipitable by a second 24-h incubation at 4°C, despite the fact that their concentrations were still high enough to induce cryoprecipitation of the initial IgG3 preparations. For example, 6-19 mAb was able to produce significant amounts of cryoprecipitates at an initial concentration of 1 mg/ml, whereas the same mAb at a concentration of 1.83 mg/ml recovered after removal of cryoprecipitates (derived from 6-19 mAb at an initial concentration of 4 mg/ml) no longer generated cryoprecipitates (Table II). Similar results were obtained with the three other cryogenic IgG3 mAb. These results suggest a heterogeneity for the cryogenic potential even among the same IgG3 proteins.

Enrichment of nonsialylated glycoforms in cryoprecipitates and of sialylated glycoforms in supernatants of IgG3 mAb

Differences in cryogenic potential among the same IgG3 mAb population could be related to the heterogeneity in sialylation levels of carbohydrate side chains. To address this question, we separated cryoprecipitates and supernatants following an incubation of 2 mg/ml 6-19, C11M, CB1, and 6-19H/9A6L mAb at 4°C for 24 h and analyzed the levels of sialylation and galactosylation in oligosaccharides isolated from cryoprecipitates and supernatants. The levels of sialylated oligosaccharides were reduced in cryoprecipitates and increased in supernatants obtained from all four IgG3 cryogenic mAb, as compared with those of unseparated total IgG3 (Table III). Consequently, the contents of sialic acids, expressed as [percentage of A1 + 2 × (percentage of A2)], were lower in cryoprecipitates and higher in supernatants than in unseparated total IgG3. Significantly, values in the supernatant fractions derived from the cryogenic IgG3 mAb became indeed comparable to those obtained with noncryogenic 6-19 variants (A6C, P11C, and Y12C), 2-6D, and J606 IgG3 mAb (Table I). In contrast, differences in the levels of galactosylation were not apparent between cryoprecipitates and supernatants, although the levels of the G2 glycoform were consistently elevated in supernatants, which could explain increases in sialylated glycoforms of the supernatants (Table III). Notably, no measurable differences in the level of fucosylation were found between cryoprecipitated and supernatant fractions, with fucosylated oligosaccharides amounting to >95% of neutral oligosaccharides (data not shown). These data indicate an enrichment of less sialylated glycoforms of IgG3 mAb in cryoprecipitates.

Stronger inhibition of IgG3 cryoprecipitation by more sialylated non-cryogenic 9-106 IgG3 mAb

We have previously shown that when cryogenic IgG3 mAb was incubated with excess amounts of non-cryogenic IgG3 mAb, cryoglobulin formation was inhibited as a result of IgG3-IgG3 complex formation between cryogenic and non-cryogenic IgG3 (22). If IgG3 cryoprecipitation is indeed associated with lower levels of sialylation, the anticryoprecipitating potential of non-cryogenic IgG3 mAb should also be dependent on their sialic acid contents. To test this possibility, 2 mg/ml cryogenic 6-19 mAb was incubated with different concentrations of non-cryogenic 2-6D, J606, and 9-106 mAb. When the amounts of cryoprecipitates were determined, the strongest inhibition of 6-19 cryoprecipitation was observed with 9-106 mAb (Table IV), which was the most abundantly sialylated (Table I). A measurable inhibition was still obtained by the presence of a 2-fold lower concentration of 9-106 mAb. In contrast, a significant reduction of 6-19 cryoprecipitation was obtained only in the presence of a 2-fold excess of 2-6D and J606 mAb. The remarkable inhibition by 9-106 mAb was not due to a more efficient interaction of this mAb with 6-19, since in vitro binding assays showed that 9-106, 2-6D, and J606 exhibited concentration-dependent complex formation with 6-19 in a quantitatively identical manner (data not shown).

