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CRYOGLOBULINS IN NEW ZEALAND BLACK MICE

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SUMMARY

In about a quarter of New Zealand Black mice (NZB mice), aged 4 months or older, cryoglobulins were found, sometimes in large amounts. The frequency was somewhat higher in females than in males. The presence of cryoglobulins was rarely observed in control mice and in these groups the cryocrit always remained low.

The majority of the cryoglobulins in NZB mice were macroglobulins with a relatively positive charge. Attempts to elucidate their significance have remained unsuccessful so far.

INTRODUCTION

The Coombs' positive haemolytic anaemia, which occurs spontaneously in NZB mice, was originally described by Bielschowski, Helyer & Howie (1959). These authors were also the first to suggest that this symptom could be regarded as a manifestation of systemic lupus erythematosus (SLE) in these animals (Helyer & Howie, 1961). The syndrome can include leucopenia, glomerulonephritis, thymus pathology and multiple autoantibodies with hypergammaglobulinaemia. Holmes & Burnet (1963) have described its natural history in detail, and the whole subject has been reviewed by Mellors (1966).

Studies of cryoglobulins in men have indicated their possible role in the pathogenesis of the haemolytic process, renal lesions and other diseases as recently reviewed by Wager, Mustakallio & Räsänen (1968). These observations have prompted us to investigate the presence of these serum proteins in NZB mice. So far they have not been reported in the literature, but recently Holmes mentioned their occurrence in a personal communication to one of the authors.

MATERIALS AND METHODS

Mice

The pedigree of the NZB mice, which were used in these studies, has been described previously (de Vries & Hijmans, 1967). In addition a number of NZB mice was studied

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from a subline, obtained in the fifty-sixth generation from the Medical Research Council Animal Centre, Carshalton, England. No differences were found between the sublines.

The control group consisted of 251 mice of different inbred and outbred strains (see Table 2), some of which had been neonatally infected with lymphochoriomeningitis (LCM) virus. These mice were kindly supplied by Dr F. Dekking, Laboratory for Hygiene, Amsterdam. Persistant tolerant infection had been developed in these mice with virus excretion for periods exceeding 20 months. The group also included forty Swiss mice, which had received streptococcal antigens in Freund's adjuvant for other purposes.

Cryoprecipitation

Blood was taken by cardiac puncture or by severing the axillary vessels. Care was taken that the blood was kept at 37°C during clotting. Separation of the serum was performed at this temperature. The serum was stored for 48 hr at approximately 0°C. The degree of cryoprecipitation was graded visually as 0, ±, + or + +. The estimated cryocrit of the last group after overnight storage at 0°C was more than 10%. Some of the cryoprecipitates were purified individually by centrifugation at 0°C, discarding the supernatant, and dissolving the precipitate in phosphate buffered saline (pH 7·2; 0·01 M-phosphate) at 37°C to the original volume. This procedure resulted in considerable losses and most cryoprecipitates were therefore washed in the cold. Both cycles were repeated four times and followed by a final centrifugation at 37°C in order to remove any denatured proteins. In some series batches of about twenty-five cryoprecipitates were pooled. After washing with distilled water at 0°C the precipitate was then dissolved in phosphate buffered saline for further analysis.

Antisera

Pooled sera of random bred Swiss mice were injected into rabbits in order to obtain an antiserum against mouse serum proteins. Another antiserum, which recognized IgG (γ_1, γ_2) and IgM globulins, as described previously (de Vries & Hijmans, 1967), was used for immunoelectrophoresis.

