Rapid cryoglobulin screening: An aid to the clinician

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SUMMARY A rapid screening method for serum cryoglobulin is reported. It requires only common laboratory equipment and is based upon the detection of light scattering (500 m μ) in the early phase of cryoaggregation. All of 28 sera negative for cryoglobulins by the conventional 5-day incubation method were negative by the screening method. Conversely, all sera containing 60 μ g/ml or more of mixed cryoglobulins were positive by the screening method. The initiation phase of cryoprecipitation in mixed cryoglobulins was found to be prompt, as reported previously for monoclonal cryoglobulins. This sensitive method of cryoglobulin detection provides results to the clinician within 2 hours, a helpful insight where consideration of possible immune complex vasculitis exists.

Recently, reports of methods for detection of circulating immune complexes have achieved a remarkable sensitivity. Radio-labelled C1q binding assays typically detect 4 μ g/ml of complexes (Gabriel and Agnello, 1977), monoclonal rheumatoid factor assays (Gabriel and Agnello, 1977) are sensitive to 500 ng/ml and the Raji cell technique may reveal as little as 200 ng/ml (Theofilopoulos *et al.*, 1974). Critical and as yet unanswered questions arise, however, as to the significance of tiny quantities of immune complexes as discussed at a recent international symposium (Soothill, 1977). The preparation of the required immune reagents or the maintenance of cell lines are also scarcely within the scope of most hospital laboratories. Nevertheless, a common diagnostic quandary in clinical rheumatology as well as general medicine, is whether immune complex vasculitis may be operative in the setting of acute multi-systemic disease, in nephritis, in acute neurological disease and with various signs such as arthritis, skin rash, haemolysis, and serositis. Mixed cryoglobulins are frequently found in post-streptococcal glomerulonephritis (McIntosh *et al.*, 1975), acute systemic lupus erythematosus (SLE) (Christian *et al.*, 1963), rheumatoid vasculitis (Weisman and Zvaifler, 1975), and other forms of systemic vasculitis in which circulating immune complexes are held to have a pathogenic role (Barnett *et al.*, 1970; Grey and Kohler, 1973; Brouet *et al.*, 1974; Meltzer

and Franklin, 1966) indicating that detection of cryoglobulins serves as a useful marker for immune complex related disease. Indeed, the detection of serum cryoglobulins may provide a better index of clinical and histological activity in immune complex renal disease than does serum complement (McIntosh *et al.*, 1975).

Traditional cryoglobulin assays require prolonged (2-5 days) incubation of serum. Conditions for sample handling, detection, quantitation, and characterisation of cryoglobulin, vary widely and are seldom known to the requesting physician. A final result may thus be long in coming to the clinician. The specific details of immunoglobulin typing and the precise quantitation of cryoglobulin protein may surpass clinical application.

The method for cryoglobulin screening described here requires common laboratory equipment; a spectrophotometer, a refrigerator, and a centrifuge, yet it is sensitive to the level of 60 μ g/ml of cryoprotein. Most importantly, a firm positive or negative result can be provided to the clinician within 2 hours of submission of sample, affording a valuable resource in suspected systemic vasculitis. The method reported here can serve to identify those sera which deserve cryoglobulin quantification and typing by the conventional methods. The assay described here depends upon detection of light scattering at 500 m μ by the cryoaggregating immunoglobulins. Whereas visible flocculant cryoprecipitates form slowly at low temperatures, the initiation of cryoprecipitation has been shown to be prompt in monoclonal cryoglobulins (Konieczny and Bobrzecka, 1976; Saluk and Clem, 1975).

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Materials and methods

A total of 62 sera from 40 Cincinnati General Hospital patients and 22 normal individuals were examined for cryoglobulins both by the new technique and conventional cryoglobulin techniques. Fasting blood was drawn into warmed sterile glass tubes and the tubes immediately returned to a styrofoam shipping container (22 × 11.5 cm with 1.5 cm wall thickness) containing 200 ml of water at 40°C, affording excellent thermal control of samples during transfer of the laboratory. Under these conditions the temperature of the water in the sealed container dropped only 2°C, in 1 hour, at room temperature. This feature is a commonly ignored but essential detail in cryoglobulin detection due to trapping of cryoprecipitate in the clot when samples are drawn on the hospital wards and allowed to coagulate at temperatures less than 37°C.

