

Pathological crystallization of human immunoglobulins

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Condensation of Igs has been observed in pharmaceutical formulations and in vivo in cases of cryoglobulinemia. We report a study of monoclonal IgG cryoglobulins overexpressed by two patients with multiple myeloma. These cryoglobulins form crystals, and we measured their solubility lines. Depending on the supersaturation, we observed a variety of condensate morphologies consistent with those reported in clinical investigations. Remarkably, the crystallization can occur at quite low concentrations. This suggests that, even within the regular immune response to infections, cryoprecipitation of Ig can be possible.

With the rapid growth in the development of antibody drugs, it has become apparent that some Igs can lose solubility at sufficiently high concentration (1–7). The resulting condensation into crystals, concentrated liquid phases, or aggregates is caused by net attractive interprotein interactions. In some diseases, the physiological concentration of Igs can also reach levels sufficient to cause their condensation. For example, Waldenström macroglobulinemia and multiple myeloma cause elevation of plasma monoclonal Ig levels. In particular, patients with multiple myeloma commonly overproduce IgGs, with blood concentrations as high as 70 mg/mL (8). Patients with these disorders occasionally develop a medical condition called type I cryoglobulinemia. Cryoglobulinemia is characterized by in vivo condensation of Ig (called cryoglobulins), which leads to various complications such as vasculitis, skin necrosis, and kidney failure (9). Cryoglobulins may also be responsible for important but poorly understood pathological entities associated with plasma cell dyscrasias, e.g., peripheral neuropathy, whereby microvascular injury may also contribute to small fiber axonal damage (10–12).

Cryoglobulins undergo reversible condensation upon changing temperature and concentration. Various morphologies of IgG cryoglobulin condensates from different patients have been reported, including crystals, amorphous aggregates, and gels (13). Extensive study on myeloma cryoglobulins (14–17) has yet to reveal the chemical or structural features responsible for their cryocondensations.

In this work, we demonstrate that crystallization of cryoglobulins underpins the various forms of cryoprecipitation observed in type I cryoglobulinemia. The morphology of cryoprecipitates and kinetics of their formation are strongly associated with the supersaturation of cryoglobulins. We measured the solubility lines of two cryoglobulins. Interestingly, we found that solubility of one cryoglobulin is quite low at body temperature. This result implies that Igs can crystallize at concentrations that could be reached in a broad range of pathophysiological conditions beyond multiple myeloma.

Results and Discussion

We have identified two patients with multiple myeloma (M23 and M31) with associated cryoglobulinemia. In addition, five patients in whom overproduction of monoclonal IgGs was seen without cryoglobulinemia symptoms (M8, M11, M12, and M14) were recruited as a control group. Upon lowering the temperature, cryoprecipitation, which produced aggregates of needle-shaped crystals, was observed in the blood plasma of patients

M23 and M31. In contrast, blood plasma of patients from the control group did not exhibit precipitation at temperature as low as -7°C . SDS/PAGE and ELISA experiments showed that the cryoprecipitates of M23 and M31 consist of the monoclonal human IgG1 (i.e., cryoglobulins). The cryoprecipitation begins at low temperature after a fixed lag time and is reversible, i.e., the crystals dissolve at high temperature. The presence of various blood components likely affects the cryoglobulin condensation. We have extracted the total IgGs from all blood plasma samples. The IgGs from the patients with cryoglobulinemia, M23 and M31, readily produce crystals in isotonic PBS buffer upon lowering the temperature. The IgGs of patients from the control group do not crystallize at concentrations as high as 90 mg/mL and temperatures as low as -5°C . We then purified cryoglobulins from patients M23 and M31 by recrystallization and determined the solubility lines (Fig. 1) of these two monoclonal cryoglobulins. Remarkably, IgG M23 crystallizes even at concentrations as low as 1 mg/mL and at temperatures that can occur in the extremities.

