Immunology, 1965, **9**, 327.

Cryoglobulinaemia in Rabbits Hyperimmunized with a Polyvalent Pneumococcal Vaccine

E. A. CATSOULIS, E. C. FRANKLIN AND M. A. ROTHSCHILD

Radioisotope Service, V.A. Hospital, New York, and Department of Medicine, Rheumatic Diseases Study Group, New York University School of Medicine, New York, N.Y.

(*Received* 13*th January* 1965)

Summary. During the anamnestic response, the sera of four rabbits immunized with a polyvalent pneumococcal vaccine contained large amounts of cryoglobulins belonging to the G and M immunoglobulin classes. These cryoglobulins appeared to possess antibody activity since they agglutinated a suspension of the pneumococci that was used for immunization.

INTRODUCTION

Serum globulins that precipitate in the cold (cryoglobulins) have been described in man in association with a variety of diseases (Lerner, Barnum and Watson, 1947; Abrams, Cohen and Meyer, 1949; Ritzmann and Levin, 1961), especially the proliferative disorders of plasma cells and lymphocytes. Not infrequently such proteins appear as the sole manifestation of a biochemical disorder and are not associated with any recognizable disease or well-defined pathologic process. Therefore, the observation by Askonas, Farthing and Humphrey (1960) of significant amounts of cryoglobulins in a single rabbit hyperimmunized with a pneumococcal polyvalent vaccine, and the finding of large amounts of such proteins in the sera of six animals hyperimmunized with pneumococcal vaccine for other purposes (Rothschild, Oratz, Franklin and Schreiber, 1962; Catsoulis, Franklin, Oratz and Rothschild, 1964) led to further studies dealing with the production and characterization of these cryoproteins.

MATERIALS AND METHODS

Eight rabbits have been immunized to date. For primary immunization, 3 ml of a formalin-treated polyvalent pneumococcal vaccine, prepared from types I, II, VI, IX, XI and XVD, containing 1×10^9 organism per ml, was given intravenously every 3 days until the development of hyperglobulinaemia, generally after 3–4 months. Thereafter, the rabbits were rested for 4–5 months until the serum proteins had returned to control levels. At this time the animals were re-exposed to the same antigenic stimulus to induce an anamnestic response. This occurred promptly and reached a maximum level in 3–5 weeks. Six rabbits developed cryoglobulins during the anamnestic response, and sera from four of them were chosen for careful studies. Each of these sera, drawn 2–3 weeks after the initiation of the secondary response, developed a precipitate when stored at 4°. This precipitate redissolved at 37°.

The cryoglobulins were isolated from the sera by precipitation at 4° (Fröhlich, Balazs and Szepesy, 1961; LoSpalluto, Dorward, Miller and Ziff, 1962; Meltzer and Franklin, 1962). They were freed of contaminating proteins by redissolving them in 5–10 ml of 0·15 M saline and repeating the precipitation in the cold at least four times until the supernatant was free of protein when tested with an antiserum to normal rabbit serum by the capillary precipitin technique.

Total serum protein was determined by the Biuret method. Protein partition was determined by boundary electrophoresis employing a Kern microelectrophoretic unit as described previously (Rothschild *et al.*, 1962). The amount of cryoglobulin present was estimated from the difference of the total proteins before and after the cryoprecipitate had been removed rather than by direct quantitation of the precipitated protein owing to significant losses during the washing procedure.

Ultracentrifugal analyses were performed in a Spinco model E analytical ultracentrifuge at 37° using double sector cells. Sedimentation coefficients were calculated as described by Trautman (1956). Agglutination reactions were carried out with all strains of pneumococci employed in the immunization at 37°, using (a) whole serum, (b) serum from which cryoglobulins had been removed and (c) a solution of cryoglobulins in 0·15 M NaCl. Immunoelectrophoresis was carried out using the micro-method of Scheidegger (1955) at pH 8·2. Sheep antisera to whole rabbit serum and against γ-globulin were kindly supplied by Dr G. J. Thorbecke.