Table II. *Loss of cryoglobulin activity of IgG3 supernatant fractions obtained after separation from initial cryoprecipitates*[a]

IgG	Concentration (mg/ml)	First ppt[b] (%)	Supernatant (mg/ml)	Second ppt
6-19	1	18.2	0.78	-
	2	38.5	1.43	-
	4	56.8	1.83	-
C11M	0.5	26.0	0.37	-
	1	43.5	0.57	-
	2	67.5	0.73	-
CB1	1	14.0	0.86	-
	2	41.0	1.18	-
6-19H/9A6L	1	9.0	0.91	-
	2	18.3	1.63	-
	4	34.6	2.57	-

[a] One milliliter of various concentrations of IgG3 mAb were incubated at 4°C for 24 h, and cryoprecipitates (First ppt) and supernatants were separated. The amounts of cryoprecipitates were estimated by measuring OD of supernatants, and results are expressed as percentages of cryoprecipitation (means of triplicates). Then the obtained supernatants were further incubated at 4°C for an additional 24 h to determine their cryoglobulin activity.

[b] ppt, Precipitate.

Discussion

The present study was designed to investigate the negative role of terminal sialic acids in oligosaccharide side chains for the cryogenic potential of murine IgG3. Comparative oligosaccharide structural analysis of cryoprecipitated and noncryoprecipitated fractions separated from four different cryogenic IgG3 mAb has demonstrated an enrichment of less sialylated IgG3 in cryoprecipitates, as compared with supernatants. In addition, the inhibitory effect of non-cryogenic IgG3 mAb on cryoprecipitation of 6-19 IgG3 mAb was correlated with the level of sialylation of these non-cryogenic IgG3 mAb. These data suggest that the content of terminal sialic acids in oligosaccharide side chains critically determines the cryoglobulin activity of IgG3 mAb, possibly through an increase of the negative charge of IgG3.

We have previously shown that in contrast to the high cryoglobulin activity of the 6-19 IgG3 mAb derived from an autoimmune MRL-*Fas*^{lpr} mouse, its variants bearing increased levels of galactosylation in the oligosaccharide side chains displayed barely detectable cryoglobulin activity (16). The terminal galactose residue of oligosaccharide side chains apparently forms a tight interaction with a lectin-like binding pocket on the CH2 domain of IgG, which plays a role in maintaining the relative geometry of the two CH2 domains (28, 29). Thus, it was speculated that the absence of terminal galactose could lead to structural alterations of IgG3 molecules, thereby promoting their cryoprecipitating potential. However, in the present study, we observed that two of three noncryogenic IgG3 mAb, 2-6D and J606, displayed low levels of galactosylation similar to those of cryogenic 6-19. These results therefore clearly indicate that galactosylation by itself does not directly determine the cryogenic activity of IgG3 mAb. This conclusion was further supported by the analysis of oligosaccharide structures of cryoprecipitated and noncryoprecipitated fractions of IgG3, which showed little difference in the extent of galactosylation in both fractions, despite the fact that the latter fraction is no longer able to generate cryoglobulins.

Instead, our analysis revealed that cryogenic IgG3 mAb had consistently lower contents of sialylated glycoforms than noncryogenic IgG3 mAb. The inhibitory role of sialic acid residues in IgG3 cryoprecipitation was further supported by the demonstration that cryoprecipitated fractions of IgG3 mAb were much less sialylated, while the corresponding noncryoprecipitating supernatants were more sialylated, as compared with unseparated total IgG3 mAb. More significantly, the extent of sialylation in the latter fraction derived from cryogenic IgG3 mAb became comparable to that of non-cryogenic IgG3 mAb. A negative role for sialic acids in IgG3 cryoprecipitation was further supported by the fact that the more sialylated, non-cryogenic 9-106 mAb most strongly inhibited cryoprecipitation of 6-19 when both types of IgG3 mAb were mixed together. Since cryogenic and non-cryogenic IgG3 mAb interact equally well to form IgG3-IgG3 complexes (10, 22), the potent anticryogenic activity of highly sialylated 9-106 IgG3 mAb is likely a result of increased content of negatively charged sialic acids in mixed IgG3 complexes formed between 9-106 and 6-19. Thus, the sialic acid content of oligosaccharide side chains apparently plays a key role in determining the cryoglobulin activity of IgG3 mAb.

