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Research Article

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Cryoimmunoglobulinemia in Rheumatoid Arthritis

SIGNIFICANCE IN SERUM OF PATIENTS WITH RHEUMATOID VASCULITIS

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ABSTRACT Cryoglobulins were examined in a standardized manner in an unselected group of 35 patients with rheumatoid arthritis (RA) and 8 patients with RA complicated by cutaneous vasculitis and neuropathy. Optimum conditions for detection and characterization of cryoglobulins were established; the proportion of resolubilized to total precipitable protein remained constant in an individual patient under these conditions. All 8 vasculitis patients and 9 of 35 other patients with RA exhibited cryoglobulins; total protein and immunoglobulin content were significantly higher in the cryoglobulins of patients with vasculitis. Immunoglobulins G and M constituted two-thirds and three-quarters of the total protein in the cryoglobulins from uncomplicated rheumatoid and vasculitis patients, respectively. Serum antiglobulin titers were higher, and serum C3 levels were lower, in vasculitis patients compared to rheumatoid patients without vasculitis.

Anti-gamma globulin activity was detected in all cryoglobulins from vasculitis patients. Cryoglobulin IgG and IgM were polyclonal. Density gradient analyses demonstrated the majority of the cryoglobulin antiglobulin activity to reside in the 19S IgM fraction. There was no evidence of a light weight (8S) IgM. A monoclonal rheumatoid factor did not detect 7S-anti-7S complexes in the cryoprecipitates, but acid eluates from some cryoglobulins absorbed with insoluble IgG revealed an antiglobulin of the IgG class.

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Serial studies performed on vasculitis patients treated with cyclophosphamide disclosed a relationship between clinical evidence of vasculitis and the presence of cryoglobulins. The antigen (IgG) and antibody (largely IgM rheumatoid factor) nature of these cryoglobulins is presented as evidence that the widespread vascular complications of RA are mediated, at least in part, by circulating immune complexes.

INTRODUCTION

Cryoglobulins, or cold-precipitable immune globulins, were first observed in the setting of reticuloendothelial malignancy (1). Subsequent studies of these proteins have focused largely on the physicochemical nature of cryoprecipitation and the immunochemical properties that determine their unique solubility characteristics (2). Fourteen years after the original observation a patient was described who exhibited a similar phenomenon in the absence of malignancy and whose clinical features suggested an immunologically mediated disease (3). In recent years the emphasis of investigations concerning these proteins has changed. Now they are being viewed as circulating immune complexes because of the demonstration that they contain appropriate antigens, antibodies, or other immunoreactants that may relate to the pathogenesis of the disease they accompany (4). For example, cryoglobulins occur regularly in the sera of patients with diseases of known infectious etiology associated with "autoimmune" features, such as infectious mononucleosis (5, 6), leprosy (7), poststreptococcal glomerulonephritis (8), cytomegalovirus syndromes (6, 9), subacute bacterial endocarditis (10, 11), and tropical splenomegaly syndrome, resulting from chronic malarial exposure (12). Cryoglobulins have, in addition, been described in rheumatic disorders

of unknown etiology such as systemic lupus erythematosus (13), the "purpura-weakness-arthritis" syndrome (14), immunologic syndromes involving the kidney (15) and in New Zealand Black mice (16), and in a primary or "essential" form (17).

In rheumatoid arthritis (RA),¹ cryoglobulins (18, 19) and immune complexes (20) are found in synovial fluid and these are thought to play a role in the pathogenesis of the articular inflammation that occurs in that disease (21). In the sera of such patients, however, immune complexes are only occasionally demonstrated (22), and spontaneous precipitates are infrequently recognized in refrigerated rheumatoid sera (23, 24). When large numbers of patients with systemic "autoimmune" diseases are screened for cryoglobulins, some patients with RA are noted (25, 26).

In order to determine the true incidence and the biologic significance of cryoglobulins in RA, a systematic analysis has been carried out. The data presented demonstrate that cryoglobulins are regularly detected in rheumatoid sera when a standardized methodology is used. The amount of cryoprecipitable protein is smaller than that usually found in other cryoglobulinemic syndromes. Rheumatoid cryoglobulins contain immunoglobulins and complement components. They have specific antibody activity, primarily anti-gamma globulin. RA patients with the largest amounts of cryoglobulins in their circulation also had systemic (i.e., vascular) complications of RA. Long-term serial studies of vasculitis patients treated with the immunosuppressive agent cyclophosphamide disclosed an association between the presence of cold-precipitable IgG-anti-gamma globulin complexes and the systemic vascular features of RA. The nature of this association is discussed.

METHODS

Patients. Samples were obtained from 38 patients with RA, who were encountered in follow-up visits to a rheumatology clinic or hospitalized for either orthopedic or medical complications of their disease. They were chosen at random over a 6-mo period (January-June 1972). Patients with rheumatoid vasculitis were chosen consecutively as they were identified over a 12-mo period (January-December 1972). Patients with RA were representative of a total population of clinic-based rheumatoids; patients with vasculitis were either discovered in our clinic based population (W. H.), referred to our rheumatology division for evaluation of vasculitis (J. S., D. A., M. H., and L. T.), or hospitalized at the Veterans Administration Hospital for vasculitis (D. M., J. S., and R. M.). All patients (including those with vasculitis) met the American Rheumatism Association criteria for definite or classical RA (27).

Collection of sera and quantitation of the cryoglobulins. All samples were handled in identical fashion. Blood was drawn into sterile glass tubes (Vacutainer, Becton-Dickinson & Co., Rutherford, N. J.) and allowed to clot at 37°C

in a water bath. Serum was obtained after centrifugation at 1,500 *g* for 10 min at room temperature. 5 ml of serum was kept at 4°C for 72 h. After centrifugation at 1,500 *g* for 10 min at 4°C the supernate was decanted and saved. The precipitate formed was washed three times with an excess of cold normal saline (0.15 M). Solubilization of the precipitate took place in two steps: first in 0.5 ml of twice normal saline (0.3 M) with incubation for 1 h at 37°C, then 0.5 ml of distilled water was added followed by incubation for another hour at 37°C. The final 1-ml solution in normal saline (0.15 M) was centrifuged at room temperature; any insoluble material was discarded. The specimens were kept at room temperature throughout the series of experiments.

