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Research Article

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Cryoprecipitation of an Anti-Pr_2 Monoclonal IgM Cold Agglutinin in the Presence of GM_3 Ganglioside

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Abstract. The mechanism of cryoprecipitation of a monoclonal IgM_κ cryoglobulin (Mou) with a cold agglutinin activity of Pr_2 specificity has been studied. By immunodiffusion this cryoglobulin reacted (by its Fab' fragment) with micellar GM_3, a ganglioside bearing the Pr_2 antigenic determinant. In contrast to previous reports that indicated a possible temperature dependent self-association of IgM molecules via an immunological interaction leading to cold precipitation, we could not detect any affinity of this cryoglobulin for IgM when we used passive hemagglutination or an indirect enzyme-linked immunosorbent assay (ELISA). However, a GM_3-like ganglioside could be extracted, by drastic methods, from the cryoglobulin studied at 22°C, whereas no GM_3 was extracted from two control cryoglobulins. Some minor gangliosides (representing <25% of total amount of bound gangliosides) were also extracted from Mou cryoglobulin and these gangliosides were shown to cross-react with GM_3, as they specifically bind to Mou cryoglobulin by ELISA. After cryoprecipitation the serum still contained a monoclonal anti-Pr_2 IgM_κ. A GM_3-like ganglioside could be extracted from this purified IgM, and cryoprecipitability could be induced by the addition of a minute amount of micellar GM_3.

These results suggest that Mou cryoglobulin circulates as an immune complex and that cryoprecipitation may depend on unique IgM-GM_3 (or IgM-GM_3 cross-reacting gangliosides) complexes.

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Introduction

Serum cryoglobulins can be classified into three types (1) according to the presence in the cryoprecipitate of monoclonal Ig (type I), both monoclonal and polyclonal Ig (type II), or polyclonal Ig with or without other serum proteins or exogenous antigens (type III). Whereas in mixed IgM-IgG type II cryoglobulins the monoclonal IgM precipitates in the cold only after interaction with polyclonal IgG (2), in type I cryoglobulins antigen binding does not seem mandatory for cold precipitation (3). In the latter case, cryoprecipitation may be related to self-association of Ig molecules via nonspecific physicochemical interactions. A specific immunological binding has, however, been suggested as the cause of cryoprecipitation in two cases of monoclonal IgM cold agglutinins with Pr_2 and Gd specificities, respectively, where cross-reactivity between carbohydrate moieties of erythrocyte (RBC)[1] antigens (Pr_2 and Gd determinants) and the IgM Fc fragment was postulated (4, 5). We

1. *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; GVB^{2+}, 5 mM veronal-buffered saline; Neu5Ac, *N*-acetylneuraminic acid; RBC, erythrocyte; SN, cryoglobulin supernatant; SPG, $\alpha(2 \rightarrow 3)$sialosylparagloboside.

describe here a type I cryoglobulin with Pr_2 cold agglutinin specificity, which shows no affinity for IgM. Cryoprecipitation may be related, in this case, to in vivo binding of the monoclonal IgM to the GM_3 ganglioside (and other cross-reacting gangliosides), which is known to contain the Pr_2 antigenic determinant (4). These results provide evidence that type I cryoglobulins may represent immune complexes, possibly playing some role in the pathogenesis of vasculitis or proliferative glomerulonephritis sometimes observed in patients with such cryoglobulins (1).

Methods

Purification and characterization of Mou macroglobulin. Waldenström macroglobulinemia was diagnosed in a 61-yr-old female patient (Mou) who presented with severe Raynaud's phenomenon and bleeding symptoms. Her serum contained a monoclonal IgM_κ at a concentration of 35 mg/ml. A heavy precipitate formed at 25°C and readily redissolved at 37°C. Isolation and purification of this cryoglobulin were performed as previously described (1). By double immunodiffusion analysis, monoclonal IgM_κ was the only serum protein detected in the cryoprecipitate at a concentration of 10 mg/ml with rabbit IgG monospecific for μ-, γ-, α-, κ-, and λ-human Ig chains and with polyvalent anti-human serum (Dakopatts A/S, Copenhagen). Cold precipitation occurred at 4°C for an IgM concentration > 1 mg/ml. Loss of cryoprecipitability appeared under pH 3.0 and above pH 9.5 (protein concentration, 1 mg/ml). Cryoprecipitability was also inhibited by NaCl at a 2 M final concentration. Fab' and $F(ab')_2$ fragments of the cryoglobulin were obtained after 4 h of pepsin digestion (6) and purified by chromatography on a 6 B Sepharose column. Although the cryoglobulin-depleted serum (supernatant [SN]) did not precipitate at 4°C even after 15 d, it still contained the monoclonal IgM_κ component at a concentration of 5 mg/ml. This IgM, hereafter referred to as SN IgM_κ, was isolated by gel filtration on a 6 B Sepharose column and did not precipitate in the cold at a concentration of 10 mg/ml.