For the production of a rabbit–anti-mouse IgG-serum 10 ml of normal mouse serum was diluted with 20 ml Tris–phosphate buffer (pH 8·5) and eluted over a Sephadex G-100 column (150 × 2·5 cm). The elution resulted in two broad peaks with a flat area between the two peaks, as determined in a Unicam spectrophotometer at 280 mμ. The flat area consisted of two regions: a flat and a slightly descending part. Fractions of these two regions were combined and subsequently led over a Kodak DEAE-cellulose column. The column was stepwise eluted with buffers of pH 8·5, 7·6, 7·2, 6·6 and 6·0, prepared by mixing Tris–phosphate buffers of pH 8·5 and 4·0 (pH 8·5:0·35 M-Tris–0·005 M-H_3PO_4; pH 4·0:0·5 M-Tris–0·59 M-H_3PO_4). The first eluted protein peak consisted of pure IgG, the following fractions were contaminated with β_{1C} protein. A last peak contained only a small amount of IgG and a major part of α- and β-globulins. IgG and β_{1C} were separated by electrophoresis in polyacrylamide gel, followed by elution of the gel sections. The rabbits were immunized by intramuscular injections of 200 μg protein in incomplete Freund's adjuvant until strong precipitating antibodies were obtained. The antiglobulin reagent was freed from anti-transferrin by absorption.

Rabbit–anti-mouse IgM was obtained accidentally in an effort to make a rabbit–anti-mouse fibrinogen serum. Although it contained anti-fibrinogen besides anti-IgM this

disturbed only the immunofluorescent technique on kidneys. Therefore this technique was performed with an anti-IgM serum produced by the immunization of rabbits with IgM myeloma proteins. Mice with IgM–plasma cell tumours were kindly supplied by Dr K. Robert McIntire (Bethesda, Maryland). Myeloma proteins were isolated by preparative 0·9% agar gel electrophoresis. After freezing and thawing of the appropriate section the proteins were dissolved and used as immunizing agent. If the protein yield was very poor, the agar particles were homogenized after the freezing and thawing procedure in a Potter–Elvehjem homogenizer and injected without further purification.

One rabbit received about 250 μg of a purified cryoglobulin preparation with Freund's adjuvant in the toe pads and intracutaneously in multiple sites. The antiserum only reacted with mouse IgM.

Quantitative determination of IgM

Another antiserum was prepared against a pool of purified cryoglobulin preparations. It showed a major line against IgM in addition to an IgG line. The latter was no more visible when the antiserum was diluted 1:100 and therefore did not interfere in the quantitative radial agar diffusion test (Mancini, Carbonara & Heremans, 1965), in which the antiserum was used in a dilution of 1:750. The results were expressed as the percentage of the IgM content of normal Swiss mouse serum and they were evaluated with the Student's *t*-test on an Olivetti–Underwood computer Programme 101.

DNA determination

The serum from a patient suffering from SLE with circulating antibodies against calf thymus deoxyribonucleic acid (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) was used for the qualitative determination of deoxyribonucleic acid (DNA) in several cryoprecipitates.

The antiglobulin test was performed on 115 heparinized blood specimens with a rabbit–anti-mouse IgG and a rabbit–anti-total mouse serum, and moreover 190 samples were studied with a rabbit–anti-mouse anti-IgG serum only. One drop of twofold serum dilutions of the antiglobulin serum was incubated with 1 drop of a 4% three times washed mouse red cell suspension during 1 hr at 37°C. The reactions were read microscopically. No differences were observed between the anti-globulin test performed with anti-total mouse serum and anti-mouse IgG.

Antibodies to bromelinized NZB red cells were determined by the following procedure: Nine parts of a 4% suspension in phosphate buffered saline (0·015 M-phosphate, pH 7·3) of three times washed red cells of young NZB mice with a negative direct antiglobulin test, were mixed with 1 part of a bromelin solution (1 g bromelin; 0·1 g NaN_3; *aqua ad* 100 ml). After incubation for 30 min at 37°C the cells were washed twice with phosphate buffered saline. To one drop of a 4% suspension of the bromelinized cells 1 drop of the serum under study, or dilutions of this serum was added. The reaction was read microscopically after incubation for 1 hr at 16°C.

To exclude the presence of cold agglutinins the positively reacting sera were re-investigated by the same test method with the exception that the incubation was performed at 37°C. In all cases the same results were obtained.

