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Macroglobulins

2. IMMUNOCHEMICAL STUDIES ON ISOLATED PATHOLOGICAL MACROGLOBULINS

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Albert & Johnson (1961) described the isolation by chromatography of the macroglobulins and other protein fractions from the serum of a single case of macroglobulinaemia. The same macroglobulin and accompanying protein fractions have been further characterized and compared by immunochemical studies, which are now reported. Previous work along these lines has given conflicting results. Thus Morton & Deutsch (1958), on the basis of cross-reactivity and absorption experiments, conclude that several pathological macroglobulins 'do not contain any immunological determinants lacking in normal human serum' and that the iodoacetamide-stabilized dissociation products as well as the reaggregated molecules possess the same antigenic groupings. However, from similar experiments, Franklin & Kunkel (1957) consider that though 7s and 19s components of γ -globulin cross-react, they are not immunologically identical. Similarly Korngold & Van Leeuwen (1957), regarding pathological macroglobulins as immunologically related to normal (γ_1 -)macroglobulins and 7s γ -globulins, report the preparation of antisera to macroglobulins, which by absorption with normal γ -globulins can be made specific for homologous macroglobulin. In substantial agreement with this view, Fahey & Horbett (1959) show that 18s and 7s γ -globulins give rise to distinct and separate precipitation lines when allowed to diffuse against anti-(whole γ) serum.

The different views held might conceivably arise from the different states of purity achieved by different workers for both macroglobulins and 7s

components. Results on chromatographically isolated fractions thus become of considerable interest.

MATERIALS AND METHODS

Serum from a single case (P) of macroglobulinaemia was fractionated on diethylaminoethylcellulose as described by Albert & Johnson (1961) and the various macroglobulin and 7s globulin fractions were utilized in both gel-diffusion (Ouchterlony, 1949) and immunoelectrophoresis (Grabar, 1958) studies.

The macroglobulins used, in both native and dissociated form, were mainly fractions 9 from both fractionations (I and II). Fractions 10.I and 11.II were very similar in composition, but since much smaller quantities were available and they had been less thoroughly characterized physicochemically, they were not much utilized in this section of the work. Of the 7s γ -globulin fractions (all from fractionation I), fraction 6 (γ -6) was mainly used but γ -2, γ -3 and γ -4 were also studied. The different proteins were used in phosphate-NaCl buffer, 1.0.1, pH 7.8, at the following concentrations (g./100 ml.): macroglobulins (native and dissociated) 0.65–0.70; 7s globulin fractions (except γ -6), 0.2–0.4; γ -6, 1.0. In addition to the P serum and derived fractions, a small sample of a further serum (S) containing macroglobulins was made available by Dr R. Arden Jones of Newmarket General Hospital. Some months earlier, the patient (S) had shown a much higher content of macroglobulin ($\approx 47\%$), but, after treatment, the macroglobulin fraction now accounted for some 11% of the total protein. The following antisera were kindly provided by Professor J. R. Marrack and Dr A. Kelus. Rabbit antiserum against macroglobulin 9.II (AM) was prepared by a single multiple subcutaneous injection together with Freund's adjuvant. Anti- γ -globulin serum (A_γ) was prepared in a rabbit by injecting some of the animal's own red cells to which human γ -globulin (contaminated with other serum proteins) had been coupled by bisdiazotized benzidine. The rabbit received ten such injections intravenously.

A further anti- γ -globulin serum ($A_{\gamma T}$) was prepared in a

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rabbit from pooled human sera from patients with cirrhosis of the liver. The γ -globulins were precipitated with thymol, washed, mixed with Freund's adjuvant and injected subcutaneously.

A rabbit antiserum against whole human serum (AW) was prepared as follows. *Salmonella typhi* were coated by exposure to a convalescent typhoid serum, washed and injected intravenously. Somewhat surprisingly the antiserum produced reacted to all the main serum components.

The agar plates for double diffusion were prepared from 1% New Zealand agar in 0.9% NaCl soln. containing 0.1% of sodium azide. The wells, of 5 mm. diam., were bored at the corners and centre of a regular hexagon of side 10 mm. with the help of a jig. After filling the wells with the appropriate solutions, development was allowed to proceed in a saturated atmosphere at room temperature ($16 \pm 2^\circ$) for some 18–20 hr., when the precipitation lines were readily visible. For permanency, the unchanged protein was dissolved by extensive washing in 0.9% NaCl soln. The plates were then dried at 37° after gently squeezing filter paper on the whole surface.

The lines were stained with 0.2% Ponceau S in 3% trichloroacetic acid or by Naphthalene Black in methanol-water-acetic acid (50:50:10, by vol.). The latter dye gave rise to more general background staining but was more successful for the detection of faint precipitation lines.