In studies examining alternative methods for solubilizing the cryoprecipitates 15 ml of fresh rheumatoid serum was divided into three equal parts. The procedure was the same as that described above up to the final wash in cold normal saline. At that point the packed cryoglobulins were solubilized with either 1.0 ml of 1 N NaOH or 1.0 ml of 0.06 M glycine HCl (pH 3.2) or the regular two-step procedure of 0.5 ml of 0.3 M NaCl followed by 0.5 ml of water. In another series of experiments cryoprecipitates solubilized by the routine method were compared to those solubilized in 1.0 ml of 0.06 M glycine HCl (pH 3.2) at 37°C for 1 h, followed by neutralization with the addition of a few drops of 1.0 N NaOH. The neutralized, solubilized cryoprecipitate was then dialyzed overnight at room temperature against a large volume of normal saline.

Characterization of the cryoglobulins. The total protein was measured by the Lowry method using an IgG standard of known protein content (28). The concentrations of specific immunoglobulins were determined by radial immunodiffusion in gel employing commercial monospecific antibodies for IgA and IgM (Hyland Div., Travenol Laboratories, Inc., Los Angeles). The lower limit of detection of IgM was 0.04 mg/ml and IgA was 0.02 mg/ml. IgG concentrations were measured in a similar manner with a rabbit anti-human IgG antibody, which, at a concentration of 2% (0.2 ml antisera to 9.8 ml 1% agarose in 0.06 M Veronal buffer, pH 8.6) could detect IgG in amounts as small as 0.03 mg/ml. Complement components C1q and C3 were recorded as present if a precipitin line was obtained in Ouchterlony analysis with monospecific antisera.

A number of procedures were examined to maximize the yield of soluble cryoprotein. Resuspending the initial precipitate in a volume of saline greater than one-fifth of the starting serum only minimally increased the total yield of proteins and significantly reduced the concentrations; suspension in volumes less than one-fifth of starting serum resulted in only a slight reduction of total protein yield. The protein concentration in the final solution remained the same regardless of the starting volume of sera, provided a 5:1 ratio of initial sera to diluent volume was maintained.

Table I demonstrates the effect of various diluents on the protein and immunoglobulin (IgG and IgM) yield as well as the antibody constituents of the cryoprecipitates. Incubation with NaOH at 56°C for 30 min was used as the standard for complete protein solubilization. This treatment denatures protein with a resultant loss of immunoglobulins and antibody activity. Use of an acidic diluent resulted in complete solubilization of the cryoprecipitate (comparable to alkali treatment) and a variable increase in immunoglobulin levels. C3 and rheumatoid factor activity were destroyed. Cryoglobulin antinuclear activities and the complement component C1q were also eliminated

¹Abbreviations used in this paper: BDB, bis-diazotized benzidine; RA, rheumatoid arthritis.

(not shown in this table). If the acidic solution was neutralized promptly and dialyzed overnight the antibody activities could be retained; however, this caused a variable portion of the previously soluble material to reprecipitate. Therefore, using 0.5 ml of twice normal (0.3 M) saline for solubilization followed by the addition of an equal volume of distilled water proved most satisfactory for optimizing the yield of soluble cryoprotein, retaining its immunologic reactivity, and ensuring its subsequent stability at room temperature. A single large serum sample obtained from a subject with a known cryoglobulin was divided into five samples of 5 ml each. Replicate analysis by the method outlined above yielded 0.59 ± 0.03 mg protein/ml.

Serial determinations were performed on a number of patients. Those patients demonstrating cryoglobulins on one occasion had them on most other examinations except when treatment with immunosuppressive drugs (cyclophosphamide) was employed. Absence of cryoglobulins on one determination usually meant they were undetectable in multiple samples.

The proportion of protein solubilized from the actual precipitate was not constant and varied from patient to patient, ranging from 15 to 40% of the total, but for any given patient this proportion was remarkably stable at different times and with disparate amounts of cryoprecipitates.

Serologic studies. Antinuclear factors were detected by an indirect immunofluorescent technique employing mouse kidneys as the source of nuclei. Antisera was made in rabbits by using IgG isolated from human serum by 50% ammonium sulfate precipitation and purification by DEAE-cellulose chromatography. Fluoresceinated conjugates were prepared with fluorescein isothiocyanate by the method of Nairn (29).

The protein-fluorescein ratio of the conjugate was 5:2. Fluorescent microscopy was performed with a Zeiss microscope (Carl Zeiss, Inc., New York) using an HBO 200 mercury vapor lamp with a BG 38 exciter filter and barrier filter setting at 53. Anti-gamma globulin (rheumatoid factor) activity was measured by the bentonite flocculation test (30). Serum C3 was measured by using commercial radial immunodiffusion plates (Hyland Div., Travenol Laboratories).

Immunologic techniques. Sucrose density gradient ultracentrifugation was usually carried out by layering 0.5-ml samples over a 4.5-ml linear 10-40% sucrose gradient constructed in phosphate-buffered saline (pH 7.2). When mild dissociating conditions were desired, the experiments were conducted with a 0.1 M acetate buffer (pH 4.2). For experiments requiring larger volumes, 30-ml gradients were constructed with sucrose concentrations ranging from 5 to 20%. All density gradient experiments were run for 18-20 h in duplicate. A third gradient containing a 1:2 dilution of normal serum was included with each run. Fractions from this control tube were analyzed by double diffusion in gel to determine the location of IgG and IgM.

Gel filtration chromatography was done with Sephadex G-200 which was washed and equilibrated with 0.1 M acetate buffer (pH 4.2). Resolution of IgG and IgM was accomplished by upward flow on a 2.5×100 -cm column. The various peaks were analyzed by their $OD_{280 \text{ nm}}$, concentrated to 1-2 ml by positive pressure dialysis, dialyzed overnight against phosphate-buffered saline (pH 7.2), and analyzed by radial immunodiffusion.

Protein concentrations were determined by a modification of the Lowry method (28) and immunoglobulin levels were determined on commercial quantitative immunodiffusion

TABLE I
Effect of Normal Saline, Acidic Buffer, and NaOH Incubation on the Yield of Soluble Cryoproteins and their Immunoglobulin, Complement, and Antibody Constituents

Diluent solution*	Protein concn	IgG	IgM	C3‡	BFT§
	mg/ml	mg/ml	mg/ml		
0.15 M NaCl	0.16	0.125	0.045	+	20
Glycine HCl¶	0.31	0.125	0.02	0	0
1.0 M NaOH	0.34	0	0	ND	ND
0.15 M NaCl	0.16	0.13	0.05	+	160
Glycine HCl	0.57	0.195	0.185	0	0
1.0 M NaOH	0.53	0	0	ND	ND
0.15 M NaCl	0.35	0.26	0.05	+	0
Glycine HCl	0.92	0.46	0.24	0	0
1.0 M NaOH	0.92	0	0	ND	ND

* Diluent used to resuspend cryoprecipitate. A 5:1 ratio of initial serum to diluent volume was maintained for all samples.