The morphology of the condensate from patient M23 varies with the degree of supersaturation (Fig. 2A). In solutions with low supersaturation (Fig. 2B), large crystals are produced. At lower temperature and higher concentration, many small crystals are formed. At substantially higher supersaturation (Fig. 2C and D), kinetically arrested amorphous aggregates or gels are generated. Similarly, M31 forms needle-like crystals in solutions at low supersaturation (Fig. 3A) and amorphous aggregates in solutions at high supersaturation (Fig. 3B).

Thus, the cause of type I cryoglobulinemia is apparently a crystallization of monoclonal Igs. This mechanism is very different from autoantibody binding of rheumatoid factors (18). Condensation of our cryoglobulins is initiated by a nucleation process characterized by a lag time. In contrast to aggregation of autoantibodies, no intermediate size IgG oligomers are produced in this process (see Fig. S2). Depending on the degree of supersaturation, the nucleation lag time for two cryoglobulins in this study ranges from 30 min to 1 d. This observation is consistent with the fact that cryoglobulinemia manifestations are associated with prolonged exposure to cold temperatures. Generally, nucleation of protein crystals is more probable at high supersaturation. Accordingly, the symptoms of cryoglobulinemia usually occur in extremities (where temperature is lower) and in kidneys (where protein is concentrated by ultrafiltration). The kinetics of cryoglobulin crystallization can also be affected by other blood components, resulting in condensates with morphologies different from that in purified solutions (Fig. 2E).

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The authors declare no conflict of interest.

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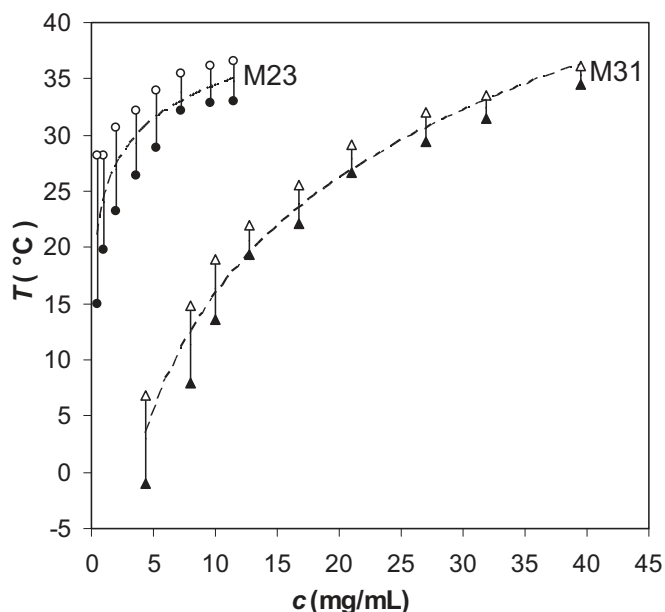


Fig. 1. Solubility of two cryoglobulins in isotonic phosphate saline buffer, pH 7.4. Crystals grow at temperatures below the solid symbols, and dissolve at temperatures above the open symbols; dashed lines represent eye guides for the solubility lines.

Protein crystallization is driven by net interprotein attraction. This net interaction can be significantly altered even by a single site mutation (19–22). Because of the diversity of Igs, they have different propensities to undergo crystallization. In the extremely large Ig repertoire in the human body, some Igs may, by chance, be able to crystallize at body temperature. Clonal selection and expression of these Igs should not be affected by their ability to crystallize. As the solubility can be quite low (Fig. 1), precipitation of such Igs might occur in a variety of circumstances such as immune response to infections, lymphoproliferative disorders, and administration of antibody pharmaceuticals. However, presence of cryoglobulins in the bloodstream, although potentially harmful, can be asymptomatic unless nucleation occurs and until crystals accumulate.

Materials and Methods

The study has been reviewed and approved by the Dana-Farber Cancer Institute (DFCI) Institutional Review Board and Massachusetts Institute of Technology

Committee On the Use of Humans as Experimental Subjects (COUHES). Patients' informed consent were obtained and documented at DFCI.