The rheumatoid factor was determined by the Waaler–Rose test (van Loghem-Langereis, 1952). After absorbtion of the sera with human red cells, they were incubated for 1 hr at 37°C and subsequently overnight at +4°C with a 0·2% suspension of human red cells, which had been previously incubated with a rabbit serum containing incomplete anti-human red cell antibodies. The reactions were read macroscopically.

Ultracentrifuge analyses were performed in a Spinco model E centrifuge at 50,740 rev/min and 37°C. The obtained sedimentation coefficients at 37°C (S_{37}) were corrected for temperature to the sedimentation coefficients at 20°C. No further corrections to standard conditions were made.

The immunofluorescent technique was used for the detection of anti-viral antibodies. The indirect method was performed as described by van Furth, Schuit & Hijmans (1966), with slight modifications.

Cryostat sections and suspensions of thymus tissue of about 1-month-old Swiss or NZB mice were used, either unfixed or fixed with acetone during 15 min at room temperature or at 37°C. These substrates were incubated with normal mouse sera, with cryoglobulin containing sera, or with isolated cryoglobulins. Antisera, prepared in rabbits with total mouse serum, a mouse immunoglobulin preparation or a macrocryoglobulin preparation, were conjugated with fluorescein isothiocyanate.

Besides, the indirect immunofluorescent technique was performed on unfixed and acetone-fixed cryostat sections of thymus glands of two NZB mice (aged 1 and 11 months), one NZW mouse (aged 1 month) and two outbred mice (aged 1 and 13 months), as described by Feltkamp & van Rossum (1968). From six cryopositive NZB mice the total serum, the isolated cryoglobulins, and the serum free from cryoglobulins, were tested for antibodies against thymic epithelial cells. As controls normal mouse sera and ANF containing mouse sera were tested. The thymic sections were incubated subsequently with the serum or serum fraction to study, a rabbit–anti-mouse-IgG- or IgM-serum, and a sheep–anti-rabbit immunoglobulin IgG, conjugated to fluorescein isothiocyanate, according to the method described previously (Feltkamp & van Rossum, 1968). The conjugated sheep–anti-rabbit immunoglobulin IgG contained 7·1 mg protein/ml; molecular fluorescein–protein ratio 2:1; standard agar precipitation test (Beutner, Holborow & Johnson, 1967) against rabbit IgG, titre 1:4; agar block titration test against rabbit IgG, titre 1:16 (Feltkamp, 1969). The conjugate was used in a final dilution of 1:32.

Mouse kidneys were studied with the indirect immunofluorescent technique for the demonstration of IgG, IgM, complement and fibrinogen. The technical details will be described elsewhere.

Antinuclear factors were determined by the indirect immunofluorescent technique, using mouse-liver as antigen and unlabelled specific rabbit anti-IgG and anti-IgM sera in combination with the conjugated sheep–anti-rabbit immunoglobulin IgG (Feltkamp & van Rossum, 1968).

Immunoprecipitation

Agar-electrophoresis and micro-immunoelectrophoresis were carried out on sera or serum fractions of individual mice at room temperature on microscope slides according to standard methods.

For the qualitative determination of DNA in some of the cryoprecipitates, the immunoprecipitation technique according to Tan *et al.* (1966) was applied as follows: At each dilution of a two-fold dilution series of a human serum, containing precipitating antibodies to DNA in a titre of 1:4, equal amounts of a cryoprecipitate were added. After incubation for 1 hr at 37°C and overnight at 4°C, the supernatants were tested for antibodies against DNA. As control phosphate buffered saline was used instead of the cryoprecipitate in the absorbtion experiments.

RESULTS

Cryoglobulinaemia, which could be graded as + or + +, was found in 155 (22%) sera out of a series of 697 which covered all age groups. The frequency was significantly ($P<0{\cdot}05$) higher in females (26%) than in males (18%). This difference was due to the fact that cryoglobulins were present in the females at an earlier age than in the males. The combined results and also the influence of age and sex are shown in Table 1. The question of a possible difference between female breeders and virgin mice was not analysed.