‡ Recorded as present or absent in Ouchterlony analysis.

§ Reciprocal of bentonite flocculation titer.

|| ND, not done; 0, not detected; +, present.

¶ Glycine HCl is 0.06 M, pH 3.2.

plates (low level) with appropriate standards (Melyo Laboratories Inc., Springfield, Va.).

An insoluble IgG immunoabsorbent was prepared by coupling heat-aggregated IgG to Sepharose 4B. 15 g of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) was washed repeatedly with a total of 3,000 ml of 0.001 N HCl (pH 3.0). After the last wash the gel was resuspended in 52 ml of a 0.1 M sodium bicarbonate-0.5 M NaCl buffer (pH 9.2) containing 9.5 mg/ml of human IgG (Cohn fraction II, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) that had been heated for 20 min at 63°C. After overnight stirring at 4°C the suspension was centrifuged at 2,500 g for 30 min. The supernate was decanted and its IgG content measured to determine the percent IgG coupled to Sepharose (63%). The gel was washed extensively with bicarbonate buffer (pH 9.2) until the $OD_{280 \text{ nm}}$ of the wash was less than 0.01 and then resuspended in 1 M ethanolamine (pH 8.0) for 2 h at room temperature. After this the immunoabsorbent was washed three times with a buffer of 0.1 M acetate (pH 4.2) and 1 M NaCl, alternating with 0.1 M borate (pH 8.0) and 1 M NaCl, and then suspended in 50 ml of phosphate-buffered saline (pH 7.2). 5-ml aliquots of this Sepharose 4B mixture containing approximately 30 mg of coupled IgG were packed by centrifugation. The supernate was discarded and the immunoabsorbent resuspended in 1 ml of cryoglobulin-containing solution. After incubating for 60 min at 37°C and overnight at room temperature, the immunoabsorbent was separated by centrifugation. The supernate was removed with a Pasteur pipette and analyzed for residual immunoglobulin, light chains, and anti-gamma globulin activity.

Determination of anti-gamma globulins. Antibody to human IgG was measured by the bentonite flocculation test as described (30). Serum dilutions were in normal saline. Cryoglobulins were diluted in normal saline containing 1.0

mg/ml of bovine serum albumin. Cold-reactive antiglobulins were determined after overnight incubation at 4°C. Precipitation reactions were performed in 1% agar with the test material in a central well surrounded by wells containing heat-aggregated (63°C for 20 min) human IgG in concentrations ranging from 1.0 to 0.125 mg/ml. The plates were maintained at room temperature and read at 24 to 48 h. The immunoglobulin class of the anti-gamma globulin factors was determined by absorption to insolubilized human IgG (Cohn fraction II). Insoluble IgG was prepared by cross linking with bis-diazotized benzidine (BDB) by the method of Ishizaka, Ishizaka, Salmon, and Fudenberg (31). Serum samples of 1.0 ml at a 1:2 dilution or undiluted cryoprecipitates in a volume of 1.6 ml were incubated with 10 mg of BDB-aggregated IgG for 1 h at 37°C and then 2 h at 4°C. Aggregates were packed by centrifugation at 500 rpm for 20 min at 4°C and the supernates decanted. The packed aggregates were resuspended in borate-buffered normal saline (pH 8.0) and incubated for 1 h at 37°C. After incubation the mixture was centrifuged, the wash discarded, and the bound protein was eluted by incubation for 1 h at 37°C with 0.5 ml of a 0.1 M acetate buffer (pH 4.2). The precipitate was repacked by centrifugation and the supernate containing the eluted immunoglobulin was carefully removed with a Pasteur pipette. The eluates were neutralized immediately with 0.1 M NaOH and analyzed for immunoglobulin content and anti-gamma globulin activity.

Reduction and alkylation of serum and cryoprecipitates with 0.1 M 2-mercaptoethanol and 0.02 M iodoacetamide was accomplished as described (32).

Statistical analysis. Statistical analyses were performed on a Burroughs 6700 computer (Burroughs Corp., Detroit, Mich.) using standard Student's *t* test and linear regression analysis techniques.

RESULTS

Optimum conditions for detection and characterization of the cryoglobulins. Serum kept at 4°C showed little visible precipitation at 24 h; from 24 to 48 h the serum became opalescent, and by 72 h precipitation was maximal. Cryoprecipitates allowed to stand from 5 to 10 days at 4°C showed little more precipitation

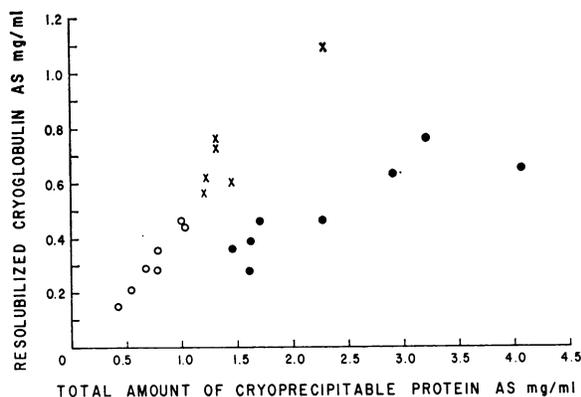


FIGURE 1 The relationship between total amount of cryoprecipitable protein and the amount resolubilized for consecutive samples in three patients.

TABLE II
Protein, Complement, and Antibody Constituents of RA Cryoglobulins

Protein	IgG	IgM	Clq*	C3*	ANA†	Anti-gamma globulins‡
mg/ml	mg/ml	mg/ml				
0.15	0.07	0.05	+	+	0	0
0.16	0.10	0.04	0	+	0	10
0.18	0.08	0.05	0	+	4	80
0.18	0.06	0	0	0	0	20
0.18	0.085	0	0	0	0	0
0.19	0.04	0.12	0	0	0	0
0.20	0.06	0.09	0	0	0	0
0.24	0.11	0.05	0	0	4	0
0.24	0.085	0	+	0	0	80
0.30	0.10	0.11	0	0	0	10
0.32	0.14	0.08	+	+	4	40
0.66	0.125	0.50	+	0	0	160

* Recorded as present (+) or absent (0).

† Reciprocal of antinuclear antibody titer.

‡ Reciprocal of bentonite flocculation titer.

and were often more difficult to solubilize. Fig. 1 displays the relationship between the total amount of cryoprecipitate and the amount resolubilized for consecutive samples, followed over time in three patients. Percentage resolubilized for each patient remained in a narrow range (14–20%, 29–36%, and 26–34%).