Mou cryoglobulin, serum, or SN, and twofold serial dilutions thereof, were added to an equal volume of human or animal RBC suspension at a concentration of 5% vol/vol in phosphate buffered saline (PBS) (10 mM phosphate, 150 mM NaCl, pH 7.2); agglutination was read microscopically after 2 h at 4°C. Human or animal RBCs were used either untreated or treated by papain and neuraminidase by standard methods (7).

Specificity of IgM Mou for gangliosides and glycoproteins. Gangliosides GM_1, GD_{1a}, GT_{1b}, and galactosylceramide were isolated from bovine brain; GD_3 (containing *N*-acetylneuraminic acid [Neu5Ac]) was obtained from buttermilk. These glycolipids were purified according to Fredman et al. (8). GM_3 (containing Neu5Ac) was obtained from human liver and purified according to Seyfried et al. (9). $\alpha(2 \rightarrow 3)$Sialosylparagloboside (SPG) (a gift from J. Portoukalian, Cêntre Léon Bérard, Lyon, France) was purified from human RBCs. Chemical structure and homogeneity were assessed as described by Ghidoni et al. (10). The purity was >99%. Gangliosides concentrations were determined by assaying bound sialic acid according to Svennerholm (11). Two different preparations of gangliosides were used to assess antiganglioside activity: a micellar suspension or an ethanolic solution. (*a*) A micellar preparation was obtained after 5 min sonication of GM_3 in PBS at a concentration of 1 mg/ml (10^{-3} M), above the known critical micelle concentration (12). Immunoelectrophoresis of Mou cryoglobulin (1 mg/ml) and SN IgM_κ (1 mg/ml) was performed with this preparation diluted at 0.1 mg/ml and the precipitation line was compared with that obtained with an anti-μ-serum. In other experiments, Mou cryoglobulin (0.25 mg/ml) and Mou SN IgM_κ (0.5 mg/ml) were incubated in capillary tubes for 15 h at 4°C with an equal volume of GM_3 or GM_1 micellar suspension at concentrations of up to 1 mg/ml. Finally, Mou cryoglobulin (1 mg/ml), and Fab' and $F(ab')_2$ IgM fragments (5 mg/ml) were also tested against micellar GM_3 (0.2 mg/ml) in a micro-Ouchterlony plate incubated at 23°C for 15 h. (*b*) Study of Mou cryoglobulin specificity for various purified gangliosides in ethanolic solution was performed by enzyme-linked immunosorbent assay (ELISA) as adapted for glycolipids by Iwamori et al. (13). Briefly, each well of polystyrene microtiter plate (Immuno 96 F; Nunc, Kamstrup, Denmark) was coated with 10^{-1} μg glycolipid. The washing and incubation media were, respectively, 0.1 and 1% bovine serum albumin (BSA) in a 5-mM veronal buffered saline 0.15 M, pH 7.5, containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 0.5 mM Mg Cl_2 (0.1% BSA and 1% BSA-GVB^{2+}). The Mou cryoglobulin concentrations ranged from 0.05 to 50 μg/ml, and the peroxidase labeled goat anti-human μ-chain antibodies (Biosys., Compiègne, France) were used at the concentration of 0.5 μg/ml. Incubation times were 30 min at 37°C or 20 h at 4°C. Bound peroxidase-conjugated antibodies were determined by the *O*-phenylenediamine procedure (14). The absorbance was measured at 492 nm in a photometer (Titertek Multiskan MC; Flow Laboratoires, Puteaux, France).

To study antiglycoprotein activity of Mou cryoglobulin, ELISA was performed according to Engvall and Perlmann (15), with glycophorin or human chorionic gonadotropin (both, Sigma Chemical Co., St. Louis, MO) coated to plastic wells. The washing medium was PBS 0.15 M, pH 7.5, containing 0.1% Tween 20, and a 0.3% gelatin solution in PBS, 0.15 M, pH 7.5, containing 0.1% Tween 20 was used as the incubation medium (these media were preferred to the BSA-GVB^{2+} media used for gangliosides, as they gave lower background levels in protein studies). Mou cryoglobulin concentrations studied ranged from 0.5 to 50 μg/ml and the incubation time was 1 h at 37°C or 2 h at 4°C. Peroxidase-labeled anti-human μ-chain antibodies were used as described above. The binding to glycophorin or human chorionic gonadotropin of three purified monoclonal IgM devoid of anti-Pr_2 antibody activity was also tested. In preliminary experiments, the coating of the glycoproteins to the plates was controlled using specific antibodies (a gift of J. P. Cartron, Centre National de Transfusion Sanguine, Paris), and the optimal coating conditions were determined; a 20 μg/ml protein concentration in 0.1 M sodium carbonate buffer, pH 9.6, was chosen.