Coagulation and clot retraction were allowed to proceed at 37°C in the laboratory and the serum was then isolated by centrifugation at 1000 rpm for 10 min at 37°C. Prior incubation of the metal centrifuge carriers at 37°C and operation of the centrifuge at high speed ensured temperature control. A second centrifugation of the serum at 2000 rpm for 15 min eliminated cellular elements.

A 1 ml portion was then diluted in an equal volume of 0.9% saline, mixed well, and split into a pair of 75 × 25 mm Pyrex glass tubes, one kept at 37°C and the other at 4°C, for 1 hour.

The optical density (OD) of the two samples was read by spectrophotometry (Beckman Model 24) at 500 mμ using normal saline as blank. These readings were performed after transfer by Pasteur pipettes to cuvettes (at room temperature). Samples were held in the Pasteur pipettes before transfer for 30 s to prevent steaming of the cuvettes. The difference in OD of sera held at these 2 temperatures was recorded ($OD\ 4^\circ C - OD\ 37^\circ C = \Delta OD$).

A second 3 ml portion was placed in a 10 ml graduated centrifuge tube (Kolmer/Corning 8360) and kept at 4°C for 5 days utilising 0.01% sodium azide as preservative. Cryoprecipitate was harvested by centrifugation at 1500 rpm for 15 min and washed 3 times with 10 ml of pH 7 phosphate buffered saline at 4°C. Washed precipitates were suspended in 0.5 ml of phosphate buffered saline (pH 7.0) and kept at 37°C with intermittent agitation for 1 hour. After centrifugation at 37°C, insoluble material was discarded. Insoluble material was usually absent or in small quantity. Total protein content was determined by the Lowry method (Lowry *et al.*, 1951). Cryoimmunoglobulins were detected by Ouchterlony immunodiffusion against monospecific antisera to

IgG, IgA, and IgM using a cryoglobulin concentration of 5–10 mg/ml.

Results

Preliminary studies showed that variation in the time required for mixed cryoglobulins to reach a maximum in optical density at 500 mμ (ΔOD) was a function of cryoglobulin concentration. As an example, Fig. 1 shows a serum with large amounts of mixed cryoglobulins (2 mg/ml of the original serum). Here the relative increase in ΔOD was at a maximum on the initial reading at 10 min following which it declined. In sera containing small amounts of mixed cryoglobulins (0.17 mg/ml of the original serum), as is shown in Fig. 2, the ΔOD was increasing even after 4 hours of incubation at 4°C. From this type of profile of time versus relative increase in turbidity at 4°C, 1 hour was selected as the most suitable incubation period. Greater sensitivity

Fig. 1 Turbidity versus the time profile of serum (50% in saline) containing mixed cryoglobulins (2 mg/ml of the original serum). $\Delta OD = OD\ 4^\circ C - OD\ 37^\circ C$.

Fig. 2 Turbidity versus time profile of serum (50% saline) containing mixed cryoglobulins (0.17 mg/ml of the original serum). $\Delta OD = OD\ 4^\circ C - OD\ 37^\circ C$.

could, however, be achieved by recording later readings. Dilution of sera in saline to a concentration of 50% reduces the amount of serum necessary for the performance of the test without untoward effect on the discrimination of sera positive or negative for cryoglobulins.

All of 28 sera negative for cryoglobulins by the conventional method showed a Δ OD of ≤ 0.020 OD units (Table 1). In 23 of these 28 sera that difference was 0–0.010. None of the sera negative by this technique were positive for cryoglobins by the conventional techniques. Eighteen of 19 sera positive for cryoglobulins by the standard method showed an increase in OD in the chilled sample ranging from 0.040 to 1.14 (Table 2). A single sample with a cryoglobulin concentration of 30 $\mu\text{g/ml}$ of the original serum by the conventional method showed an OD difference of 0.020 (1 hour reading).