The cryoglobulins of RA. For all studies reported herein, patients were designated to have cryoglobulins if the protein concentration in the final cryoglobulin solution was equal to or greater than 0.15 mg/ml. The reason for selecting this value is explained in detail in the discussion. Of the initial 38 rheumatoid patients studied, 12 had significant amounts of cryoglobulins.

Protein constituents of the cryoglobulins. Table II shows an analysis of the RA cryoglobulins. Protein concentrations ranged from 0.15 to 0.66 mg/ml with a mean of 0.25 mg/ml. Three patients with RA complicated by vasculitis (two had dermal infarctions, all three sensory-motor neuropathy) are included in this group. They had the greatest amounts of protein: 0.30, 0.32, and 0.66 mg/ml.

All the cryoglobulins contained a measurable amount of IgG, 9 of 12 contained IgM, and none had IgA. When both classes of immunoglobulins were detected in a cryoglobulin, the relative amount of IgM compared to IgG was significantly greater than found in serum. Immunoglobulins G and M made up 67% of the total protein. The complement components, Clq or C3, were detected in six precipitates.

For comparison, sera from 14 control subjects were studied. 3 of 14 developed cryoprecipitates with protein concentrations equal to or greater than 0.15 mg/ml (0.18, 0.20, and 0.20 mg/ml). In contrast to the rheumatoids, however, barely detectable levels of IgG were

TABLE III
Clinical Description of Eight Patients with Rheumatoid Vasculitis

Patient	Age	Sex	Duration of RA*	Steroid dose‡	Neuropathy		Vasculitis		Rheumatoid nodules
					Peripheral sensory	Severe sensory-motor§	Dermal infarctions	Digital gangrene	
D. A.	46	F	3	40	+	2	+	0	+
M. H.	72	F	>25	10	+	3	+	+	+
L. T.	50	F	25	15	0	1	+	+	0
W. H.	72	M	30	5	+	1	+	0	+
J. S.	60	M	3	15	+	3	+	0	0
R. M.	46	M	8	30	+	?	+	+	+
J. S.	58	M	12	0	+	3	+	0	+
D. M.	76	M	23	15	+	4	+	0	+

* Years preceding vasculitis.

‡ Milligrams prednisone per day at time of vasculitis.

§ Denotes number of major sensory-motor nerves involved.

|| Milligrams prednisone every other day at time of vasculitis.

the vasculitic event ranged from 3 to 30 yr with an average of 16 yr. All but one patient was receiving steroids at the time of the appearance of vasculitis; five of the seven were taking prednisone in a daily dose equal to, or greater than, 15 mg.

The clinical presentation of rheumatoid vasculitis in all eight patients included neuropathy. Seven had clear-cut major sensory-motor nerve damage in one or more extremities; the eighth patient had such extensive extremity infarction and gangrene that it was impossible to document peripheral nerve involvement. Two patients had a mononeuropathy limited to one extremity; the rest had mononeuritis multiplex: one involving two major motor nerves, three patients each with three nerves involved, and one with motor nerve lesions in all four extremities (Table III).

All patients had dermal vasculitis with skin infarction. Three had frank digital gangrene. Rheumatoid nodules were palpated in six patients.

Immunologic features. Rheumatoid factor was present in significantly higher titers in the serum of patients with vasculitis than in those without vasculitis ($P < 0.001$) (Fig. 4). Although there appears to be a trend, there was no significant statistical difference ($P = 0.06$) between the rheumatoid factor titers of vasculitis patients and the nine rheumatoids with cryoglobulins without vasculitis. Antinuclear factor was detected in a few members of each group, but the titer elevations were not different.

Serum C3 levels (Fig. 5) were not significantly lower in patients with vasculitis (mean, 129 mg/100 ml) when compared to rheumatoids with cryoglobulins but without vasculitis (mean, 148 mg/100 ml), but the mean

C3 concentration of the vasculitis patients was significantly lower than the mean of 154 mg/100 ml for the entire group of 35 patients with RA without vasculitis ($P = < 0.01$).

Comparison of RA cryoglobulins and RA vasculitis cryoglobulins. As noted above, rheumatoid patients were said to have cryoglobulins if the protein concentration of the cryoglobulin is equal to or greater than 0.15 mg/ml. By this definition, all of the eight vasculitis patients exhibited cryoglobulins; they will be compared to the nine cryoglobulins from rheumatoids without vasculitis.

Composition. The total protein of the vasculitis cryoglobulins ranged from 0.15 to 0.66 mg/ml with a mean of 0.37 mg/ml (Fig. 6 and Table IV). This was significantly higher than found in the cryoglobulins from rheumatoid patients without vasculitis ($P = 0.01$). All eight vasculitis cryoglobulins contained measurable amounts of IgG and IgM, accounting for more than 75% of the total protein. The mean IgG and IgM concentrations were 0.14 and 0.15 mg/ml both of which were significantly higher than found in the rheumatoid cryoglobulins ($P = 0.01$ for IgG, $P = 0.03$ for IgM). There was no difference between the two groups of patients in the frequency of antinuclear factors or complement components (C1q or C3) detected in their cryoglobulins.

Anti-gamma globulin activity. All eight vasculitis cryoglobulins contained anti-gamma globulin activity (Table IV); in comparison only four of nine rheumatoid cryoglobulins had measurable amounts of this antibody. Vasculitis cryoglobulin rheumatoid factor titers ranged from 1:10 to 1:512; these titers were not in-

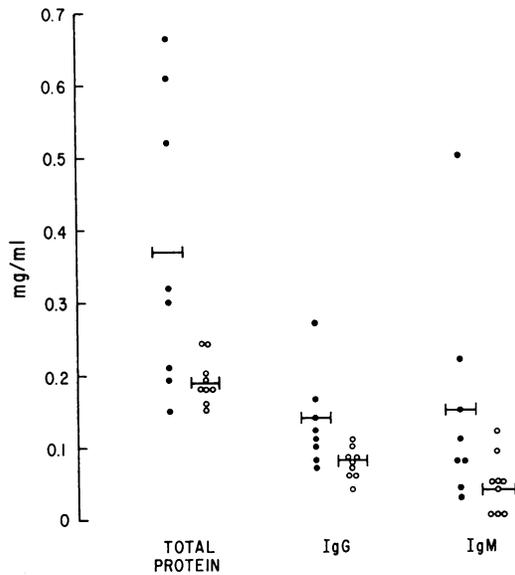


FIGURE 6 A comparison of total protein, IgG, and IgM content between vasculitis cryoglobulins (●) and cryoglobulins from rheumatoids without vasculitis (○).

to IgG, proportionally more IgM is in the cryoprecipitate than in the companion serum.