Anti-IgM activity of IgM Mou. Six monoclonal IgM, purified as previously described (16), as well as untreated and neuraminidase-treated Mou cryoglobulin, were used to coat sheep RBCs by the chromium chloride technique (17). Hemagglutination tests were performed in microtiter plates with either untreated or neuraminidase-treated Mou cryoglobulin and dilutions thereof starting at 1 mg/ml. The plates were read after an overnight incubation at 4 or 22°C. ELISA experiments were also performed as described above with three different purified monoclonal IgM_λ coated to plastic wells. The binding of IgM_κ Mou to IgM_λ was revealed using a second layer of peroxidase conjugated sheep anti-human K-chain antibodies (Institut Pasteur Production, Paris) at a concentration of 0.4 μg/ml.

Detection of gangliosides in Mou macroglobulin. The presence of ganglioside in Mou cryoglobulin was first studied by ELISA: the wells were directly coated by incubation for 1 h at 37°C, then for 2 h at 4°C, with concentrations of Mou cryoglobulin in GVB^{2+} ranging from 0.1 to 100 μg/ml. After being washed as described above, the plates

were incubated with a rabbit anti-GM_3 serum, raised as previously described (18), and revealed with goat anti-rabbit Ig antibodies labeled with β-galactosidase (a gift of J. L. Guesdon, Institut Pasteur) using 4 methyl-umbelliferyl-βd-galactopyranoside (Sigma Chemical Co.) as the fluorogenic substrate, as described by Labrousse et al. (19). In other experiments, gangliosides were extracted from Mou cryoglobulin and Mou SN IgM_κ as previously described (20) by a modification of the technique of Cham and Knowles (21). Briefly, to 2 ml of the protein solution were added 0.2 mg EDTA and 4 ml of a water-saturated solution of *n*-butanol diisopropylether (40:60 vol/vol). After thorough mixing and centrifugation, all the gangliosides were found in the lower phase. A second extraction with the same solvents was performed in the lower phase, which contained the proteins and the gangliosides. Under those conditions, most proteins were not denatured. More drastic conditions for extraction were also used: to the 2 ml of the lower phase (lipid free, except for gangliosides), 10 ml of chloroform-methanol (2:1 vol/vol) was added and a partition was performed. The upper phase, containing all the gangliosides, was purified further using Sep-Pak cartridges (Waters Associates, Milford, MA) (22). The detection of gangliosides on thin-layer chromatography and the determination of their relative percentages by densitometry of neuraminic acid resorcinol positive spots were performed as previously described (23).

Study of control monoclonal cryoglobulins. Seven monoclonal cryoglobulins were isolated and purified as Mou cryoglobulin. They included five type I (two IgM_λ, two IgM_κ, and one IgG_κ) and two type II (IgM_κ-IgG) and were tested for cold agglutinin activity and cryoprecipitation sensitivity to neuraminidase. Anti-GM_3 activity was checked by precipitation in capillary tubes in the presence of micellar GM_3, and lipid extraction was performed in two cases.

Results

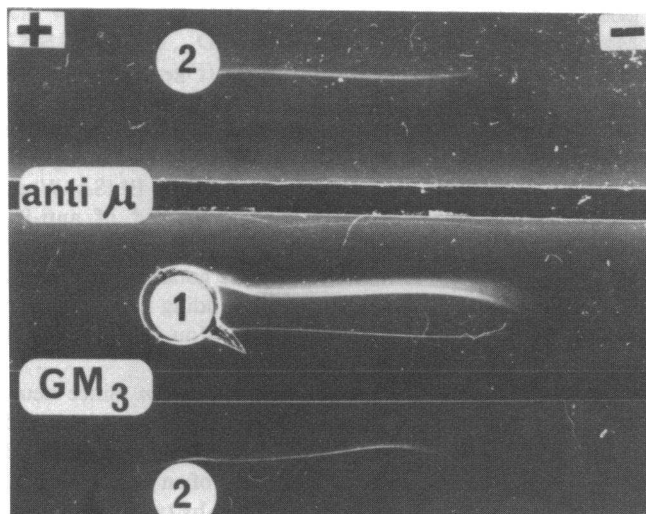
Cold agglutinin activity of IgM Mou. Mou serum and isolated cryoglobulin contained a monoclonal IgM_κ (35 and 10 mg/ml, respectively) with the same electrophoretic mobility and had a high titer (2048) cold agglutinin activity with Pr_2 specificity, as indicated by the absence of hemagglutination after neuraminidase or protease treatment of human RBCs as well as by the absence of hemagglutination of dog RBCs, which was unaffected by protease but abolished by neuraminidase treatment of the cells. Mou SN also contained the monoclonal IgM_κ (5 mg/ml), which exhibited the same cold agglutinin activity (titer 256). To confirm that the cold agglutinin activity was carried by monoclonal IgM_κ, Mou serum and cryoglobulin were absorbed at 4°C on untreated human RBCs. A heat eluate prepared thereof at 37°C contained the cold precipitating and agglutinating monoclonal IgM_κ, whereas the heat eluate obtained after using papainized RBCs were used was free of Ig. Neuraminidase treatment of Mou serum and Mou cryoglobulin abolished cold precipitability without altering cold agglutinin activity.

