The incidence of cryoglobulinaemia as determined by a turbidimetric method

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SYNOPSIS The incidence of cryoglobulinaemia was determined in 34 normal persons and in 56 sera from 52 patients with miscellaneous disorders. The turbidity of a sample of serum after 18 hours' refrigeration at 4°C. was compared with a sample of the same serum incubated at 38°C. for the same period of time. All refrigerated sera showed a greater turbidity than incubated sera. The abnormal sera showed a high degree of statistically significant correlation between total protein, γ globulin, and the amount of cryoglobulinaemia. There was no significant correlation between cryoglobulinaemia and the age and sex of individuals or any of the other protein fractions. The results suggest that some degree of cryoglobulinaemia is of almost universal occurrence in various unrelated disorders and that this tendency to precipitate out in the cold is a characteristic of the γ globulin group of proteins, especially when present in excess.

Lerner and Watson in 1947 first gave the name of cryoglobulin to an abnormal serum protein which precipitated spontaneously in the cold.

Such proteins are now known to form a heterogeneous group similar to the heterogeneous specificity of myeloma proteins (Osserman and Lawlor, 1955). Cryoglobulins are most commonly found in disorders with increased γ globulin fractions, such as chronic infections, hepatitis, myelomatosis, the leukaemias, and 'collagen' diseases. Rarely it may be an isolated phenomenon without overt disease.

In some disorders with a raised plasma fibrinogen level cryofibrinogens may precipitate out spontaneously and these should be distinguished from the cryoglobulins of serum. Macroglobulins may or may not be associated with a part or whole of the abnormal cold precipitated protein. The whole subject has been comprehensively reviewed by Mackay, Eriksen, Motulsky, and Volwiler (1956).

Dreyfuss and Librach (1952) showed that 61.5% of 180 sera from 50 patients with subacute bacterial endocarditis had globulins which precipitated in the cold, suggesting that cryoglobulins, especially in small quantities, may occur more commonly than supposed. This has been confirmed by other workers for various disorders (Wertheimer and Stein, 1944; Lerner, Barnum, and Watson, 1947; Barr, Reader, and Wheeler, 1956).

The purpose of the present investigation was to

ascertain the incidence of cryoglobulinaemia in healthy persons and in persons suffering from disorders associated with serum protein abnormalities. The present work deals only with the cryoglobulins of serum.

METHODS

Cryoglobulins are difficult to separate from other serum proteins in pure form for paper electrophoresis unless present in amounts of 1 g./100 ml. serum or over, as other serum proteins tend to become adsorbed to the precipitate. Thus an estimation of the total protein content of unwashed cryoglobulin precipitate will be inaccurate as other adsorbed protein fractions will be present.

Experience has shown that for saline-soluble cryoglobulins at least three washings with physiological saline are necessary for the cryoglobulin to be electrophoretically pure. If the amount present is much less than 1 g./100 ml. serum the washings inevitably cause some loss of the precipitate and insufficient remains for paper electrophoretic analysis.

Mackay and his colleagues (1956) centrifuged the serum in haematocrit tubes and determined the height of cryoglobulin present as a 'cryocrit'. This method, however, is unsatisfactory for very small amounts of cryoglobulins. Moreover, most methods of cryoglobulin estimation are inaccurate since maximum precipitation of the abnormal protein occurs slowly over a period of hours, days, or weeks, depending on the serum.

It was therefore decided to determine the incidence of cryoglobulinaemia under standard conditions by measuring the turbidity of a sample of serum, expressed as

optical density after refrigeration at 4°C. for 18 hours and comparing this with the turbidity of a sample of the same serum kept at 38°C. for the same period of time. It was impossible to compare curves of optical density for varying concentrations of a standard γ globulin with cryoglobulins from patients, as the latter appear to be virtually different and specific for each individual patient and do not correlate with standard γ globulin.