TABLE 1. Influence of age and sex on presence of cryoglobulinaemia in NZB mice

Age (months)	Male	Positive	Female	Positive	Total	Positive
0–8	61	1 (2%)	65	11 (17%)	126	12 (10%)
9–20	272	60 (22%)	299	83 (28%)	571	143 (25%)
All age groups	333	61 (18%)	364	94 (26%)	697	155 (22%)

No cryoglobulins were found during the first 3 months and thereafter the incidence rose to about 25%. There was no correlation between age and the amount of cryoprecipitate in the individual mice. About two-thirds could be graded as +, and one-third of the cryoprecipitates were graded as + +. Three mice, aged 5, 12 and 12 months, respectively, showed cryoprecipitates with a cryocrit of about 50%.

TABLE 2. Cryoprecipitates in mice other than NZB

Strain	No. investigated	Cryoprecipitate present
Swiss	60	2
Swiss immunized with streptococcal antigens	40	2
C57/BL	22	0
A/Jax (> 1 year)	47	0
Inbred not registered	32	1
Outbred	30	0
Outbred infected with LCM virus	20	0
Total	251	5* = 2%

* The cryocrit was maximally +.

The time, which was necessary to obtain a cryoprecipitate, varied widely. Some cryoglobulin solutions became turbid directly when cooled, but most had to be kept in the cold overnight to obtain a visible precipitate. In addition to the first series of 697 mice, which covered all age groups and which was also analysed for other parameters, another series consisting of 802 NZB mice was only analysed for the presence of cryoglobulins. In sixty-five animals the amount of cryoglobulins was sufficient for further analysis. Cryoglobulins were also found in some of the control strains (Table 2). The percentage of positive samples was

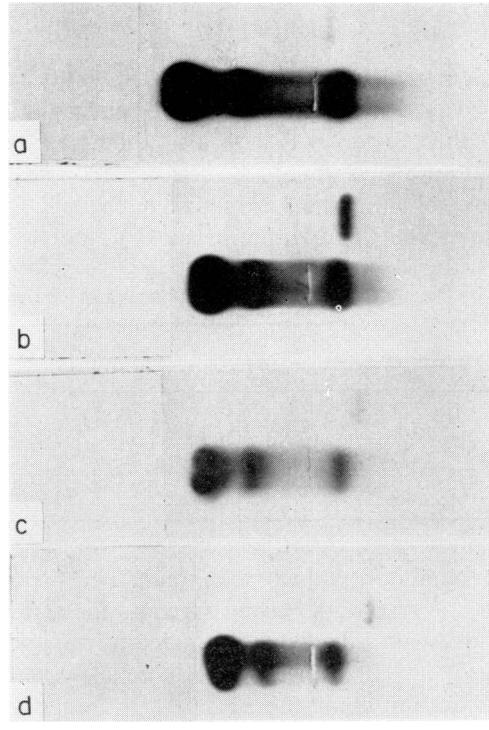

FIG. 1. Agar electrophoresis of four purified cryoglobulin preparations from individual NZB mice. The location of the cryoglobulin indicates a relatively positive charge. The reference sera were derived from the Swiss strain of mice.

far less than in the NZB mice and in none of these the quantity exceeded grade one. This amount proved to be less than the minimal amount which was necessary for the detection in immunoelectrophoresis. On agar-electrophoresis of the purified cryoglobulins (Fig. 1) single bands were seen, which were located on the cathodal side of the place of application of the sample. The immunoelectrophoresis slides (Fig. 2) showed precipitin curves, which indicated the presence of macroglobulins, but which never extended into the anodal part. Some patterns were very suggestive for a monoclonal gammopathy, others were heterogeneous. Occasionally two precipitin curves (Fig. 3) were observed with a reaction of identity, which were compatible with the presence of IgM. The macroglobulin character was apparent in the ultracentrifuge pictures of all eleven samples of individual sera and of all seven pooled samples. These pools were made up of three to fourteen sera of individual mice of a total series of forty-eight animals. The S_{20} values varied from 18 to 20 (Fig. 4).