Purification of Myeloma IgG. Peripheral blood samples from patients with multiple myeloma (patients M31 and M23 with cryoglobulinemia and patients M14, M12, M11, and M8 without cryoglobulinemia) were collected in green-top tubes with sodium heparin at DFCI. To avoid possible precipitation of cryoglobulins, all of the following purification procedures were conducted at 37 °C. Blood plasmas were separated from the samples by centrifugation at $1,000 \times g$ for 5 min. Total IgGs were separated by using an affinity column (Chromatography Cartridge Protein G, 5 mL; Pierce). The purified IgGs were dialyzed into isotonic PBS solution, pH 7.4, and concentrated by using ultrafiltration membranes (Ultra, 10 kDa; Amicon). The two IgG cryoglobulins (from patients M23 and M31) were further purified by recrystallization at 4 °C, repeated four times. The purity of monoclonal IgGs was tested by SDS-PAGE under reducing [10 mM DTT, 12.6% (wt/vol) gel] and nonreducing [7.5% (wt/vol) gel] conditions (Fig. S1). The homogeneous bands signify the high purity of the monoclonal IgGs. IgG concentration was determined by UV absorbance at 280 nm by using an extinction coefficient of 1.4 L/g-cm (23). The total IgG and cryoglobulin concentrations in the plasma sample were estimated by the amount of purified protein and the initial volumes of plasma sample. The total IgG concentrations in the plasma samples were 26 mg/mL for patient M23 and 13 mg/mL for patient M31. Considering the loss of cryoglobulins during recrystallization, the cryoglobulins concentrations in plasma can be estimated to be no less than 16 mg/mL for patient M23 and 7 mg/mL for patient M31. The subclasses of the IgGs were determined by human IgG subclass ELISA (Novex; Life Technologies). The two cryoglobulins belong to the IgG1 subclass.

Solubility Measurements. The solubility of the two cryoglobulins was measured by using the following procedures. A total of 30 μ L of IgG solutions was prepared at concentrations indicated in Fig. 1. A 2- μ L aliquot of each solution were pipetted into a well on a multiwell chambered microscope slide (Culture Well; Grace Bio-Labs) and quenched to a relative low temperature to grow needle-like crystal seeds in a short time. Then, 20 μ L of the same solution was added into the well, which was sealed with a glass coverslip. After incubated at a fixed temperature for 48 h, the solution was checked under an optical microscope for growth or dissolution of the crystals. The experiment was repeated at a series of temperature with a 1 °C interval until the highest temperature for growth and the lowest temperature for dissolution were found. The average of the two temperatures was taken as the temperature at which the solubility is equal to the initial IgG concentration.

IgG Condensation at Different Degrees of Supersaturation. IgG solutions (from patients M23 and M31) with different concentrations were quenched to several temperatures lower than the solubility line. The morphology of the IgG condensate was observed under an optical microscope several times during incubation. For patients M23 and M31, crystals were observed in solution at a low degree of supersaturation (Figs. 2B and 3A), and aggregates were observed at high degree of