Fifty-six samples of serum were collected from 52 patients with the disorders listed in Table I. Normal blood was collected from 34 healthy blood donors. The blood was allowed to clot either at room temperature or at

38°C. depending on the precipitation temperature of the cryoglobulin. The blood was then centrifuged and the serum pipetted off into sterile test tubes which were immediately well stoppered. One was refrigerated and the other incubated as described above. At the same time a paper electrophoretic analysis of a sample of the whole serum was carried out, and the total protein estimated by the Kjeldahl and Biuret method (Bagratuni, 1957). After 18 hours the optical density of the well-shaken refrigerated sample was compared with that of the control incubated serum in a Spekker photoelectric absorptiometer, model H.760, using 1 ml. cells and neutral filters. The light transmission of the incubated control was set to 100%.

In those sera in which sufficient cryoglobulin was present, simultaneous electrophoretic runs were done on whole serum, on supernatant serum after removal of the cryoglobulin in a refrigerated centrifuge at 0°C., and on cryoglobulin dissolved in physiological saline. Total protein concentrations were measured by the Kjeldahl or Biuret methods.

The optical density of the refrigerated sera was correlated with the age and sex of the normal controls and patients as well as with the concentrations of total protein, albumin, and α_1 , α_2 , β and γ globulins in the two groups using the correlation coefficient r .

RESULTS

All refrigerated sera showed a greater turbidity than incubated sera.

In the 34 normal male sera r was significant at 1% for total protein and α_2 globulin only. For combined male and female normal sera this was achieved only at the 2% level for the same components (Table II).

In the combined 56 abnormal sera, however,

TABLE I

DIAGNOSIS, SEX, AND NUMBER OF SERUM PROTEIN ANALYSES PERFORMED ON 52 PATIENTS

Diagnosis	♂	♀	No. of Serum Protein Analyses
Ankylosing spondylitis	1	—	2
Anarthritic rheumatoid syndrome (polymyalgia rheumatica)	1	10	11
Amyloidosis	—	1	1
Carcinomatosis	2	—	2
Dermatomyositis	1	—	2
Giant cell arteritis	1	—	1
Gout	1	—	1
Haemolytic anaemia	2	2	4
Hashimoto's thyroiditis	—	2	2
Hepatitis	—	1	1
Hepatitis and ulcerative colitis	1	—	1
Lymphatic leukaemia	1	—	1
Periarteritis nodosa	1	1	3
Purpura, drug	1	1	3
Pyelonephritis	—	2	2
Rheumatoid arthritis	3	8	11
Sarcoidosis	—	2	2
Scleroderma	—	1	1
Systemic lupus erythematosus	1	4	5
Total	17	35	56

TABLE II

AGE, SEX, TOTAL PROTEIN ELECTROPHORETIC ANALYSIS, AND OPTICAL DENSITY AFTER REFRIGERATION OF SERA FROM 34 NORMAL PERSONS

	Age (yr.)	Total Protein (g./100 ml.)	Albumin + α_1 Globulin (%)	α_2 Globulin (%)	β Globulin (%)	γ Globulin (%)	Optical Density
Males (24)							
Mean	30.4	6.3	58.8	7.5	13.1	19.9	0.172
S.D. $\pm$	10.0	0.4	4.3	1.9	2.7	4.6	0.163
Range	19-58	5.9-7.8	45.3-69.5	3.5-12.3	9.0-17.1	10.5-26.4	0.038-0.58
Correlation coefficient r	0.369	0.490	0.161	-0.542	0.218	-0.154	
P	10%	1%	N.S.	1%	N.S.	N.S.	
Females (10)							
Mean	35.7	6.4	64.2	8.0	10.4	17.5	0.063
S.D. $\pm$	10.2	0.3	5.5	2.3	2.0	3.5	0.030
Range	19-49	6.0-7.1	57.7-76.2	4.3-11.1	7.6-13.4	9.5-21.4	0.035-0.13
Correlation coefficient r	0.638	-0.236	-0.411	-0.392	0.699	0.520	
P	5%	N.S.	N.S.	N.S.	N.S.	10%	
Males and Females (34)							
Mean	31.8	6.3	60.5	7.7	12.4	19.3	0.142
S.D. $\pm$	10.3	0.4	5.7	2.0	2.8	4.4	0.148
Range	19-58	5.9-7.8	45.3-76.2	3.5-12.3	7.6-17.1	9.5-26.4	0.035-0.058
Correlation coefficient r	0.249	0.384	-0.048	-0.458	0.342	-0.020	
P	N.S.	2%	N.S.	2%	5%	N.S.	