The antiserum prepared against one of the IgM-cryoglobulins only reacted with the IgM component of normal mouse serum and it formed only one line with the cryoglobulin, which was used for immunization. This sample showed only one peak in the analytical ultracentrifuge (Fig. 4). The limited amount interfered with a detailed investigation, but the location of the peak indicated the presence of a macroglobulin with an S_{20} value of 18·4 (uncorrected). A further five samples were shown to contain IgG and IgM on immunoelectrophoresis (Fig. 5).

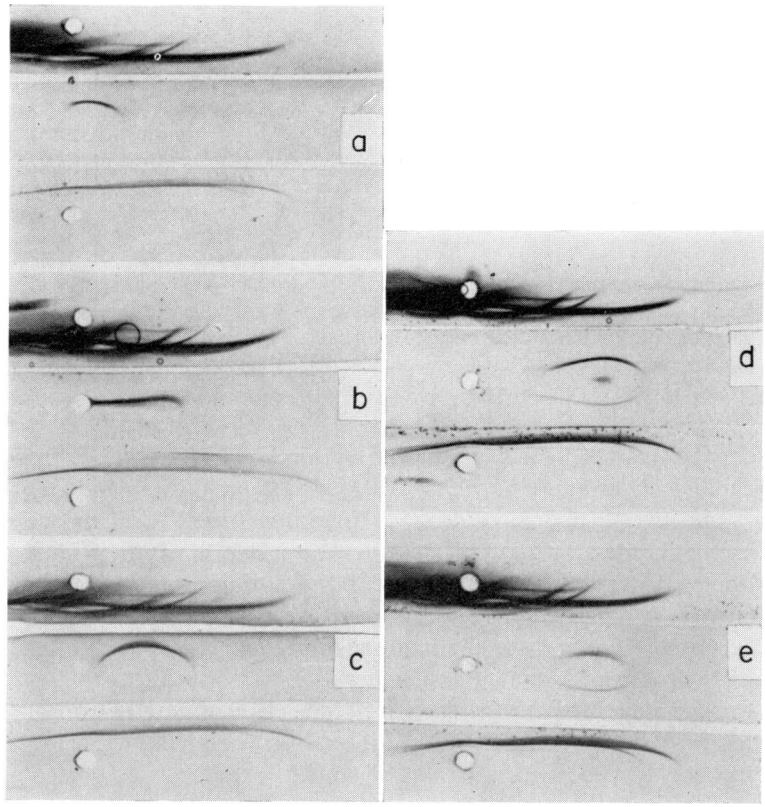

FIG. 2. Immunoelectrophoresis of five purified cryoglobulin preparations from individual NZB mice. In each slide (a to e) the top well and the bottom well were filled with the reference serum, derived from the Swiss strain of mice. The cryoglobulin sample was applied to the centre well. The antiserum in each upper trough was prepared against whole mouse serum, the antiserum in the lower trough recognized γ_1, γ_2 and IgM.

The average IgM level, determined in eighty NZB animals without cryoglobulins and older than 4 months, was 337% of the level in normal Swiss mouse serum and the average level, determined in eleven animals with cryoglobulins, was 475%. The difference is highly significant ($P<0{\cdot}001$).