Early on it was noted that when post prandial sera and sera from hyperlipidaemic individuals were turbid at 37°C, due to the presence of chylomicrons or very low density lipoproteins, there was a significant increase in OD on cold incubation in the absence of cryoglobulins by the conventional technique. It can be seen from Table 3 that these types of sera increased in OD on cold incubation, ranging from 0.04 to 0.28. All sera with this type of

Table 1 Results of cryoglobulin screening by the optical density method in normal sera or sera without cryoglobulins

Case	Diagnosis	37°C	4°C	Δ OD*
1	N	0.27	0.27	0
2	N	0.37	0.39	0.02
3	N	0.19	0.20	0.01
4	N	0.25	0.25	0
5	N	0.24	0.24	0
6	N	0.26	0.26	0
7	N	0.22	0.20	0.02
8	N	0.25	0.25	0
9	N	0.27	0.28	0.01
10	N	0.18	0.18	0
11	N	0.39	0.39	0
12	N	0.26	0.26	0
13	N	0.23	0.23	0
14	N	0.25	0.24	0.01
15	SLE	0.15	0.16	0.01
16	SLE	0.32	0.33	0.01
17	SLE	0.23	0.23	0
18	SLE	0.35	0.35	0
19	SLE	0.33	0.33	0.02
20	RA	0.31	0.33	0.02
21	RA	0.38	0.38	0
22	RA	0.19	0.20	0.01
23	RA	0.24	0.23	0.01
24	RA	0.23	0.21	0
25	RA	0.23	0.21	0.02
26	RA	0.24	0.25	0.01
27	JRA	0.17	0.18	0.01
28	JRA	0.22	0.21	0.01

*The difference in OD at 500 m μ between the sample of serum (50% in saline) incubated at 4°C and the sample incubated at 37°C for 1 hour. ($\text{OD } 4^\circ\text{C} - \text{OD } 37^\circ\text{C} = \Delta \text{OD}$.)

N=normal, SLE=systemic lupus erythematosus, RA=rheumatoid arthritis, JRA=juvenile rheumatoid arthritis.

false positivity were grossly turbid at 37°C, unlike non-lipaemic cryoglobulin containing sera, allowing ready distinction of sera appropriate to testing by the techniques described here. Filtration of the serum (at 50% concentration) at 37°C through Millipore filters (0.22 μ pore diameter) clarified 7 of 10 tested hyperlipidaemic sera allowing use of the screening technique. Millipore filtration of turbid hyperlipidaemic sera at 37°C lead to a 50% greater reduction in Δ OD (Table 3) in all sera, whether or not clarification occurred. There was little or no effect upon samples containing cryoglobulins (Table 4), another point of differentiation.

Table 2 Results of cryoglobulin screening by the optical density method in sera with cryoglobulins by the conventional technique

Case	Diagnosis	Amount of cryoprotein ($\mu\text{g/ml}$)*	Δ OD†	Cryoglobulin content of cryoprecipitate
1	RA vasculitis	400	0.06	IgM-IgG
2	SLE	80	0.06	IgM-IgG
3	SLE	150	0.12	IgM-IgG
4	MCTD	250	0.21	IgM-IgG
5	Ess. Cryo.‡	5000	1.14	IgM-IgG-IgA
6	RA	80	0.04	IgM-IgG
7	RA	30	0.02	IgM-IgG
8	SLE	150	0.06	IgM-IgG
9	RA	90	0.04	IgM-IgG
10	Ess. Cryo.	1900	0.78	IgM-IgG-IgA
11	Vasculitis	60	0.04	IgM-IgG
12	MCTD	120	0.05	IgM-IgG
13	SLE	253	0.26	IgM-IgG-IgA
14	SLE	90	0.06	IgM-IgG
15	RA	155	0.18	IgM-IgG
16	SLE	360	0.10	IgM-IgG
17	SLE	144	0.05	IgM-IgG
18	RA	120	0.06	IgM-IgG
19	SLE	160	0.07	IgM-IgG

*Protein content of the cryoprecipitate by the Lowry method ($\mu\text{g/ml}$ of original serum).

†See Table 1.

‡Ess. Cryo. = essential mixed cryoglobulinaemia.