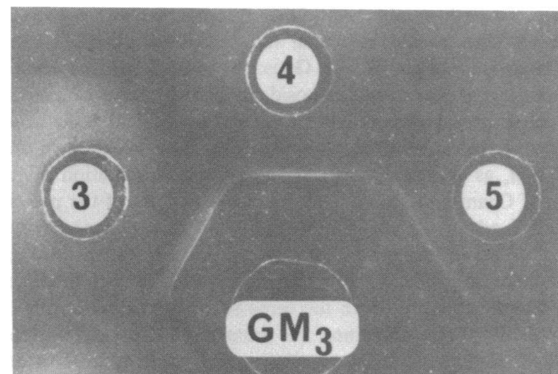
Anti-GM_3 ganglioside activity of IgM Mou. By immunoelectrophoresis, a precipitin line developed between Mou IgM_κ cryoglobulin and GM_3; it was similar to that obtained with an anti-μ-serum (Fig. 1). To check further the immune nature of GM_3 binding to Mou cryoglobulin, double immunodiffusion studies were performed using peptic fragments of Mou cryoglobulin; purified Fab′ and $F(ab')_2$ Mou cryoglobulin fragments did not cryoprecipitate at 5 mg/ml in PBS; however, when tested in immunodiffusion plates at 22°C, $F(ab')_2$ precipitated with GM_3, whereas Fab′ could inhibit the precipitation reaction between IgM Mou and GM_3 (Fig. 1).

The specificity of Mou IgM_κ for various gangliosides or galactosylceramide was studied by ELISA. Results show that Mou cryoglobulin binds to GM_3, GD_3, GD_{1a}, GT_{1b}, and SPG, but not to GM_1 or galactosylceramide (Fig. 2). Performing ELISA at 4 rather than at 37°C did not enhance IgM Mou binding to GM_3. Because the carbohydrate moiety of certain glycoproteins shares some epitopes with GM_3, an antiglycoprotein activity of IgM Mou was investigated by ELISA. When compared with three monoclonal IgM devoid of anti-Pr_2 antibody activity, a specific binding of Mou cryoglobulin to glycophorin, but not to human chorionic gonadotropin, could be demonstrated (Fig. 3). The specific binding to glycophorin was observed at a concentration of IgM Mou 10 times higher than that needed to react with GM_3.

Study of the mechanism of cryoprecipitation. Since neuraminidase treatment of the monoclonal IgM abolished cold precipitation, as shown for an anti-Pr_2 cold agglutinin described by Tsaï et al. (4), these authors' hypothesis of an immunological IgM self-association in the cold was tested by two immunological methods. First, passive hemagglutination tests using sheep RBCs coated with a panel of seven monoclonal IgM were performed with Mou cryoglobulin, before and after neuraminidase treatment, and with Mou SN. Neuraminidase treatment was performed with the aim of releasing antibody sites possibly bound to Neu5Ac-containing antigen. No significant binding was observed at 4 or 22°C, though an anti-μ-serum gave a high agglutinating titer (16,000). Second, when plastic wells coated with three different purified monoclonal IgM_λ were used, no binding of Mou IgM_κ could be detected by ELISA with a second layer of peroxidase conjugated anti-κ-antibodies, whether performed at 37 or at 4°C.