N.S. = Not significant

TABLE III

AGE, SEX, TOTAL PROTEIN, ELECTROPHORETIC ANALYSIS, AND OPTICAL DENSITY AFTER REFRIGERATION OF 56 SERA FROM 52 PATIENTS WITH VARIOUS DISORDERS

	Age (yr.)	Total Protein (g./100 ml.)	Albumin (%)	α_1 Globulin (%)	α_2 Globulin (%)	β Globulin (%)	γ Globulin (%)	Optical Density
Males (17)								
Mean	56.9	6.8	43.8	5.3	9.2	13.7	25.9	0.291
S.D. $\pm$	14.4	0.7	6.5	1.6	3.1	4.8	7.0	0.549
Range	14-78	5.8-8.0	29.5-52.2	2.2-8.5	4.5-13.7	5.0-22.2	11.7-38.5	0.01-2.0
Correlation coefficient r	-0.248	0.397	0.345	-0.451	-0.523	-0.560	0.505	
P	N.S.	10%	N.S.	5%	2%	1%	2%	
Females (35)								
Mean	59.9	6.7	42.1	6.2	11.3	13.2	25.2	0.154
S.D. $\pm$	15.3	0.8	12.0	2.3	3.5	3.5	11.7	0.284
Range	18-81	5.8-7.4	18.5-61.5	2.6-11.6	6.0-17.3	6.3-18.9	8.3-46.3	0.005-1.5
Correlation coefficient r	-0.165	0.721	-0.373	0.366	0.013	-0.106	0.384	
P	N.S.	0.1%	5%	5%	N.S.	N.S.	2%	
Males and Females (52)								
Mean	58.7	6.7	42.8	5.8	10.5	13.4	25.6	0.206
S.D. $\pm$	15.1	0.8	10.3	2.1	3.5	4.1	10.2	0.413
Range	14-81	5.8-8.0	18.5-61.5	2.2-11.6	4.5-17.3	5.0-22.2	8.3-46.3	0.005-2.0
Correlation coefficient r	-0.209	0.516	-0.052	-0.055	-0.276	-0.371	0.440	
P	N.S.	<0.1%	N.S.	N.S.	5%	1%	<0.1%	

N.S. = Not significant.

increased optical density correlated highly with total protein ($P = < 0.1\%$) and the γ globulin ($P = < 0.1\%$). Both these figures are highly significant, the correlation with total protein being no doubt due to the increased γ globulin (Table III).

Figure 1 shows the scatter and regression curve of the results on abnormal sera.

DISCUSSION

The results show that the optical density of refrigerated sera may vary from 0.005 to 2.0 in various disorders, the optical density of refrigerated sera always being greater than that of incubated sera.

There was no evidence that in the incubation

period of 18 hours any or sufficient bacterial growth had occurred to produce any visible turbidity in the serum which had been collected with full sterile precautions. Any turbidity due to lipaemia was overcome by using the same patient's serum each time as control and unknown rather than using as control an arbitrary normal serum.

Although lipoproteins are unstable and dissociate readily at temperatures below 0°C ., the sera in the present series of experiments were never cooled below 4°C . Lipoproteins appear to be stable at this temperature (del Gatto, Nichols, and Lindgren, 1959) or dissociate only very slightly (Oncley, Gurd, and Melin, 1950).

Cold centrifugation of many samples of abnormal sera at 4°C . invariably showed the precipitation of cryoglobulin to be greater than the flotation of lipids and the turbidity remained on shaking the serum after removal of the supernatant lipid layer. Under the circumstances it was felt that any increase in turbidity due to dissociation of lipoproteins at 4°C . was slight. Statistically the correlation between optical density and protein concentration in abnormal sera was 10 times greater for γ globulin when compared with β globulin.

Although in the past note was only taken of large quantities of cryoglobulin which either precipitated spontaneously at room temperature or formed large masses from refrigerated sera, the results reported here show that some degree of reversible turbidity may be present in normal sera and in those from a variety of disorders when cooled to 4°C . There is indeed a whole gradation from scarcely visible flocculation to massive precipitation of protein.