Table 3 Results of cryoglobulin screening by the optical density method in turbid hyperlipidaemic sera negative for cryoglobulin by the conventional technique

Case	Diagnosis	Δ OD before filtration*	Δ OD after filtration*
1	N-PP*	0.25	0.06
2	N-PP	0.22	0.02
3	N-PP	0.28	0.14
4	N-PP	0.06	0.01
5	N-PP	0.05	0.01
6	N-PP	0.22	0.06
7	N-PP	0.08	0.02
8	N-PP	0.06	0.03
9	N-PP	0.08	Not tested
10	N-PP	0.04	Not tested
11	Diabetes mellitus	0.08	0.02
12	Diabetes mellitus	0.06	0.02
13	Diabetes mellitus	0.04	Not tested
14	Diabetes mellitus	0.04	Not tested
15	Diabetes mellitus	0.06	Not tested

*As in Table 1.

†Normal - post prandial.

Table 4 The effect of millipore filtration upon cryoglobulin screening by the optical density method using sera with cryoglobulins by the conventional technique

Case	Diagnosis	Amount of protein (µg/ml)*	Δ OD before filtration†	Δ OD after filtration†
3	SLE	150	0.12	0.10
4	MCTD	250	0.21	0.19
5	Ess. Cryo.	5000	1.14	1.15
6	RA	80	0.04	0.04
8	SLE	150	0.06	0.05
11	Vasculitis	60	0.04	0.04
13	SLE	253	0.26	0.20
15	RA	155	0.18	0.17

*As in Table 2.

†As in Table 1.

Discussion

There are a number of elegant new techniques for detection of circulating immune complexes. These remain, for the most part, procedures for research laboratories. A simple, sensitive, and clinically proven method, is not now available for detection of all circulating immune complexes (Agnello, 1976). Methods utilising C1q as a precipitating reagent for complexes can be applied only to those complexes which activate the classical complement pathway. Complement binding methods do not detect small or low affinity immune complexes with small degrees of lattice formation (Agnello *et al.*, 1971). Substances which may be found in patient sera, yet not in immune complexes, such as free DNA or lipopolysaccharide, react with C1q giving false positive results (Hyslop *et al.*, 1970) in C1q binding techniques. The use of monoclonal rheumatoid factor (RF), will detect smaller complexes, but these techniques are limited to the detection of IgG complexes and require the availability of a rare monoclonal immunoglobulin with potent rheumatoid factor activity. Preparing and finding these immunoreagents poses considerable problems for most clinical laboratories. Neither assay differentiates immune complexes from non-specifically aggregated immunoglobulins (Gabriel and Agnello, 1977). The elegant Raji cell assay is perhaps the most sensitive available assay but it requires the expense and labour of maintenance of a tissue culture cell line as well as radioisotopes (Theofilopoulos *et al.*, 1974). Anti-lymphocyte antibodies interfere with this test. Finally, the remarkable sensitivity of these specialised new techniques is not clearly relevant to the clinician since miniscule amounts of immune complexes have been detected in a remarkable diversity of pathogenetic settings and in presumably normal individuals (Soothill, 1977).

The method for cryoglobulin screening reported here is based upon the spectrophotometric detection of a difference in optical density (Δ OD at 500 mμ)

between a sample of serum incubated at 4°C and another incubated at 37°C. This difference is due to light scattering in the initial phase of cryoaggregation. The test was recorded after 1 hour of cold incubation and requires no more than 1 ml of serum. None of the 28 sera negative for cryoglobulins by the conventional method demonstrated an increase in OD upon incubation at 4°C. Conversely 18 of 19 sera containing as little as 60 µg/ml were positive by the screening techniques, well within the level of below 80 µg/ml which has been considered to be the upper limit of normal (Cream, 1972) as performed by the conventional method.

The present study shows that the initiation of cryoprecipitation by mixed cryoglobulins is prompt. This has been reported previously for monoclonal cryoglobulins (Konieczny and Bobrzecka, 1970; Saluk and Clem, 1975). The phenomenon depends upon the decrease in light transmission generated as colloidal particles are formed in the cold from interaction of previously soluble immunoglobulins. In low concentrations cryoreactive proteins were observed, in the studies reported here, to slowly approach a maximal light scattering value whereas in the samples with much cryoprotein, larger aggregates formed rapidly, presumably lessening colloidal light dispersion and fostering sedimentation. Cryoglobulin protein concentration was thus the most important variable for the time course of this phenomenon.

This screening method will provide to the clinician a prompt means of screening for possible immune complex disease and may be of particular assistance in the diagnosis of undefined multi-systemic disease or possible vasculitis.

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