The cryocrit was determined by placing the serum in a haematocrit tube, cooling it for 24 hours, and reading the level of precipitated proteins.

RESULTS

Three weeks after the initiation of the second course of immunization, the concentration of total protein rose from a mean value of 6·6 g/100 ml to 8·2 g/100 ml, and the γ-globulin concentration increased from a mean of 0·9 g/100 ml to 3·3 g/100 ml. As shown in Table 1, the concentration of cryoglobulins ranged from 0·7 to 1·4 g/100 ml with a mean value of 1·1 g/100 ml serum. In these four sera, therefore, almost one-third of the total γ-globulins consisted of cryoglobulins.

Immunoelectrophoretic analyses of the isolated cryoglobulins from all four animals showed that they had a mobility in the γ-globulin region. The precipitin line appeared somewhat more homogeneous than that of the rest of γ-globulin in two animals, but

TABLE 1

GAMMA-GLOBULINS AND CRYOGLOBULINS IN FOUR RABBITS HYPERIMMUNIZED WITH A POLYVALENT PNEUMOCOCCAL VACCINE

Rabbit No.	Total γ-globulins (g per cent)		γ-Globulins minus cryoglobulins (g per cent)		Cryoglobulins (g per cent)		Cryocrit (per cent)
	C*	E*	C	E	C	E	
319	0·9	3·0	0·9	1·6	—	1·4	10·0
320	0·9	2·2	0·9	1·3	—	0·9	5·0
322	0·9	3·4	0·9	2·0	—	1·4	10·0
324	1·0	4·8	1·0	4·1	—	0·7	5·0
Mean value	0·9	3·3	0·9	2·2	—	1·1	7·5

*C = Control; E = experimental.

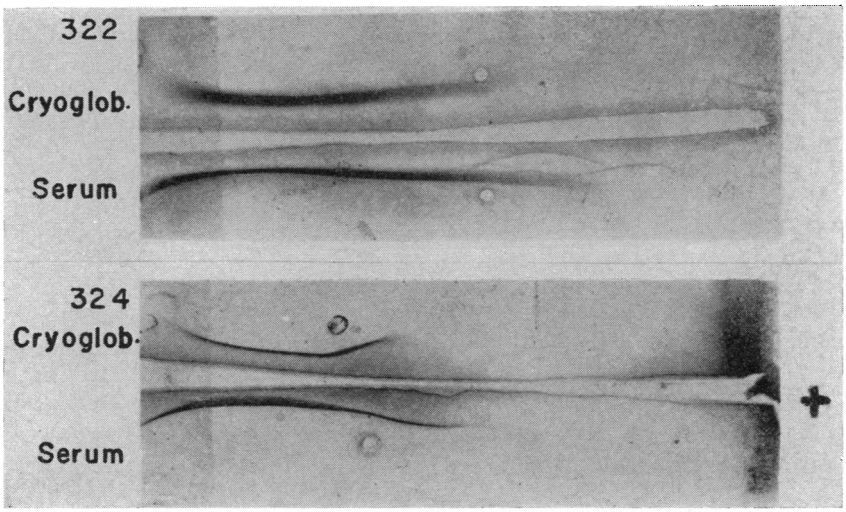

FIG. 1. Immunoelectrophoresis of isolated cryoglobulins and sera from two rabbits immunized with a polyvalent pneumococcal vaccine. The antisera used were: top, against whole rabbit serum and, bottom, against rabbit γ-globulin.

corresponded in electrophoretic mobility to the remaining γ-globulin in two others (Fig. 1). Ultracentrifugal analyses performed at 37° of three of the cryoglobulins showed that 80–95 per cent of the protein had a sedimentation coefficient of 6·4S and that from 5–20 per cent of the protein had a sedimentation coefficient of 18·6–19S (Fig. 2).