FIG. 1. Regression curve for 56 abnormal sera showing correlation between percentage γ globulin and optical density of refrigerated sera. O.D. = optical density.

FIG. 2. *Washed cryoglobulin from a patient (No. 55) with haemolytic anaemia. This amount was present in 10 ml. serum (1.5 g./100 ml. serum).*

FIG. 3. *Whole serum electrophoresis from patient No. 55.*

FIG. 4. *Electrophoresis of washed cryoglobulin from serum of patient No. 55.*

FIG. 5. *Electrophoresis of serum from patient No. 55 after removal of cryoglobulin.*

Cryoglobulins are usually associated with the γ globulin fraction of serum or move in an intermediate position between the β and γ fractions. Exceptionally they may occur in the α_2 fraction (Craig, Waterhouse, and Young, 1952). In the present series the mean γ globulin level was 25.6% (mean total serum protein 6.7g./100 ml.). The largest quantity of cryoglobulin was found in the serum from a patient with haemolytic anaemia which contained 35.6% γ globulin (total serum protein 7.7g./100 ml.). The cryoglobulin formed 1.5g. of total protein, the optical density of the refrigerated serum being 2.0. In this patient the blood literally gelled as it was withdrawn into the syringe and it was necessary to keep all equipment at 38°C. Figure 2 shows a washed sample of this patient's cryoglobulin and Figs. 3-5 the serum before and after removal of the cryoglobulin which formed part of the γ globulin band.

This was the only patient who experienced symptoms from cryoglobulins, in his case paraesthesiae and cyanosis of the extremities when these were cooled.

A patient with systemic lupus erythematosus showed a serum optical density of 1.5 associated with a γ globulin of 33.5% (total serum protein 6.4g./100 ml.), and the cryoglobulin was again incorporated in the γ globulin fraction.

In contrast a patient with rheumatoid arthritis whose serum showed the high optical density of 0.47 had a γ globulin level of only 18.4% (total serum protein 7.2g./100 ml.) with the cryoglobulin associated with this fraction.

The highest γ globulin of 59.2% (total serum protein 7.0g./100 ml.) was found in a woman with hepatitis whose serum gave the lowest optical density of 0.005.

A patient with chronic pyelonephritis whose serum contained 46.3% γ globulin (total serum protein 5.7g./100 ml.) showed an optical density of only 0.175.

In seven patients whose serum showed an optical density of 0.47 or over, electrophoresis showed the washed cryoglobulin as moving with the mobility of γ globulin or mid-way between the γ and β globulins.

Overall the present results show a correlation between the amount of γ globulin present and the amount of cryoglobulin when measured in terms of

the optical density of the serum (Fig. 1). The correlation extends to the amount of total protein present, no doubt due to the increased globulins, especially γ globulin. There was no correlation between type of disease and amount of cryoglobulin.

Very high levels of γ globulin may, however, show minimal cryoglobulinaemia and low and moderate levels may show great amounts. This suggests that the abnormal protein is separate and distinct from normally occurring γ globulin and its synthesis is independent of it. Indeed, as Figs. 2-5 show, the abnormal fraction may be completely incorporated in the γ globulin and have the identical mobility yet only form part of it. This is in keeping with the concept of normal and abnormal γ globulin being a heterogeneous aggregate of proteins forming complexes (Cooper, 1960), all or part of which, as the present work shows, tend to precipitate out in the cold over a whole range of critical concentrations.

Some degree of cryoglobulinaemia is thus of almost universal occurrence in various unrelated disorders. When present in small amounts it is frequently overlooked; when present in large quantities it may become a striking abnormality leading to clinical symptoms.

I am grateful to Professor G. W. Pickering, Professor L. J. Witts, Dr. A. M. Cooke, Dr. P. Mallam, Dr. P. Bedford and the late Dr. F. G. Hobson for access to patients under their care, and to Mr. J. R. P. O'Brien for helpful criticism in connexion with this paper.

I am especially grateful to Mr. N. T. J. Bailey of the Institute of Statistics for help with the statistical analyses.

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