In view of these negative results, we tested the possibility that cryoprecipitability of IgM Mou depended upon the presence of GM_3. A 15-h incubation of Mou cryoglobulin at 4°C at a concentration (0.25 mg/ml) that prevents spontaneous precipitation, with micellar GM_3 at a concentration of $>6 \times 10^{-3}$ mg/ml in capillary tubes, induced a precipitate which was not solubilized after a further incubation for 1 h at 37°C. Conversely, incubation at 4°C of Mou SN IgM_κ (0.5 mg/ml) yielded a precipitate that was dissolved after additional incubation at 37°C, provided that GM_3 was used at a concentration of $<12 \times 10^{-3}$ mg/ml; for higher GM_3 concentrations, the precipitate formed at 4°C was stable at 37°C. No precipitation was obtained with micellar GM_1 at any concentration. Since these experiments support the possibility that GM_3 is responsible for cold precipitability of IgM Mou, one might expect that GM_3 could be detected in Mou cryoglobulin. When the modified Cham and Knowles technique for lipid extraction was performed, the cryoglobulin was not denaturated and no ganglioside was extracted from Mou cryoglobulin. However,





A

B

Figure 1. Anti-GM_3 activity of Mou IgM_κ. (*A*) Comparative immunoelectrophoretic pattern of Mou cryoglobulin (1 mg/ml) (*1*) and Mou SN IgM_κ (1 mg/ml) (*2*) with anti-μ-serum and micellar GM_3 solution (0.1 mg/ml). (*B*) Double immunodiffusion reactions of Mou SN IgM_κ at 1 mg/ml (*3*), Mou cryoglobulin at 1 mg/ml (*4*) and 2 mg/ml (*7*), Mou cryoglobulin peptic fragments (5 mg/ml) $F(ab')_2$ (*5*) and Fab' (*6*), with micellar GM_3 (0.2 mg/ml) in central wells; well 8 contains PBS, pH 7.2. $F(ab')_2$ precipitates weakly with GM_3, whereas Fab' inhibits the precipitation of Mou cryoglobulin with GM_3.

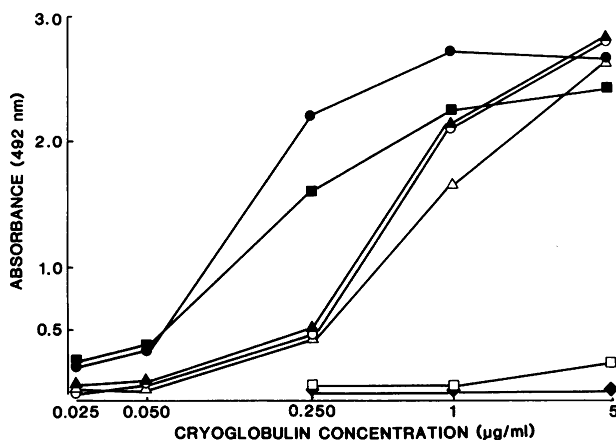


Figure 2. Specificity of Mou cryoglobulin for various gangliosides. The reactivity of Mou cryoglobulin is studied by an immunoenzymatic assay in which wells are coated with GM_3 (●), GD_3 (○), GD_{1a} (▲), GT_{1b} (△), SPG (■), GM_1 (□) ganglioside, or galactosylceramide (◆) at a concentration of 0.1 μg/well. The binding of Mou cryoglobulin is revealed by goat anti-human μ-chain antibodies labeled with peroxidase and by the transformation of a chromogenic substrate.

when a more drastic extraction was performed by the use of a mixture of chloroform and methanol and leading to protein denaturation, gangliosides could be extracted. As shown in Fig. 4, a GM_3-like ganglioside was present in Mou cryoglobulin. Its migration on thin-layer plate was slightly lower than that of the standard. Some minor gangliosides migrating, as the standard GD_3, GD_{1a}, and GT_{1b}, were also detected. Because the molar ratio of Neu5Ac/ganglioside was 1 for GM_3 and 2 for GD_3 and GD_{1a}, it was clear that, in molar quantities, GM_3 represented at least 75% of gangliosides present. GM_3 was also the main ganglioside detected in Mou SN IgM_κ, where all gangliosides were more concentrated than in Mou cryoglobulin.

In view of these results, we attempted to detect GM_3 in Mou cryoglobulin by immunological methods. No binding of anti-GM_3 antibodies was observed by ELISA on plates coated with various concentrations of Mou cryoglobulin. Under the same conditions, binding of rabbit anti-human μ-chain antibodies was readily demonstrated (data not shown).

Studies on control monoclonal cryoglobulins. Six of the seven control cryoglobulins had no cold agglutinin activity, and cold precipitation was not abolished after neuraminidase treatment. None of the six precipitated at 23°C in capillary