Table 2 shows the agglutination titres obtained when the sera and cryoproteins were tested, using a mixture of all types of pneumococci as antigens. The sera agglutinated the cells at dilutions ranging from 1 : 512 to 1 : 1 024. After the removal of the cryoglobulin, the titres diminished and ranged from 1 : 128 to 1 : 512. The pure cryoglobulins at protein concentrations ranging from 0·5 to 0·75 g/100 ml gave agglutination titres ranging from 1 : 64 to 1 : 128. Thus, although precise quantitation of the cryoglobulins proved

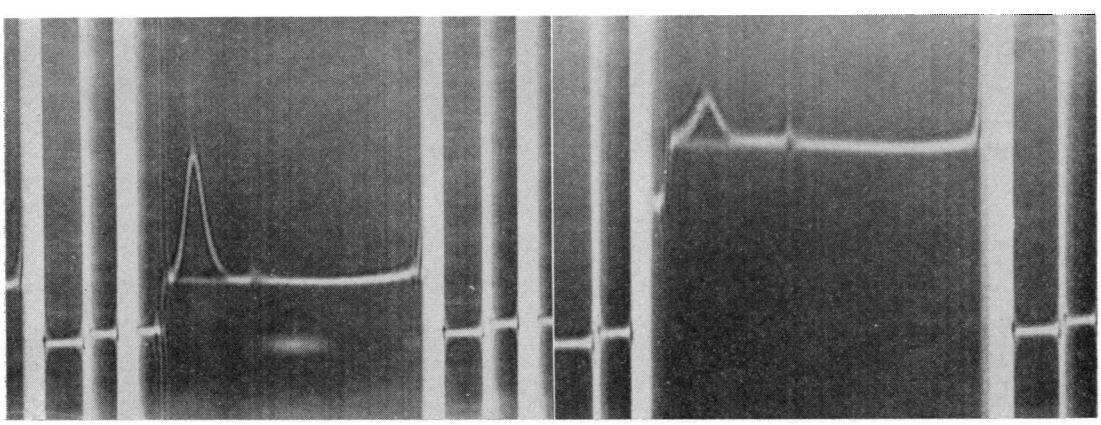

FIG. 2. Ultracentrifugal patterns of cryoglobulins isolated from the sera of two hyperimmunized rabbits. Time 20 minutes. Speed 52,640 rev/min. The sedimentation is from left to right.

TABLE 2

AGGLUTINATION TITRES TO PNEUMOCOCCI (ALL TYPES USED) OF SERA AND CRYOGLOBULIN OF FOUR HYPERIMMUNIZED RABBITS

Rabbit No.	Whole serum		Whole serum minus cryoglobulins		Cryoglobulins	
	Titre	(g per cent γ-globulins)	Titre	(g per cent γ-globulins)	Titre	(g per cent)
319	1 : 512	3·0	1 : 128	1·6	1 : 128	0·72
320	1 : 512	2·2	1 : 128	1·3	1 : 64	0·50
322	1 : 512	3·4	1 : 512	2·0	1 : 64	0·76
324	1 : 1024	4·8	1 : 512	4·1	1 : 128	0·56

difficult, in each instance the agglutinating ability per mg of γ-globulin was approximately equal for each of the fractions tested. Attempts to detect antibody activity after separation of the 7S and 19S fractions by density gradient centrifugation proved unsuccessful.