There was no correlation between the presence of cryoglobulins and the Coombs' positivity, as determined in 305 blood samples from NZB mice, aged 9 months or more (Table 3). Neither could a difference be detected in the histological and immunofluorescent patterns between the kidneys of four mice with cryoglobulins, as compared with four NZB

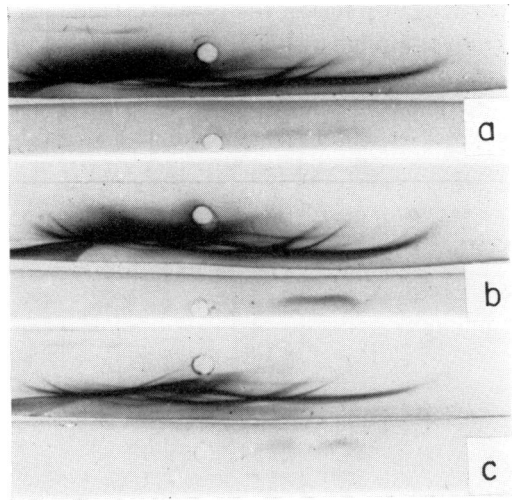

FIG. 3. Immunoelectrophoresis of three purified cryoglobulin preparations from individual NZB mice. In each slide (a, b and c) a reference serum was applied to the top well and the cryoglobulin sample to the bottom well. The troughs contained antiserum against mouse serum. In each slide two cryoglobulin curves could be distinguished, which gave a reaction of identity in slide (a) and (b) and which were separate in (c).

animals which were matched for age and sex but who lacked cryoglobulins. The kidneys were histopathologically examined by Dr Thea M. Feltkamp-Vroom and Dr A. W. Helder. Twenty sera were investigated for antibodies to bromelinized NZB red cells. Nine sera contained these antibodies. Two out of these nine sera, and three other sera revealed cryoglobulins. None of the twenty sera contained cold agglutinins. Sixty sera were investigated for the presence of a rheumatoid factor. In no case could this factor be demonstrated. Cryoglobulins reacted negatively in the immune-adherence test (Melief *et al.*, 1967) using human group O red cells or human granulocytes as indicator cells.

Attempts to determine DNA in the cryoprecipitates by immunologic means have so far failed, as antibodies to DNA could not be absorbed by cryoprecipitates. With eight different cryoprecipitates such a negative result was obtained, in contrast to the total absorption by calf thymus DNA of the anti-DNA factor which was present in a case of human SLE.

TABLE 3. Absence of correlation between the presence of cryoglobulins and a positive direct Coombs' test

		Cryoglobulin		Total
		Present	Absent	
Direct Coombs' test	Positive	39	113	152
	Negative	46	107	153
	Total	85	220	305

$\chi^2 = 0{\cdot}53$; $0{\cdot}3 < P < 0{\cdot}5$.

The total serum of three out of eight NZB mice, containing cryoglobulins, showed a weak antinuclear activity using an anti-IgM serum. The same sera showed stronger antinuclear factors when using an anti-IgG serum. However, the purified cryoprecipitates failed to show any antinuclear activity.

The fluorescent antibody technique was also applied to investigate whether the cryoglobulins had antibody activity against the virus-like particles, which have been demonstrated