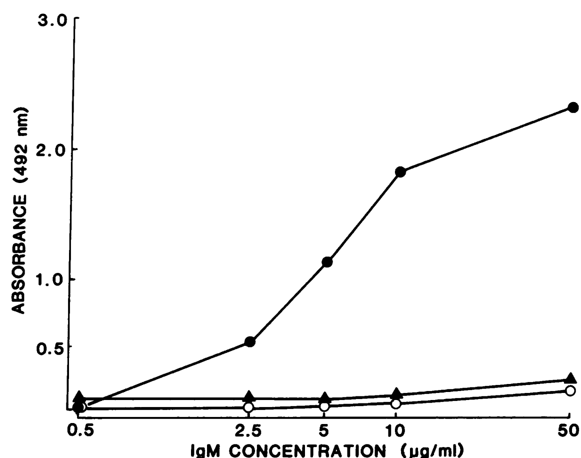


Figure 3. Specificity of Mou cryoglobulin and control IgM for glycoproteins. Wells coated with glycophorin are tested with IgM Mou (●) or three different monoclonal IgM devoid of anti-Pr_2 cold agglutinin activity (▲; the mean values are plotted) by ELISA. The binding of IgM is revealed as indicated in the legend for Fig. 2. No binding to human chorionic gonadotropin is observed with IgM Mou (○) or control IgM (not shown).

tubes in the presence of GM_3 at a concentration up to 1 mg/ml, and no ganglioside was extracted from the two purified cryoglobulins tested. In contrast, one other type I IgM_κ cryoglobulin (Rol) had a Pr_2 cold agglutinin activity (titer 2048). This cryoglobulin also disclosed an anti-GM_3 activity with a precipitate formed at 23°C in capillary tubes after incubation with micellar GM_3 at a concentration $>40 \times 10^{-3}$ mg/ml. Absorption experiments performed on human RBCs gave results similar to those obtained with Mou cryoglobulin, and cryoprecipitation, but not cold agglutinin activity, was abolished after neuraminidase treatment. Not enough material was available for lipid extraction.

Discussion

Cryoglobulins are usually classified according to the Ig (and sometimes other serum proteins) they contain (1). Although such classification is quite useful to establish clinical correlations between the type of the cryoglobulin and patient's symptoms or underlying disease, it may not be accurate since it does not take into account the presence of exogenous antigen or minute amount of autologous antigen which may be very important in getting insights into the mechanism of cryoprecipitability. In selected cases, the patient's clinical history (HBs hepatitis) or the antibody activity of one component of the cryoglobulin (monoclonal IgM rheumatoid factors) allowed a comprehensive study and pointed to a critical role for antigen-antibody immune complexes (2, 24). We therefore examined here the role of GM_3 ganglioside, which is known to carry the Pr_2 antigenic determinant (4), in the cryoprecipitability of a monoclonal IgM that was both an anti-Pr_2 cold agglutinin and a cryoglobulin.

As expected from this Pr_2 specificity, IgM Mou could react with GM_3; anti-GM_3 activity was shown by double immunodiffusion technique with GM_3 and cryoglobulin Mou or its Fab′ or $F(ab')_2$ fragments. Among eight cryoglobulins tested at 23°C, only Mou and Rol cryoglobulins, both with Pr_2 cold agglutinin activity, gave a precipitin line with GM_3; under the same conditions Mou $F(ab')_2$ also gave a precipitin line, whereas Fab′ could inhibit the precipitation between GM_3 and cryoglobulin Mou. GM_3 binding was thus considered to be mediated by the antibody site. The above results confirm and extend those reported by Tsaï et al. (4) on the MKV anti-Pr_2 cold agglutinin. As in this study, neuraminidase treatment of Mou and Rol cryoglobulins abolished the cold precipitation, whereas four type I and two type II cryoglobulins were unaffected.

The fine specificity of Mou cryoglobulin towards various gangliosides was assessed by ELISA. When the gangliosides that react with Mou cryoglobulin are considered, the results suggest that the ganglioside structure that binds to IgM Mou could be the terminal sequence Neu5Ac (α, 2 → 3)-Gal, and these findings agree with those published by Roelcke et al. (25) on the antigenic determinants that react with various cold agglutinins. Binding of Mou IgM to glycophorin, but not to human chorionic gonadotropin, was also demonstrated by ELISA. Both types of molecules contain Neu5Ac (α, 2 → 3)-Gal terminal sequences, and glycophorin, the major human erythrocyte membrane sialoglycoprotein, had already been shown to react with anti-Pr_2 human antibodies (26). We have no specific interpretation for the lack of binding of IgM Mou to human chorionic gonadotropin, which has been observed for other human antibodies (27). Considering that the Neu5Ac containing carbohydrate moieties of human IgM resemble the Pr_2 and Gd targets, Tsaï et al. (4) and Weber et al. (5) suggested that cryoglobulins with Pr_2 or Gd cold agglutinin activity would self-associate at low temperature, leading to cryoprecipitation. This hypothesis may be supported by the findings of Weber et al. (5), who showed that Fc fragments of an IgM cryoglobulin with anti-Gd cold agglutinin activity coprecipitated with the whole IgM at 4°C. In this report we could not detect, by ELISA performed at 4 or 37°C, any interaction between Mou IgM_κ and three purified monoclonal IgM_λ when labeled anti-human κ-chain antibodies were used for revelation. Negative results were also obtained with a passive hemagglutination assay in which sheep RBCs coated with a panel of seven monoclonal IgM were reacted with IgM Mou, whether treated with neuraminidase or not. A comparison between structures of oligosaccharide moieties present on μ-chain and on the two distinct (27) human RBC Gd or Pr_2 specificities indicates that cross reactivity may be rather poor; indeed, the sequence Neu5Ac-Gal-Glc is present with a Neu5Ac (α, 2 → 3)-Gal liaison in GM_3 carrying Pr_2 specificity, and in Gd (5) and a Neu5Ac (α, 2 → 6)-Gal liaison in oligosaccharide moieties associated with μ-chain (28). Furthermore, Roelcke showed that Neu5Ac (α, 2 → 3)-lactose, but not Neu5Ac (α, 2 → 6)-lactose, inhibits a cold agglutinin anti-Pr_2 activity (25). In view of these negative results, we explored the