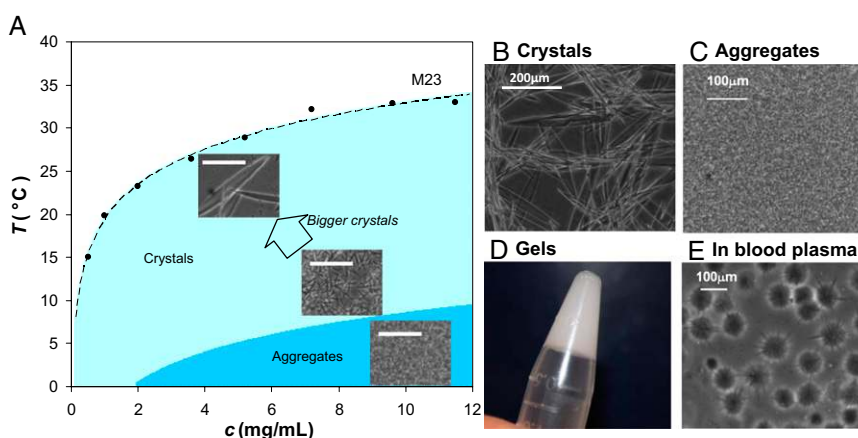


Fig. 2. (A) Correlation between morphologies of patient M23 condensates and the degree of supersaturation. (Scale bars: 100 μ m.) (B–E) Various forms of patient M23 condensates produced under different conditions: (B) 3.5 mg/mL at 19 °C, (C) 10 mg/mL at 4 °C, (D) 50 mg/mL at 4 °C, and (E) in plasma at 19 °C.

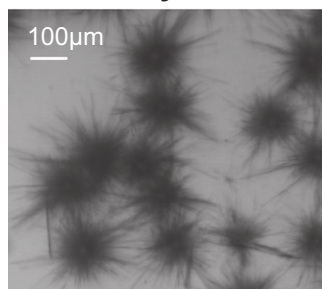
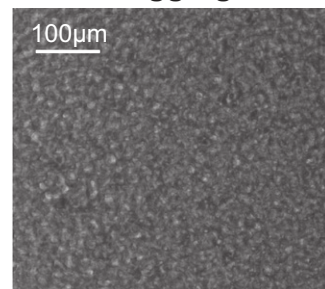
A M31 crystals**B M31 aggregates**

Fig. 3. Condensates of patient M31 cryoglobulin formed in solution with different supersaturation: (A) crystals grown from a 17 mg/mL solution at 22 °C and (B) aggregates grown from a 32 mg/mL solution at –3.6 °C.

supersaturations (Figs. 2C and 3B). A solution of IgG (from patient M23) was concentrated to 50 mg/mL by centrifugal ultrafiltration at 37 °C and

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immediately quenched to 4 °C. In this case, a gel-like solid phase was observed (Fig. 2E).

Quasielastic Light Scattering Measurements. The samples were filtered through 0.1- μ m Millipore filter. Quasielastic light scattering experiments were performed at a scattering angle of 90° by using a custom-built optical setup, a PD2000DLS^{PLUS} correlator (Precision Detectors), and a He-Ne laser (35 mW, 632.8 nm; Coherent). The hydrodynamic radii of proteins were calculated from the correlation functions by using Precision Deconvolve 5.5 software (Precision Detectors). The results are shown in Fig. S2.

CD Spectrometry Measurements. The IgGs were dialyzed into 10-mM sodium phosphate buffer, pH 7.4. CD spectra were measured with an Aviv Model 202 Circular Dichroism Spectrometer. Far UV and near UV spectra of the two cryoglobulins were measured at 37 °C and 20 °C (Fig. S3). The spectra at two temperatures overlap within experimental error. This indicates no apparent changes in protein structure upon changing the temperature.

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Supporting Information

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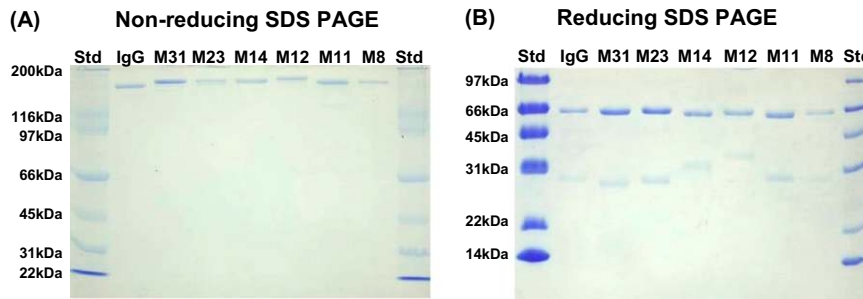


Fig. S1. SDS-PAGE of myeloma IgGs at broad range (A) and low range (B) with Bio-Rad Molecular Weights Standards (Std), IgG (monoclonal IgG control), patients M31 and M23 (IgG cryoglobulins), and patients M14, M12, M11, and M8 (noncryoglobulin myeloma IgGs).