Immunoelectrophoresis was performed in a gel formed from 1% of agar in 0.06M-boric acid-NaOH buffer, pH 8.25, containing 0.1% of sodium azide. Parallel slots, 2.5 mm. wide and 7 cm. long, were cut 1.5 cm. apart in a rectangular plate (9 cm. $\times$ 7.5 cm.) and cylindrical wells, 2.5 mm. diam., were bored centrally between them and half-way along the length of the slots. A potential of 220 v (d.c.) was applied between platinum-foil electrodes immersed in the above-mentioned boric acid buffer. Washing and staining procedures were as already described for gel diffusion.

RESULTS

The macroglobulin (9.I or 9.II) gave two or possibly three precipitation lines against its own serum (Plate 1, A, C); the γ -globulin fractions (3, 4, 5 and 6) gave no detectable response against the same antiserum (Plates 1, B; 2, A). The absence in the macroglobulin fractions of material sedimenting at about 7s (S_7 material) and of macroglobulin in S_7 fractions was thus strongly indicated, demonstrating the success of the column fractionation (see below, however). The slowly sedimenting fraction 8.II gave a feeble response against anti-M serum, indicative of slight impurity of macroglobulin.

Dissociated macroglobulin gave a less well-defined precipitation pattern than the parent macroglobulin (Plate 1, C and D), a precipitation line (near the antigen well) being missing. The one broad band occurring gave a reaction of partial identity with 9.II (Plate 2, A), in conformity with previous observations (Korngold & Van Leeuwen, 1957). In Plate 1 (C) the lower well containing dissociated macroglobulin had not been freed from

excess of mercaptoethanol. The effect of rapidly diffusing mercaptoethanol on 9.I can be seen by comparing the lower right with upper left well, which contains 9.I in the absence of reducing agents. No such effect occurs when the latter are removed and the protein is stabilized by iodoacetamide.

In Plate 2 (A) and (B), a comparison of the precipitation patterns of macroglobulin with AM and with the two different anti- γ -globulin sera is made. In interpreting these patterns it is to be recalled that the γ -globulins against which the antisera were prepared undoubtedly contained macroglobulins and probably traces of β - and α -globulins as well as the main γ component. The occurrence of an antibody common to AM and anti- γ sera is clear, but an extra precipitation line with the latter is also clearly visible. This line is apparently to be identified with the strong line occurring between γ -6 and anti- γ serum (Plate 2, B), and it is of note that dissociated macroglobulin gives an apparently identical line against anti- γ serum. It would therefore appear that the macroglobulin contains an impurity of S_7 material in low concentration which was not antigenic when injected with the macroglobulin into the rabbit (since γ -6 does not react with AM sera). When the antibody to this material is otherwise stimulated (as by injection at higher concentration in whole γ -globulin), the S_7 impurity, occurring alongside native or dissociated macroglobulin, reacts with it to give the extra precipitation line.

Immunoelectrophoresis was undertaken to investigate these reactions further (Plate 2, D). The macroglobulins were placed in the wells, and subjected to electrophoresis alongside the total globulins (see Albert & Johnson, 1961) from P serum and total serum from a different case (S) of macroglobulinaemia. In the troughs were rabbit antisera against macroglobulins, γ -globulins and total human serum. The precipitation-line system due to the macroglobulins accounts for only a small portion of that occurring between total γ -globulins and total human serum. Against AM sera, the macroglobulins (both 9.I and 9.II) give rise to two precipitation lines in the γ -globulin region, to be identified with those of Plate 1 (A), and an extra line near the origin against A γ serum. The antigen responsible for this line is probably of somewhat higher mobility at pH 8 than those responsible for the main macroglobulin precipitation lines, but no exact electrophoretic description can be made from motion in an electrical field in a gel medium. The extra line here is most probably to be identified with that observed in double-diffusion plates against anti- γ sera (Plate 2, A, B and C), where it has been shown that the antigen responsible is related to γ -globulin. The occurrence of

(A) Centre well: anti-macroglobulin (AM) serum. Top well: Macroglobulin (9.II) at concentration (c) 0.65 g./100 ml. Clockwise from the right of the top well, other wells contain macroglobulin at concentrations $3/4$ c, $1/2$ c, $1/4$ c, $1/6$ c and $1/7$ c.

(B) Centre well: anti-macroglobulin serum. Outer wells: macroglobulin (9.II) and various 7s chromatographic fractions.