A number of experiments were done to define the immunoglobulin class of the anti-IgG antibodies. Sera and cryoglobulins were examined for anti-gamma globulin activity by the bentonite flocculation test before and after treatment with 2-mercaptoethanol. All activity in the serum was removed by the treatment. Initially, it appeared that the cryoprecipitates were resistant to mercaptoethanol, with at most a one or two tube reduction in titer. Subsequently, it was found that reduction fol-

lowed by alkylation eliminated the rheumatoid factor activity, suggesting that recombination occurred in the absence of the alkylating agent (iodoacetamide). This was further supported by the observation that the anti-gamma globulin activity was destroyed by reduction alone if the cryoglobulin (0.1 ml) was added to normal human serum (0.4 ml) before reduction.

Sucrose density gradients were run in acid buffers to prevent reassociation of the cryoprecipitates in the cold. In more than a dozen studies, with but one exception, the anti-gamma globulin activity was limited to the 19S region. In that one instance, only very low titers (1:2 to 1:4) were detected in the light fractions from the gradient.

The absence of IgG in the heavier fractions of the gradients was not unexpected since the acid buffer should dissociate complexes of IgG and IgM anti-gamma globulins. If IgG and 7S (IgG) anti-gamma globulin complexes were present they also would be separated, but after neutralization they might recombine and form heavier complexes. In two experiments the cryoglobulin with the most IgG was separated on a 30-ml acid density gradient. The fractions containing IgG were combined, concentrated, dialyzed extensively against a phosphate saline buffer (pH 7.2), and run through a 5-ml neutral (pH 7.2) density gradient. Analysis of the fractions from the neutral density gradient showed IgG limited entirely to the lighter (7S) region. In a similar type of experiment the cryoprecipitate was acidified by dialysis and chromatographed over Sephadex G-200 with 0.1 M acetate buffer (pH 4.2). The fractions from the peak containing IgG were combined, dialyzed, and concentrated to 1.0 ml. The IgG (0.5 ml of this solution) was separated on a

TABLE IV
Immunological Characteristics of Patients with Rheumatoid Vasculitis

Patient	Cryoglobulin						Serum			
	Protein	IgG	IgM	Clq*	C3*	ANA‡	Anti-globulins§	Anti-globulins§	ANA‡	C3
	mg/ml	mg/ml	mg/ml							mg/100 ml
D. A.	0.66	0.125	0.50	+	0	0	512	160	<10	180
M. H.	0.19	0.12	0.08	0	+	0	160	2,560	<10	140
L. T.	0.61	0.165	0.22	0	0	0	160	640	50	92
W. H.	0.30	0.10	0.11	0	0	0	10	320	10	155
J. S.	0.32	0.14	0.08	+	+	4	40	2,560	400	120
R. M.	0.52	0.27	0.15	0	0	0	80	5,120	10	120
J. S.	0.21	0.08	0.03	+	+	0	40	10,240	10	105
D. M.	0.15	0.07	0.05	0	0	0	40	2,560	<10	120

* Recorded as present (+) or absent (0).

‡ Reciprocal of Antinuclear antibody titer.

§ Reciprocal of bentonite flocculation titer.

TABLE V
Absorption of Cryoglobulins with an IgG Immunoabsorbent*

Subject	Preabsorption				Postabsorption		
	Protein	IgM	RF‡	Precipitin§	IgM	RF‡	Precipitin§
	mg/ml	mg/ml		mg	mg/ml		mg
M. R.	0.34	0.18	640	0.25	0	±10	0
D. A.	0.85	0.47	320	0.50	0	0	0

* Heat-aggregated (63°C for 20 min) human IgG coupled to Sepharose 4B. 5 ml of packed immunoabsorbent containing approximately 30 mg of IgG was incubated with 1 ml of concentrated cryoglobulin solution for 60 min at 37°C overnight at room temperature.

‡ Reciprocal of the rheumatoid factor titer by the bentonite flocculation method.

§ Precipitin reaction = the smallest concentration of heat-aggregated (63°C for 20 min) human IgG giving a precipitin line by Ouchterlony double diffusion method in agarose gel.

5-ml neutral gradient (pH 7.2). No IgG was detected in fractions heavier than 7S.

A purified monoclonal rheumatoid factor (kindly supplied by Dr. R. Winchester) that detects IgG anti-IgG complexes was used to examine vasculitis cryoglobulins. Experiments were carried out with two precipitating systems—double diffusion in gel and layering the monoclonal rheumatoid factor over the test solution in capillary tubes. Neither system demonstrated gamma globulin complexes in the cryoglobulins, while some RA sera and most RA synovial fluids tested were positive.

Concentrated cryoglobulin solutions from two patients with vasculitis were incubated with BDB-insolubilized IgG, washed, and eluted with acid. The results were compared to two normal sera and a rheumatoid serum containing a high titer of rheumatoid factor (1:20,000 by the bentonite flocculation method). As seen in Table VI, this technique detects IgG anti-gamma globulins in normal serum and IgG and IgM antiglobulins in the

rheumatoid sample. Under the same conditions, the two cryoglobulins released amounts of IgG and IgM comparable to RA serum despite the fact that there were much smaller amounts of immunoglobulin (especially IgG) in the starting samples. The agglutinating activity in the eluates probably was accounted for by the IgM in the cryoprecipitates.

Clinical studies. Because of the potential life-threatening consequences of rheumatoid vasculitis, combinations of intravenous and/or oral cyclophosphamide were administered to all of the five patients with mono-neuritis multiplex and to the one patient with widespread extremity gangrene. The two patients with neuropathy limited to one extremity were not treated with cytotoxic drugs. Serial cryoglobulin determinations were performed on five of the six treated and one of the two untreated patients.

Two patients were administered a single intravenous (500 mg) dose followed in 2 wk by continuous oral

TABLE VI
Absorption of Serum or Cryoglobulin Samples with Aggregated (BDB) IgG Followed by Acid Elution

Sample*	Starting material				Eluates		
	Protein	IgG	IgM	RF‡	IgG	IgM	RF‡
	mg/ml	mg/ml	mg/ml		mg/ml	mg/ml	
NHS no. 1	—§	10.0	0.33	0	0.083	0	0
NHS no. 2	—	10.2	1.5	0	0.057	tr§	0
RAS	—	10.8	4.9	8,192	0.093	0.10	512
Cryo no. 1	1.40	0.62	0.42	1,280	0.093	0.14	512
Cryo no. 2	0.60	0.16	0.28	1,024	0.032	0.056	256
Saline	—	—	—	—	0	0	0

* NHS, normal human serum; RAS, rheumatoid arthritis serum; Cryo, vasculitis cryoglobulin.