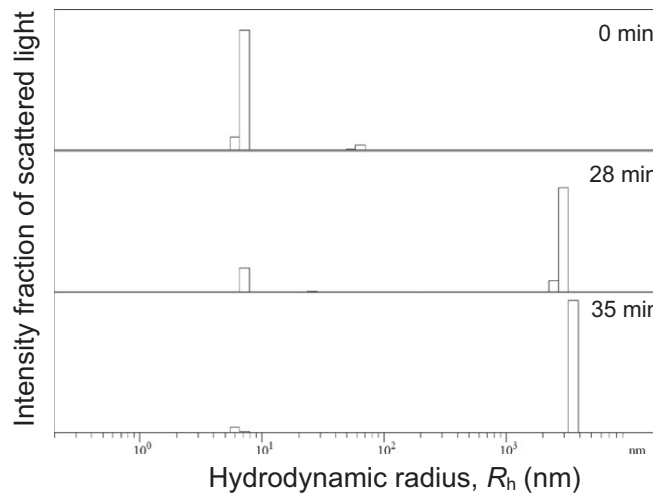


Fig. S2. Nucleation of crystallization in patient M23 monitored by quasielastic light scattering. The IgG concentration is 11 mg/mL. As incubated at 19 °C, the hydrodynamic radii of the protein in the solution at 0, 28, and 35 min are shown. The peaks at 7.2 nm indicate the IgG monomer and the peaks above 1 μm indicate large particles (crystalline) of size beyond the limit of quasielastic light scattering instrument.

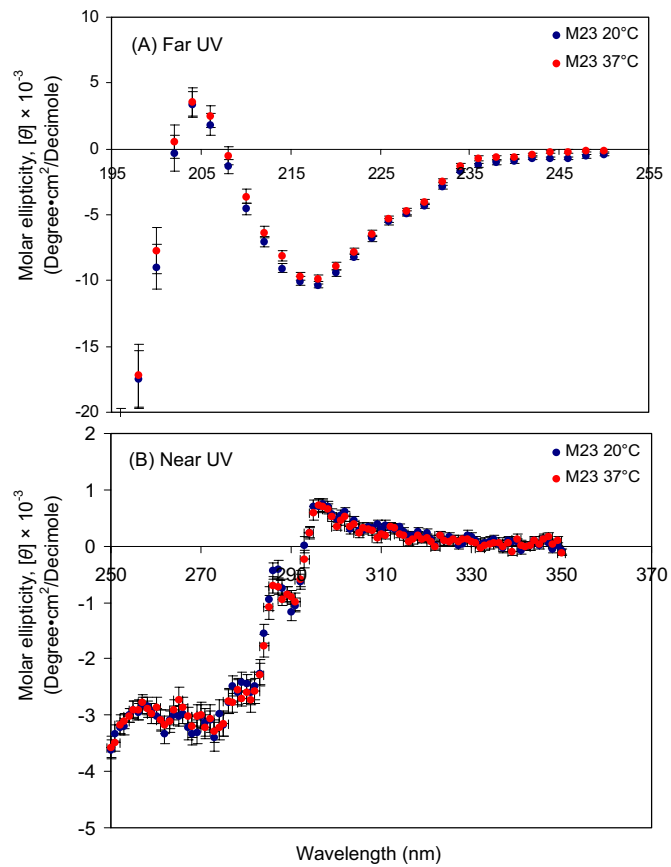


Fig. S3. Circular dichroism spectra from patient M23 at two different temperatures shows no apparent temperature dependence of the protein conformation.