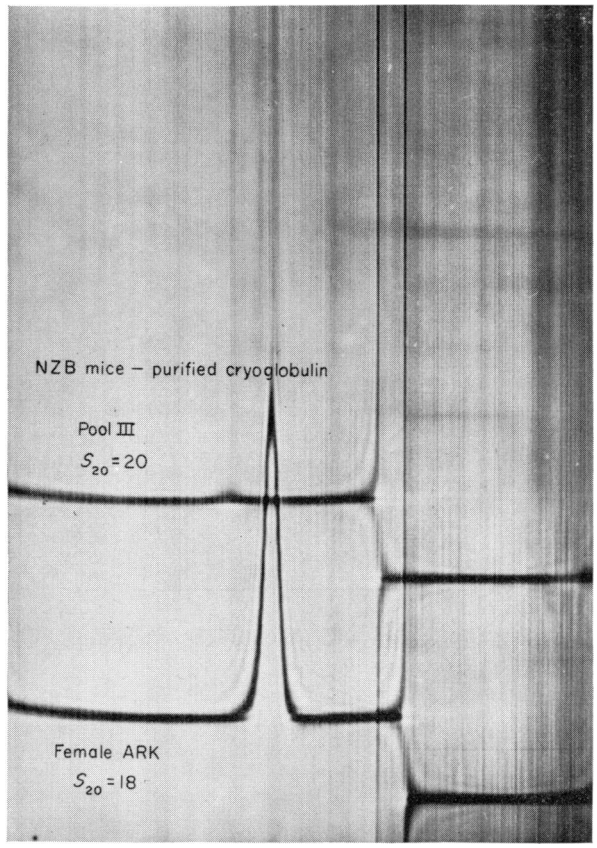

FIG. 4. Ultracentrifugation of purified cryoglobulin preparations from NZB mice. The top line shows the pattern of a pool of seven cryoglobulin samples and the bottom line of the cryoglobulin preparation of a single mouse. The sedimentation coefficients at 20°C (S_{20}) of, respectively, 20 and 18 are uncorrected.

to be present in large numbers in the NZB mice. These particles are present among others in the thymus of these animals (Mellors & Huang, 1966; East *et al.*, 1967; Hollmann & Verley, 1967; Yumoto & Dmochowski, 1967; Helder, personal communication), and this tissue was therefore chosen as substrate. No difference could be detected between the control and the test series.

DISCUSSION

Cryoglobulins in men are usually composed of either one or two classes of immunoglobulins, *viz.* IgG and IgM, and they can accordingly be classified as belonging to the single or mixed

type, respectively. The great majority of the cryoglobulins in NZB mice were found to belong to the first category and these were furthermore always immunoglobulins of the IgM class.

Another subdivision is determined by the fact that these proteins are either heterogeneous or paraproteinaemic. The latter implies antigenic homogeneity, which results in homogeneity with respect to charge. The question of antigenic homogeneity of the L-chain type has not been investigated, but a few cryoglobulins showed distinct bands on agar

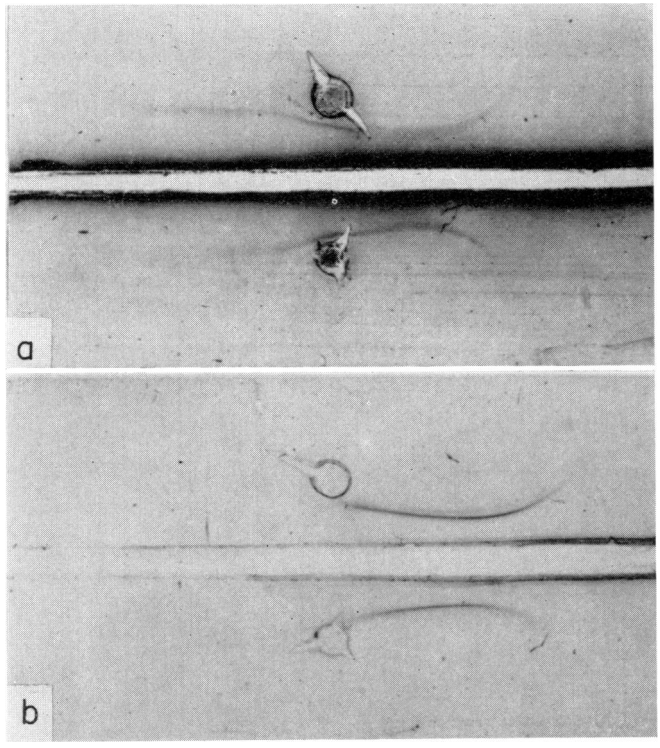

FIG. 5. Immunoelectrophoresis of purified cryoglobulin preparation from a NZB mouse. This sample represents the mixed type, because it reacts with an antiserum which was specific for IgM (a) and an antiserum, which recognized IgG (b).

electrophoresis and sharp curves on immunoelectrophoresis. Since it is known, however, that purified antibodies are not necessarily heterogeneous (Yount *et al.*, 1968), but can behave as myeloma proteins, this observation may have no bearing on the question of the antibody nature of these proteins.