The precise molecular mechanism responsible for the generation of monoclonal cryoglobulins has not yet been well defined. As we have previously shown, murine IgG3 interact because of the unique physicochemical property of their H chain constant region (5, 10, 22). Consequently, all murine IgG3 mAb are able to form self-associating complexes, independently of their cryoglobulin activity. Thus, the formation of self-associating IgG3 complexes is likely to be the first step required for cryoglobulin formation. The second step would be the charge interaction of these IgG3 complexes, which would lead to the generation of larger complexes to promote precipitation at a lower temperature. The presence of an excess of negatively charged amino acid and/or sialic acid residues in IgG3 molecules would interfere with this process, thereby inhibiting the generation of cryoglobulins. Since the CH2 domain of each H chain bears an *N*-linked oligosaccharide chain, an IgG molecule contains zero to four sialic acid residues. Thus, it would be of interest to determine how many sialic acids are necessary to achieve a change in cryogenic potential of IgG3 mAb. In addition to the charge effect of sialic acids, we cannot exclude the possibility that the presence of sialic acid residues could lead to a structural change in the Fc region, thereby modulating cryogenic activity of IgG3 mAb.

Our demonstration that terminal sialic acid residues of oligosaccharide side chains inhibit IgG3 cryoglobulin activity is consistent with several previous findings. First, the cryoglobulin activity of IgG3 anti-DNP mAb was inhibited following the binding of negatively charged DNP-coupled amino acid conjugates (9). Second, the analysis of a panel of IgG3 mAb has shown that cryogenic IgG3 mAb are in general more positively charged at V_H residues 6 and 23 than their noncryogenic counterparts (15, 16). Third, the cryoglobulin activity of IgG3 6-19 and 5-7B mAb was substantially reduced in mutants bearing more negatively charged residues at V_H 6 and 23 (15, 16). Thus, the cryoglobulin activity of murine IgG3 mAb is apparently controlled by at least two factors: the level of sialylation on oligosaccharide side chains and the V region's amino acid sequences.

It is clear that the addition of terminal sialic acids is dependent on the presence of galactose residues on nascent oligosaccharides, since a markedly increased sialylation in the 9-106 mAb was associated with greatly elevated contents of the digalactosylated glycoform. However, the level of galactosylation does not directly regulate the level of sialylation, because the extent of sialylation among 6-19, 2-6D, and J606 mAb was substantially different, despite comparable levels of galactosylation. The sialylated oligosaccharides of these mAb were sensitive to *A. ureafaciens* sialidase, which can cleave both sialyl α2,3 and α2,6 linkages (30), but not to *Salmonella typhimurium* LT2 sialidase, which preferentially hydrolyzes sialyl α2,3 linkage (Ref. 31 and Y. Kuroda, T. Funase, and M. Nakata, unpublished data). Thus, the observed differences

Table III. *Structural analysis of* N*-linked oligosaccharide chains of cryoprecipitates and supernatants separated from cryogenic IgG3 mAb*