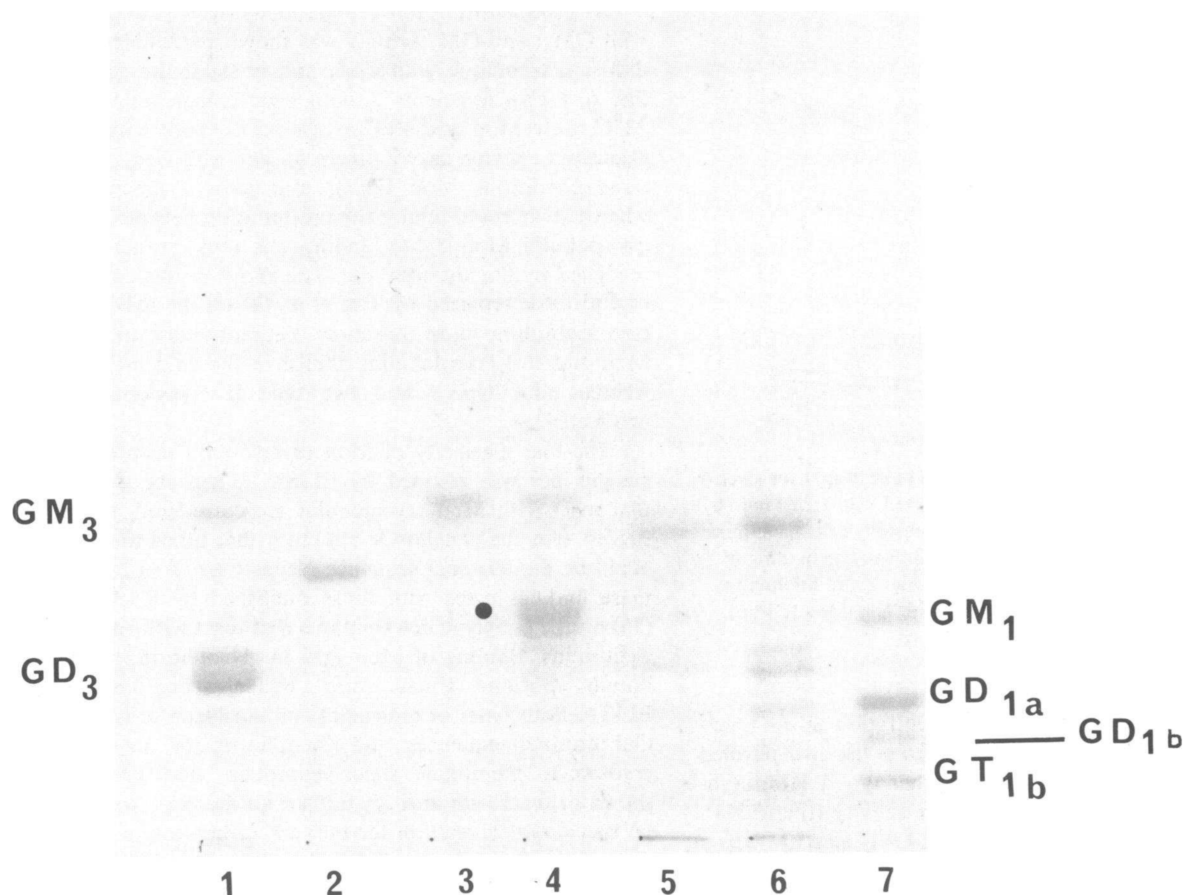


Figure 4. Thin-layer chromatography of gangliosides extracted from Mou cryoglobulin and SN IgM_{κ}. Lanes *1* to *4*: GD_3 and GM_3 (*1*), GM_2 (*2*), GM_3 (*3*), sialosylparagloboside (see dot), and GM_3 (*4*) standards; lanes *5* and *6*: gangliosides extracted from 48 mg of Mou cryoglobulin and 6 mg of SN IgM_{κ}, respectively; lane *7*: GM_1, GD_{1a}, GD_{1b}, and GT_{1b} standards.