(C) Centre well: anti-macroglobulin serum. Outer wells: macroglobulin (9.II), dissociated macroglobulin (D.II) (containing mercaptoethanol) and fraction 8.II.

(D) Centre well: anti-macroglobulin serum. Outer wells: macroglobulin (9.I and 9.II) and dissociated macroglobulin (D.II).

(A) Top and bottom wells: anti- γ -globulin serum ($A\gamma_T$). Centre well: anti-macroglobulin (AM) serum. Other wells contain macroglobulin (9.I and 9.II), dissociated macroglobulin (D.II) and the 7s fraction (6.I).

(B) Top well: anti-macroglobulin (AM) serum. Centre well: anti- γ -globulin serum ($A\gamma$). Other wells contain macroglobulin (9.I and 9.II), dissociated macroglobulin (D.II) and the 7s fraction (6.I).

(C) Top well: anti-macroglobulin (AM) serum. Centre well: anti- γ -globulin ($A\gamma$) serum. Other wells contain macroglobulin fractions (9.I, 10.I, 11.II) and 7s fraction (6.I).

(D) Immunoelectrophoresis. Solutions of macroglobulins (9.I and 9.II), total γ -globulins (from same P serum) and total serum proteins from S serum (see text for details) were placed in various wells and subjected to electrophoresis. Troughs were then filled with one of the following antisera: anti-macroglobulin, anti- γ -globulin ($A\gamma$) and anti- (total) human serum.

such material in small concentration occurring to the extent of up to 5% of the total protein alongside the main macroglobulin is not in any sense ruled out by previously published sedimentation diagrams. The absence of the corresponding line between the total globulins of P serum and AM serum confirms the absence in the AM antiserum of the relevant antibody (see above), which apparently requires higher antigen concentrations for its formation than are present in the macroglobulin preparation.

The whole serum (S), while showing certain lines common to the P macroglobulins against AM serum, shows definite differences and its precipitation lines are relatively weak, indicating relatively low concentration of the macroglobulins. The ultracentrifuge gave a similar indication about concentration, but no qualitative differences between the macroglobulins occurring and those of P serum.

In Plate 2 (C), chromatographically different fractions containing macroglobulin (9.I, 10.I, 11.II) are compared alongside γ -6 in their reactions with AM and $A\gamma$ antisera. Although the different macroglobulin fractions all give the typical macroglobulin line and the extra line against $A\gamma$, it is clear from the strengths of the lines that 10.I and 11.II both contain the γ -6 type of impurity in higher concentration than 9.I.

DISCUSSION

The results confirm the great resolution possible in the chromatography of proteins on diethylaminoethylcellulose. Thus the major macroglobulin fractions contain 7s γ -globulin impurity in such small amounts that the latter did not stimulate antibody production when injected into a rabbit in the macroglobulin fraction. That the impurity did occur, however, in amounts too small to be observable in the ultracentrifuge, was shown by the reaction of these macroglobulin fractions with anti-whole- γ -globulin sera, when a precipitation line, additional to those occurring with anti-macroglobulin sera, occurred. Such impurity also occurred in larger concentration in the minor macroglobulin fractions.

Similarly, if macroglobulin occurred in 7s globulin fractions, it was in too low a concentration to give rise to a visible precipitation line with anti-macroglobulin sera. These conclusions are in substantial agreement with those of Fahey & Horbett (1959), who chromatographically isolated 7s and 18s γ -globulins and showed that they gave rise to distinct and different precipitation bands against anti-whole- γ -globulin. The claim by Korngold &

Van Leeuwen (1957) to have produced antisera specifically against macroglobulins is also in accord with the above conclusions.

Reports of cross-reactivity and antigenic identity between macroglobulins and 7s γ -globulins may have arisen from imperfect separations, based usually upon solubility differences between the two types of molecule.

SUMMARY

1. The main chromatographically separated macroglobulins gave two well-defined precipitation lines against anti-macroglobulin sera produced in the rabbit.

2. Chromatographically separated γ -globulin fractions did not give detectable precipitation lines against the same antisera.

3. Against anti- (whole γ -globulin) sera ($A\gamma$), macroglobulin fractions gave rise to the same lines as against anti-macroglobulin sera and an extra weaker line related to that produced when γ -globulin fractions reacted with $A\gamma$ sera. Apparently a γ -globulin-type impurity occurred in the main macroglobulin fractions at a concentration such that it did not, on injection, stimulate the formation of its respective antibody. Subsidiary macroglobulin fractions contained such an impurity in higher concentration. The absence in the anti-macroglobulin serum of the antibody corresponding to the 'extra' precipitation line was shown by absence of the line when whole γ -globulins diffused against anti-macroglobulin sera.

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