IgG	Sample[a]	Sialylated Glycoforms[b]				Galactosylated Glycoforms[b]			
		N	A1	A2	A1 + 2A2	G0	G1	G2	G1 + 2G2
6-19	Total	88.9	9.9	1.2	12.3	52.6	39.2	8.2	55.6
	Supernatant	86.3	11.1	2.6	16.3	51.8	38.8	9.4	57.6
	ppt[c]	90.5	8.8	0.7	10.2	50.6	41.7	7.7	57.1
C11M	Total	93.8	4.1	2.1	8.3	49.7	40.0	10.3	60.6
	Supernatant	88.7	6.9	4.4	15.7	49.3	38.5	12.2	62.9
	ppt	95.6	3.0	1.4	5.8	50.9	40.9	8.2	57.3
CB1	Total	93.2	4.7	2.1	8.9	57.3	34.0	8.7	51.4
	Supernatant	90.2	6.5	3.3	13.1	54.4	34.2	11.4	57.0
	ppt	97.5	1.9	0.6	3.1	57.5	35.3	7.2	49.7
6-19H/9A6L	Total	89.2	9.4	1.4	12.2	49.0	44.2	6.8	57.8
	Supernatant	86.4	8.6	5.0	18.6	55.4	36.9	7.7	52.3
	ppt	96.0	2.9	1.1	5.1	66.4	29.8	3.8	37.4

[a] One milliliter of 2 mg/ml IgG3 mAb were incubated at 4°C for 24 h before cryoprecipitates and supernatants were separated.
[b] Percent molar ratios of neutral (N) and two acidic sialylated (A1 and A2) oligosaccharides from total oligosaccharides, and of nongalactosylated (G0) and two galactosylated (G1 and G2) glycoforms in desialylated, neutral oligosaccharides were calculated on the basis of their UV absorbance at 304 nm. The analysis was carried out in duplicate, and results of mean values are shown.
[c] ppt, Precipitate.

in IgG3 sialylation among these three IgG3 mAb are likely to be a result of differential expression of α2,6-sialyltransferase involved in the terminal sialylation of IgG oligosaccharides. It is also striking to see that the 9-106 mAb is abnormally highly sialylated, as compared with other IgG3 mAb. cDNA sequence analysis has revealed the presence of an additional potential *N*-linked glycosylation site in its V_H region, but no mutations in its H chain constant region (S. Izui, unpublished data). Therefore, it can be speculated that additional V_H oligosaccharide chains present in the 9-106 mAb may be aberrantly sialylated, as compared with the CH2 oligosaccharides. This possibility is currently under investigation.

Our demonstration that a decrease in the relative content of sialic acid residues in the oligosaccharide chains is associated with IgG3 cryoglobulin activity suggests that the level of IgG sialylation may be an important factor in determining the pathogenic potential of autoantibodies with cryoglobulin activity (10–13, 23, 32, 33). In addition, our present findings could provide the molecular base responsible for the possible pathogenic role of nongalactosylated IgG, the proportion of which has been shown to be markedly increased in some diseases, most notably rheumatoid arthritis (19). The increased sialylation may be a mechanism that reduces the cryogenic potential of autoantibodies, thereby avoiding deleterious immunopathological consequences. Apparently, multiple mRNA isoforms are generated from the α2,6-sialyltransferase gene in B cells, which results from the usage of differential exon, promoter, and/or polyadenylation site (34, 35), and its transcription can be modulated by glucocorticoids and cytokines (36, 37). Clearly, further identification of the molecular basis of IgG sialylation and elucidation of its protective role would help develop new diagnostic and therapeutic approaches for cryoglobulin- and autoantibody-mediated disorders.

Table IV. *Inhibition of 6-19 cryoprecipitation by non-cryogenic 9-106, 2-6D, and J606 IgG3 mAb*[a]

IgG3	Concentration (mg/ml)	6–19 ppt (μg)	% Inhibition
9-106	1	27.2	11.7
	2	20.6	33.1
	4	1.8	94.2
2-6D	2	30.2	2
	4	14.4	53.2
J606	2	32.0	0
	4	20.6	33.1
PBS		30.8	

[a] One humdred microliters of 2 mg/ml 6-19 mAb was incubated with 0.1 ml of different concentrations of 9-106, 2-6D, or J606 mAb at 4°C for 24 h. The amounts of 6-19 mAb precipitated (means of triplicates) were determined by ELISA. Results are expressed as percent inhibition of the 6-19 cryoprecipitation, as compared to coincubation of 6–19 mAb with PBS.

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Disclosures

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