possibility that cryoprecipitation of IgM Mou is related to an immunological interaction with GM_3. We tried to detect GM_3 in IgM Mou by two kinds of techniques. No GM_3 ganglioside could be demonstrated in Mou cryoglobulin when ELISA was used. However, this negative result may be due to the unavailability of the antigenic sequence of GM_3 to anti-GM_3 antibodies when bound to Fab Mou cryoglobulin. The availability of the same sequence to neuraminidase might be due to the smaller size of this molecule, or to different sites of action. We therefore tried to extract GM_3 from purified Mou cryoglobulin, according to Cham and Knowles (21); no ganglioside was detected after thin-layer chromatography of the lipid phase, and the cryoprecipitability of the IgM was unaffected. However, a further drastic lipid extraction led to the denaturation of IgM Mou cryoglobulin and revealed the presence of gangliosides firmly bound to the cryoglobulin; semiquantitative analysis of the resorcinol-revealed gangliosides indicated that a GM_3-like ganglioside was prominent, accounting for >75% of the extracted gangliosides. Its reference front was slightly different from that of *N*-acetylneuraminic GM_3, and this favors a modified GM_3, either a *N*-glycolyl form or a fatty acid- or sphingosine-modified GM_3. Some cross-reacting gangliosides were also detected, whereas unrelated gangliosides were not found in Mou cryoglobulin, though some are known to be present in human plasma (29). These results strongly argue against nonspecific (hydrophobic) binding of some glycosphingolipids to Mou cryoglobulin. Though unlikely, the possibility that these specific immune complexes could have only been formed in vitro cannot be completely ruled out; however, the existence of cold-induced clinical symptoms in such patients suggests that cryoprecipitation is not merely an incidental in vitro finding.

The results of lipid extraction experiments suggest that Mou cryoglobulin circulates as an immune complex where GM_3, or a cross-reacting ganglioside, is the antigen. No ganglioside could be extracted from two other purified IgM cryoglobulins treated under the same conditions.

Mou SN IgM shared the same electrophoretic mobility and cold agglutinin activity as Mou cryoglobulin had. However, unlike Mou cryoglobulin, it did not spontaneously precipitate

at 4°C, even after concentration. We have observed this phenomenon for some other type I and II cryoglobulins and it has been noted by others (30). Studies on Mou SN IgM$_\kappa$ ganglioside content after drastic lipid extraction demonstrated the presence of a GM_3-like and related gangliosides, which were more concentrated than in Mou cryoglobulin in a semiquantitative estimation. Finally, reversible cryoprecipitation at 4°C of this SN IgM$_\kappa$ could be obtained after incubation with a low concentration of micellar GM_3, but not with GM_1. This finding is reminiscent of those reported by Feizi and Marsh (31) and Roeckle et al. (32), who observed that the precipitate formed at 4°C between some cold agglutinins and solubilised extracts from RBC membranes can be dissolved at 22°C. As a whole, these results suggest that Mou cryoglobulin and Mou SN IgM$_\kappa$ are very similar and could differ only in their molar antigen/antibody ratio, with GM_3 being in molar excess in the cold-soluble IgM$_\kappa$ as compared with its cold-insoluble counterpart.

Whereas these results provide strong evidence that the creation of IgM Mou-GM_3 immune complexes forms the basis of the cryoprecipitation phenomenon, a number of questions remain unanswered. Since the extraction of gangliosides led to a denaturation of the monoclonal IgM, the stoichiometric interaction between IgM Mou and GM_3 could not be studied. Based on a semiquantitative colorimetric analysis of resorcinol-revealed gangliosides in thin-layer chromatography of Mou SN IgM$_\kappa$-extracted lipids, ~3 mol of ganglioside were estimated to be bound to 1 mol of IgM. That the addition of various concentrations of GM_3 to soluble IgM Mou (though containing GM_3) could lead either to an irreversible precipitation of Mou cryoglobulin or to a reversible cryoprecipitation of Mou SN (which is very rich in GM_3) indicates that only unique IgM-GM_3 complexes can cryoprecipitate. Similar observations were noted for IgM-IgG cryoglobulins (33).

Aside from their applicability in the clarification of the mechanism of cryoprecipitation, our findings may have some clinical significance: immune complex-type lesions had been reported in patients with type I cryoglobulins, and, in such cases, unrecognized antigen may combine with monoclonal IgM and lead to cryoprecipitation and tissue deposition.

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