‡ Rheumatoid factor titer determined by bentonite flocculation method.

§ —, not done; tr, trace (less than 0.030 mg/ml).

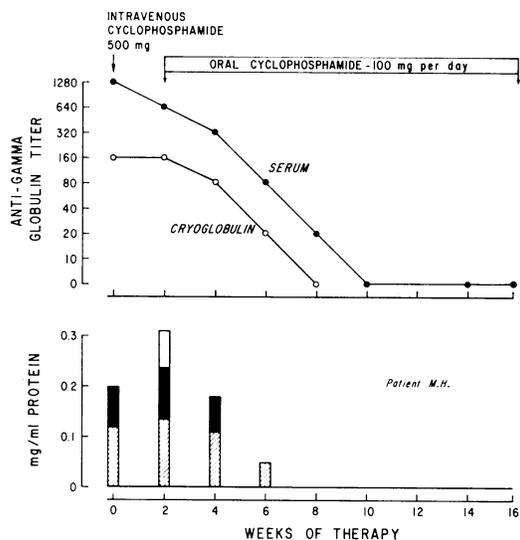


FIGURE 7 The upper graph depicts serum (●—●) and cryoglobulin (○—○) anti-gamma globulin (rheumatoid factor) titers in patient M. H., who received both intravenous and oral cyclophosphamide therapy. The lower graph depicts the total protein (entire bar), IgG (hatched bar), and IgM (solid bar) content of M. H.'s cryoglobulin.

cyclophosphamide at 100–150 mg/day. The course of one of them (M. H.) is shown in Fig. 7. In this patient the cryoglobulin disappeared from her serum by the 8th wk of therapy and never returned during the 4-mo observation period. The other patient (not shown) had a small cryoglobulin initially (0.15 mg/ml), which disappeared within 1 wk of therapy and did not return during 1 yr observation. M. H.'s serum rheumatoid factor activity diminished and was no longer detectable at 10 wk. In the other patient, it was significantly reduced (from 1:2,560 to 1:80) at 12 wk. Neither patient exhibited new signs of vasculitis once treatment had begun.

R. M. (Fig. 8) was treated with two intravenous doses of cyclophosphamide (480 mg each), but concomitant infection prevented the use of oral maintenance therapy. The amount of cryoglobulins diminished only slightly, and the serum rheumatoid factor fell two tube dilutions. However, the anti-gamma globulin activity of the cryoglobulin declined to zero and was only detected once after the 11th wk after intravenous therapy. IgM could not be measured in the cryoglobulin after the 15th wk. Evidence of further vasculitis was not observed during the 24 wk of follow-up.

A fourth patient (D. A.) was treated with oral cyclophosphamide, 150 mg/day. Her serum rheumatoid factor remained unchanged during the 8-mo observation period, but the cryoglobulin disappeared. The clinical signs of vasculitis regressed.

An additional patient (J. S.) received two intravenous doses of cyclophosphamide (500 mg) each. Oral therapy was attempted, but could not be continued because of gastrointestinal and bladder toxicity. Throughout the 19 wk of observation, his cryoglobulins tended to increase in amount and the rheumatoid factor activity of the cryoglobulin and the accompanying serum was unchanged. New dermal vasculitic lesions appeared during the first 8–10 wk of his hospital course.

Finally, one patient (L. T.) was followed who did not receive any cyclophosphamide (Fig. 9). During the 22 wk of observation the cryoglobulin content of her serum did not change appreciably and the rheumatoid factor activity of her cryoglobulin and serum remained constant. She did not exhibit any additional signs of vasculitis despite the persistence of these abnormalities.

DISCUSSION

Conditions for detection, quantification, and characterization of cryoglobulins vary considerably from one laboratory to another. A number of these variables were

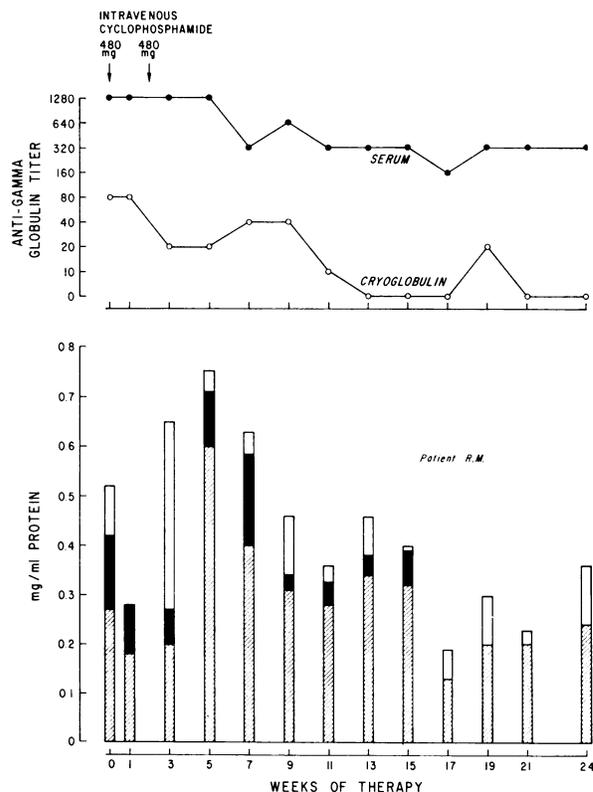


FIGURE 8 The upper graph depicts serum (●—●) and cryoglobulin (○—○) anti-gamma globulin (rheumatoid factor) titers in patient R. M., who received only intravenous cyclophosphamide therapy. The lower graph depicts the total protein (entire bar), IgG (hatched bar), and IgM (solid bar) content of R. M.'s cryoglobulin.

examined in an attempt to develop a uniform procedure for detecting and quantifying rheumatoid cryoglobulins. Optimum solubilization (approaching 90% of the total protein in the cryoprecipitates) was obtained with acid solutions, but unless these solutions were neutralized immediately, there was a loss of antinuclear and anti-IgG activity. Neutralization followed by overnight dialysis was most satisfactory in terms of yield and retention of antibody activity but proved too cumbersome when handling multiple samples. Therefore, to facilitate comparisons between different groups of patients and in serial determinations in the same patient we elected to standardize the conditions as follows: a 72-h incubation at 4°C, a final volume of 1 ml per 5 ml starting serum, and expression of all protein amounts as concentration per final 1 ml solution.