Another property of the cryoglobulins in these NZB mice is their relatively positive charge. Even in the three instances, in each of which two cryoglobulins with different mobility were observed, the less basic component was not located in the high-speed range. Sela & Mozes (1966) have reported that the chemical nature of the antibody can be related to the net electrical charge of the antigen. In view of the widespread presence of virus-like particles in NZB mice (Mellors & Huang, 1966; East *et al.*, 1967) and also of antibodies against DNA in NZB/W mice (Lambert & Dixon, 1968), it was logical to examine the possibility that polynucleotides could form the antigenic stimulus. However, the immunofluorescent

technique revealed no antibodies in the purified cryoprecipitates against NZB thymic glands, known to contain virus-like particles, while also no ANF could be demonstrated in the cryoprecipitates.

Further attempts to investigate the antibody of these cryoglobulins are justified, because in the few publications on such serum proteins in experimental animals, the cryoglobulins resulted from intense antigenic stimulation. Stein & Wertheimer (1942) describe cold-susceptible protein in the blood of dogs, infected with kala-azar. Askonas, Farthing & Humphrey (1960) administered large doses of pneumococcal antigens in rabbits and 'the fact that antibody cryoglobulin was formed in an animal during hyperimmunization suggests that cryoglobulins also may be the products of exaggeration of a normal synthetic mechanism, rather than new and abnormal proteins'. Finally there is a communication by Davie *et al.* (1968), who report on a rabbit immune serum, prepared against a streptococcal vaccine. This serum contained two electrophoretically distinct cryoglobulins, both with antibody activity. One of the peaks was of the 'monoclonal' type, the other was heterogeneous.

In our series there were only five examples of the mixed type. In humans these mixed types usually consist of IgM, which can react with normal IgG and this complex precipitates reversibly in the cold. The macroglobulin component can be of the monoclonal or the polyclonal type (Klein *et al.*, 1968). The rarity of the mixed type in NZB mice interfered with a more detailed analysis and attempts to separate such mixed cryoprecipitates by gel filtration were unsuccessful. It could, therefore, not be proven whether the observed IgG was the result of coprecipitation or if it formed an integral part of the IgG–IgM complex. It is, therefore, not possible to state whether the mixed type in these animals can be considered the analogon of the mixed type in humans. The absence of any activity in the immune adherence reaction supports the contrary.

It has been suggested that these complexes can be of pathogenetic importance (Peetoom & van Loghem-Langereis, 1965). Although this aspect has not been fully investigated, the absence of a positive correlation between the presence of cryoglobulins and a positive antiglobulin test and between cryoglobulins and antibodies to bromelinized red cells argues against this hypothesis as a general mechanism. Besides no correlation between the presence of cryoprecipitates and kidney lesions was demonstrated.

The finding that the IgM level in the mice with cryoglobulins is significantly higher than the level in animals without cryoglobulins supports the argument that the cryoprecipitation is a result of saturation at a temperature, at which the serum proteins in normal concentration are still entirely soluble. This increase of the IgM level was first reported by East, de Sousa & Parrott (1965), who also mentioned the presence of a 'narrow-band hypergammaglobulinaemia' in the macroglobulin line in one of their immunoelectrophoretic analyses.

As cold-agglutinins are IgM antibodies the cryoglobulins were tested for this activity. However, no cold-agglutinins were found.

In cryoprecipitates DNA could not be demonstrated by means of absorption experiments. On the other hand no antinuclear factor activity of the cryoprecipitates was observed. Although it is not ruled out that the pertinent antigenic determinant on the DNA molecule in the cryoprecipitates was fully neutralized by antibodies and that the antinuclear factors were completely bound to DNA, this is unlikely because in no case could any free antigen or antibody from such a complex be demonstrated. The demonstration of antinuclear activity in three out of eight sera containing cryoprecipitates may stimulate further investigation on this subject.

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