DISCUSSION

Polyvalent pneumococcal vaccine is known to be a potent stimulus for the production of hypergammaglobulinaemia in rabbits. Askonas *et al.* (1960) have reported that one rabbit immunized with type III pneumococci developed cryoglobulins. This cryoglobulin was associated with antibody activity towards the antigen employed. In man, cryoglobulins are generally devoid of antibody activity and only a small number of cryoglobulins with rheumatoid factor activity have been reported (LoSpalluto *et al.*, 1962; Meltzer and Franklin, 1962). The appearance of cryoglobulins that have antibody properties in four of the hyperimmune animals shows that, during intense immunization, immunologically competent cells can be stimulated to produce a heterogeneous spectrum of antibodies that differ from each other not only in avidity, sedimentation coefficient, electrophoretic mobility and other parameters generally studied, but also in their solubility at different temperatures. The presence of both G (7Sγ) and M (19Sγ) immunoglobulins in the cryoglobulins isolated from the sera of these rabbits, and the finding of antibody activity in these proteins, suggest that more than one population of cells may be involved in their synthesis, and that their production may be governed by factors similar to those controlling the formation of the usual type of antibodies. In spite of the presence of rather large amounts of cryoglobulins, these rabbits did not suffer any ill effects, and in all animals cryoglobulin disappeared 5 weeks after the course of vaccination had been completed.

ACKNOWLEDGMENTS

We should like to thank Mrs Jane Bulger for her excellent secretarial assistance and Miss Josephine Neglia for the illustrations.

This investigation was supported in part by U.S.P.H.S. Grants AM-02489, AM-02592 and AM-01431 and by the Arthritis and Rheumatism Foundation.

Dr E. C. Franklin is a career scientist with the Health Research Council of the City of New York, and Dr E. A. Catsoulis is the recipient of a Research Fellowship from the Arthritis and Rheumatism Foundation.

REFERENCES

ABRAMS, A., COHEN, P. P. and MEYER, O. O. (1949). 'The physical properties of a cryoglobulin obtained from lymph nodes and serum of a case of lymphosarcoma.' *J. biol. Chem.*, **181**, 237.

ASKONAS, B. A., FARTHING, C. P. and HUMPHREY, J. H. (1960). 'The significance of multiple antibody components in serum of immunized rabbits.' *Immunology*, **3**, 336.

CATSOULIS, E. A., FRANKLIN, E. C., ORATZ, M. and ROTHSCHILD, M. A. (1964). 'Gamma globulin metabolism in rabbits during the anamnestic response.' *J. exp. Med.*, **119**, 615.

FRÖHLICH, M. M., BALAZS, V. and SZEPESY, G. L. (1961). 'Physiochemical and immunological properties of some isolated cryoglobulins.' *Protides of Biological Fluids; Proceedings of the 9th Colloquium, Bruges (Belgium)*, p. 276.

LERNER, A. B., BARNUM, C. P. and WATSON, C. J. (1947). 'Studies of cryoglobulins. II. The spontaneous precipitation of protein from serum at 5°C in various states.' *Amer. J. med. Sci.*, **214**, 416.

LOSPALLUTO, J., DORWARD, B., MILLER, W. and ZIFF, M. (1962). 'Cryoglobulinemia based on interaction between a gamma macroglobulin and 7S gamma globulin.' *Amer. J. Med.*, **32**, 142.

MELTZER, M. and FRANKLIN, E. C. (1962). 'Interaction of γ-globulins. A study of cryoglobulins with and without rheumatoid factor activity.' *Arthr. and Rheum.*, **5**, 117.

RITZMANN, S. E. and LEVIN, W. C. (1961). 'Cryopathies: A Review.' *Arch. intern. Med.*, **107**, 754.

ROTHSCHILD, M. A., ORATZ, M., FRANKLIN, E. C. and SCHREIBER, S. S. (1962). 'The effect of hypergammaglobulinemia on albumin metabolism in hyperimmunized rabbits studied with albumin I^{131}.' *J. clin. Invest.*, **41**, 1564.

SCHEIDEGGER, J. J. (1955). 'Une micro-methode d'immunoelectrophorese.' *Int. Arch. Allergy*, **7**, 103.

TRAUTMAN, R. (1956). 'Operating and comparating procedures facilitating Schlieren pattern analysis in analytical ultracentrifugation.' *J. phys. Chem.*, **60**, 1211.