Comparisons of our data with others are difficult to make for a number of reasons. For example, in some studies the duration of serum incubation at 4°C ranged from overnight to 7 and 15 days (33–35). These very short or long time periods could influence the amount of cryoprecipitable material available as well as the solubility and immunochemical properties of the individual proteins. In most reports resuspension and solubilization of the cryoprecipitate is accomplished at 37°C in a neutral saline solution; however, it has been done in buffers at pH 5.5 (35) and pH 3.2 (36). There is often no fixed relationship between the starting volume of sera and the final volume of resuspended protein (34). Alternatively, the final cryoglobulin protein concentration may be adjusted to a standard amount that is suitable for immunochemical studies but makes comparisons of samples from individual patients difficult (6, 35, 37). Finally, it is often not stated (12, 34, 35) if the cryoglobulin concentration (expressed in milligrams per milliliter) refers to the final volume in which the protein is resuspended or to the volume of starting material.

It has recently been suggested (2) that cryoglobulins might be more appropriately termed “cryoimmunoglobulins” since the vast majority contain either monoclonal immunoglobulins or mixtures of IgG and IgM. Our data are consistent with this concept. On the average, two-thirds of the total protein in the rheumatoid cryoglobulins and three-quarters of the protein in the vasculitis cryoglobulins can be accounted for by immunoglobulins G and M. This could be an underestimate of the immunoglobulin concentrations because, theoretically, if they existed as IgG-IgM complexes their migration through the gel might be retarded, resulting in smaller rings in the immunodiffusion measurements. However, in studies specifically analyzing this point Bokisch, Bernstein, and Krause found that 19S and 7S anti-IgG’s

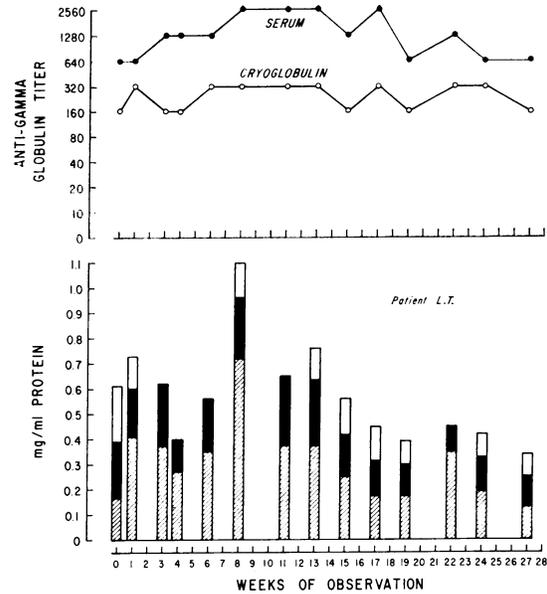


FIGURE 9 The upper graph depicts serum (●—●) and cryoglobulin (○—○) anti-gamma globulin (rheumatoid factor) titers in patient L. T., who received no cytotoxic drug therapy. The lower graph depicts the total protein (entire bar), IgG (hatched bar), and IgM (solid bar) content of L. T.'s cryoglobulin.

had little influence on the determination of IgG or IgM (38).

We selected the 0.15-mg/ml protein concentration to designate patients as having cryoglobulins. The reason for this decision was that in the cryoprecipitates with lesser protein concentrations, the immunoglobulins G and M, when detected, made up less than one-third of the total protein. Therefore all vasculitis patients, one-third of rheumatoids without vasculitis, and none of the controls are designated as having cryoglobulins, or “cryoimmunoglobulins.” In normal subjects cryoglobulins are either absent (8, 10, 26, 35) or present in limited quantities (5, 12, 34), comparable to our data. We are unaware of any other systematic analysis of a group of rheumatoid patients for comparison.

Of the initial 38 rheumatoids encountered in this study, the 12 with cryoglobulins had higher rheumatoid factor titers when compared to the 26 without cryoglobulins. There was a trend but no significant reduction in serum C3 was noted in the 12 patients when compared to rheumatoids without cryoglobulins. The differences between the groups might have been more impressive if C4 rather than serum C3 had been measured. When five additional patients with rheumatoid vasculitis were identified, we then studied vasculitis as a group (eight patients). It is evident that rheumatoid factor titers in the vasculitis group were even

higher when compared to either the remaining rheumatoids with cryoglobulins but without vasculitis (9 patients) or to the entire rheumatoid population (35 patients). Serum C3 was significantly lower in the vasculitis group when compared to the rheumatoids without vasculitis; there was no statistically significant difference when compared to RA with cryoglobulins but without vasculitis.

RA is primarily a joint disease, but lesions affecting various organs, such as nerves, heart, lungs, and eyes, have long been recognized (39, 40). Two pathologic entities account for almost all of the extra-articular features of RA, namely, vascular lesions and the rheumatoid nodule (41, 42). The vascular changes are variable ranging from a necrotizing panarteritis of medium-sized muscular arteries, indistinguishable from polyarteritis nodosa, to a bland noninflammatory mucoid-like degeneration of the intima of small digital arteries and arterioles (39, 43).

The cause of these vascular lesions and their relationship to one another has not been defined, but a number of observations suggest that circulating immune complexes are present in patients with extra-articular features of RA and that the underlying vascular injury is mediated by these complexes. Findings that support this idea include: (a) the generally held view that greater amounts of 19S rheumatoid factor correlate with systemic manifestations of the disease (44, 45); (b) IgM rheumatoid factor probably circulates as a macromolecular (22S) complex with IgG (46); (c) small aggregates of IgG are detectable in rheumatoid sera (22); (d) more of these patients have in their circulation an immunoglobulin-containing material that releases histamine from guinea pig lung tissues (rheumatoid biologically active factor (47)); (e) depression of serum complement activity, as well as decreased concentration of several components (C4, C2) are correlated with clinical signs of vasculitis (45, 48, 49), as is hypercatabolism of C3 (50).

Peripheral neuropathy in RA correlates with other organ involvement (39, 51). Histologic findings of occlusive vascular disease in the vasa nervorum is taken as evidence for a vascular etiology of rheumatoid neuropathy (52, 53), and immunoglobulins G and M and complement (C3) have been demonstrated in an epineural artery from a sural nerve in a patient with rheumatoid neuropathy (54). Thus, conventional histologic and immunofluorescent data support the concept that circulating immune complexes may lodge in vessel walls and set into motion a sequence of events which are recognized clinically as necrotizing vasculitis.

Data presented in this paper examine the notion that the cryoglobulins found in patients with vasculitis are the immune complexes responsible for these tissue

lesions. The antigen-antibody nature of the vasculitis cryoglobulins is suggested by the finding of disproportionately large amounts of IgM and a significant concentration of anti-gamma globulin activity in the precipitates. A variety of anti-gamma globulin factors have been implicated in the pathogenesis of rheumatoid vasculitis, including conventional 19S rheumatoid factor (44, 45), a low molecular weight (8S) IgM (55, 56), and an IgG with antiglobulin activity (56). Because of these associations, it was anticipated that the vasculitis cryoglobulins might contain more than one class of anti-gamma globulin. Clearly, IgM rheumatoid factor was present since: (a) the finding of anti-gamma globulin activity was limited to the heavy (19S) fractions in acidic density gradients, (b) a loss of reactivity followed reduction and alkylation of the cryoglobulins, and (c) all, or almost all, of the polyclonal IgM was removed by absorption with insoluble IgG. Indeed, the latter observation implies that all the IgM in the cryoprecipitate is antibody to IgG. The absence of IgM or anti-globulin activity from the lighter region of the acid gradients argues against a significant amount of 8S IgM in the cryoprecipitate.

The status of an IgG anti-gamma globulin is less clear despite the fact that a number of different experiments were performed to define its presence. These may have been less than satisfactory because IgG rheumatoid factor is said to be a poor agglutinator, especially when compared to IgM, and could be overlooked by conventional testing with IgG-coated bentonite particles. Undoubtedly, this holds true for serum, but, at least in the case of patients with Waldenström's hypergammaglobulinemic purpura—a disease with large amounts of circulating IgG-anti-IgG complexes, the isolated IgG rheumatoid factor has good agglutinating activity (57, 58). Therefore, an IgG rheumatoid factor isolated by cold precipitation might be a good agglutinator as well. Evidence that IgG anti-gamma globulins were not present in significant amounts includes: (a) Winchester, Kunkel, and Agnello were able to detect complexes of gamma globulin in the serum of some patients with RA by precipitation reactions using monoclonal IgM rheumatoid factors (22). This method was successful when C1q precipitation failed to demonstrate complexes. Winchester generously provided us with serum from H. A., a patient with a strongly reactive monoclonal rheumatoid factor. It did not form a precipitate with the vasculitis cryoglobulins, either by the sensitive capillary tube method or in double diffusion in gel. (b) The isolated IgG fractions from vasculitis cryoprecipitates showed no tendency to "self-association" as judged by our inability to detect intermediate or heavier complexes containing IgG. The dilution of the sample that occurs in the density gradient, how-

ever, could preclude identification of IgG if the amounts were small.

Torrigiani and Roitt demonstrated the quantitative distribution of antiglobulin factors among the major immunoglobulin classes. This was accomplished by absorbing the antiglobulins to insoluble (cross-linked) gamma globulin followed by elution with acid and estimation of the eluate by radial immunodiffusion using specific anti-immunoglobulins (59). The method is sufficiently sensitive to detect antibodies to IgG in normal serum. When two isolated cryoglobulins were analyzed in this manner (Table VI), IgG was identified in the eluates despite the very low concentration of this immunoglobulin in the starting material. One cannot exclude the possibility that a limited number of sites on the immunoabsorbent nonspecifically bind a small amount of IgG. This would explain the similar concentrations of IgG in the various eluates, despite very great differences between the test serum and cryoglobulins. But, Heimer and Abruzzo have shown in a similar system that the protein concentration of the eluted IgG is directly proportional to its anti-IgG activity, whether it comes from normal or rheumatoid serum (60). Thus, there is conflicting evidence about the antibody nature of the IgG in the cryoprecipitates.

These discrepancies can most likely be ascribed to differences in the sensitivities of the various techniques we used to search for IgG antiglobulins. The simplest explanation is that IgG rheumatoid factor is a minor constituent of the cryoglobulins, probably constituting less than 20% of the IgG in the precipitates. It is important to point out, however, that less than one-third of the total cryoprecipitable protein is extracted by the method we have employed (Fig. 1). Perhaps the most avid antigen-antibody complexes are not resolubilized and hence not detected. Alternatively, a family of anti-IgG complexes may exist in the circulation, but a decreased cold solubility of IgM rheumatoid factor favors its disproportionate representation in the cryoglobulins. In either instance, the cryoglobulins we measure could serve as faithful indicators of the potential for vasculitis while the actual phlogogenic complexes escape recognition.

The natural history of vascular complications of RA is quite variable. This makes evaluation of any form of treatment difficult (40). However, it has been shown that the presence of extra-articular disease in RA increases mortality twofold (61), and the presence of mononeuritis multiplex involving three or four extremities is associated with a greater than 40% mortality from vasculitis (62). Because of these considerations, intravenous cyclophosphamide was given to all five patients with three- or four-extremity mononeuritis multiplex, and oral therapy with the same drug was em-

ployed in one patient with two-extremity involvement. Serial observations of the patients treated with cyclophosphamide demonstrated clinical improvement in the vasculitis concomitant with diminution or disappearance of cryoglobulins. This finding supports the view that there is a causal relationship between the cryoglobulins and rheumatoid vasculitis. It must be emphasized however, that the number of subjects that we observed was small, and the prognosis of individual patients who survive an initial vasculitic event is largely unknown; therefore, improvement might have occurred regardless of therapy. This latter point is reinforced by patient L. T., who improved clinically without treatment and continued to exhibit cryoglobulins in unchanged amounts during the same period. Hence, an alternative explanation might be that cryoglobulins are a necessary, but not sufficient cause for the development of vasculitis.

Vascular lesions in RA are distributed in an unusual fashion, especially when compared to other diseases characterized by circulating antigen-antibody complexes or cryoglobulinemia. Most remarkable is the sparing of vital organs, such as the kidney and brain, whereas in systemic lupus erythematosus, periarteritis nodosa, and the mixed cryoglobulinemia described by Meltzer, Franklin, Elias, McCluskey, and Cooper (15) they are frequently involved. It is tempting to speculate that the lesser solubility of the RA complexes in the cold accounts, at least in part, for their tendency to produce lesions in acral parts, where the body temperatures are lowest. Perhaps, this temperature dependence, or the smaller quantities, or a less efficient complement-fixing ability of the RA complexes explains the relative infrequency of renal vasculitis. Studies in progress are addressing these questions.

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