The Homogeneous γ G-Immunoglobulin Produced by Mouse Plasmacytoma 5563 and its Subsequent Heterogeneity in Serum

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The mouse plasma cell tumour 5563 has been shown to synthesize and secrete a single molecular species of γ G-immunoglobulin, which was identified by labelling with ^{14}C -labelled amino acids. The heterogeneity of G-myeloma globulin as it is found in serum of tumour-bearing mice is due to subsequent changes in the charge properties of the newly secreted molecules. These changes have been reproduced *in vitro*. On incubation with sterile serum, the newly formed radioactive myeloma protein changed its chromatographic and electrophoretic properties to coincide with those of myeloma protein isolated from serum. Incubation of purified myeloma protein band α , under a variety of conditions, led to the characteristic pattern of serum myeloma protein showing multiple electrophoretic bands. The chemical nature of the molecular changes is not yet known. It is suggested that serum-induced changes could contribute to the electrophoretic heterogeneity of specific antibodies within the γ G-class of immunoglobulins. This demonstration of the production of a single molecular species of immunoglobulin by a plasma cell tumour provides support for the concept of 'one-cell-one antibody'.

Mice bearing plasma cell tumour 5563 form large amounts of G-myeloma globulin, which is present in serum in high concentrations (Potter, Fahey & Pilgrim, 1957). The serum G-myeloma globulin is heterogeneous on starch-gel electrophoresis at pH 8.9, showing at least five discrete bands differing slightly in net charge. These myeloma protein components can be partly separated from each other by chromatography on DEAE-cellulose; they all have sedimentation coefficient 6.6S on ultracentrifugation, and run true on repeated starch-gel electrophoresis (Askonas, 1961). No differences in antigenic properties could be detected. The serum pattern of G-myeloma globulin in mice bearing this tumour line had apparently stayed constant over many transplant generations, so that a single clone of cells appeared to be producing several very similar molecular species of γ G-immunoglobulin. This type of electrophoretic heterogeneity is characteristic of G-myeloma globulins in the serum of mice carrying other lines of plasmacytomas (Fahey, 1961) and of the myeloma protein found in 70% of G-myeloma patients (Fahey, 1962); it is not observed with A-myeloma globulins.

Recently we have observed variation in the relative concentrations of 5563 G-myeloma protein bands and this has led us to question the origin and nature of heterogeneity of myeloma protein. After

prolonged storage at -16° (with occasional thawing and freezing) of reference sera from mice bearing tumour 5563 it was noticed that a shift in concentration of the differently charged myeloma proteins occurred; the myeloma proteins migrating faster on electrophoresis at pH 8.9 became more concentrated relative to the slowest-migrating component. Moreover, serum, collected in the cold, from mice with rapidly growing tumours showed mainly the two slowest-migrating components and only very low concentrations of the more negatively charged myeloma proteins (see Plate 1).

We have reinvestigated the problem of G-myeloma protein heterogeneity and have found that the 5563 tumour plasma cells produce and secrete a single molecular species of γ G-immunoglobulin that differs slightly in its chromatographic properties from any of the heterogeneous components isolated from serum. The newly formed G-myeloma globulin was characterized by incubating tumour cells *in vitro* with ^{14}C -labelled amino acids and subsequent chromatography of the labelled myeloma protein from the intracellular and extracellular fluids with carrier myeloma protein prepared from serum. On incubation with serum the homogeneous, newly formed, radioactive myeloma protein becomes heterogeneous and acquires chromatographic and charge properties

similar to the myeloma protein components found in serum. We also found that incubation at 37° of serum from mice carrying plasmacytoma 5563, or of partially purified myeloma fractions with serum, caused partial shift of the slower-migrating bands to the more negatively charged bands migrating faster on starch-gel electrophoresis at pH 8.9.

It would appear that the G-myeloma globulin becomes heterogeneous in serum as a result of intramolecular conformational changes, or removal or addition of charged groups, without alteration of the sedimentation coefficient. A variety of conditions were found to mediate the conversion into heterogeneous γ G-components but the molecular basis of the change is not yet known.

MATERIALS AND METHODS

Solutions and reagents. Solution E contains tris (10mM), KCl (10mM) and $MgCl_2$ (1mM), pH 7.5. Uniformly ^{14}C -labelled protein hydrolysate was purchased from The Radiochemical Centre, Amersham, Bucks. Sodium deoxycholate was obtained from British Drug Houses Ltd., Poole, Dorset. Bovine serum albumin, fraction V, was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Plasma cell tumour 5563. This tumour line was originally obtained through the kindness of Dr M. Potter, National Cancer Institute, Bethesda, Md., U.S.A. It was maintained in C_3H/He mice in two forms: (a) as solid tumour by subcutaneous transplantation and (b) as ascitic cells by intraperitoneal transfer. The G-myeloma protein stayed constant in both forms of the tumour over many years of transplantation.

Starch-gel electrophoresis. This was carried out by the method of Smithies (1955), with 0.05M-glycine buffer, pH 8.9, in the gel, and 0.1M-sodium borate buffer, pH 8.9, in the electrode vessels.

Purification of G-myeloma protein. The 5563 myeloma protein was purified from serum or ascitic fluid of tumour-bearing mice by chromatography on DEAE-cellulose as described by Askonas (1961). For preparation of a sample containing mainly the slowest-migrating myeloma protein, the myeloma protein was eluted from DEAE-cellulose with a slow gradient (0.01–0.05M-phosphate buffer, pH 6.5) as described below for chromatography of labelled myeloma protein.

Partial purification of G-myeloma protein for use as non-radioactive carrier myeloma protein was obtained from serum by precipitation with $(NH_4)_2SO_4$ at 35% saturation at 0°. The precipitate was dissolved in water and reprecipitated at the same $(NH_4)_2SO_4$ concentration. It was dialysed against 0.01M-phosphate buffer, pH 6.5, and stored frozen.

Carboxypeptidase treatment. Crystallized carboxypeptidase A treated with di-isopropyl phosphorfluoridate (to eliminate tryptic and chymotryptic activity) and carboxypeptidase B (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) were a kind gift from Dr C. Milstein, Medical Research Council Laboratory of Molecular Biology, Cambridge.

The purified myeloma protein (containing mainly the

band α migrating most slowly on electrophoresis at pH 8.9) at a concentration of 5mg./ml. was incubated at 37° with carboxypeptidase B (20 μ g. of enzyme/mg. of myeloma protein) in 0.025M-tris buffer, pH 7.6, containing NaCl (0.1M), under a layer of toluene.

Carboxypeptidase A was solubilized before use in dilute alkali at 0° and immediately neutralized and added to a sample of the same preparation of myeloma protein in 0.025M-tris buffer, pH 7.6, containing NaCl (0.3M).

Incubation of plasma cells with radioactive amino acids. Ascitic 5563 tumour cells were washed with Hanks medium (Hanks, 1948) and the erythrocytes lysed differentially by a 2min. exposure to hypo-osmotic medium. Then 1.4×10^8 tumour cells were suspended in 3ml. of Hanks medium containing 10mg. of bovine serum albumin/ml. and incubated for 2hr. at 37° with 15 μ C of uniformly ^{14}C -labelled amino acids (protein hydrolysate, specific activity 600 μ C/mg.). The cells were spun down and the supernatant represented the extracellular fluid. The intracellular protein was extracted by resuspending the cells in 0.8ml. of solution E and disrupting them by addition of 0.2ml. of 5% (w/v) deoxycholate. The lysed cells were layered on a sucrose density gradient [8ml. of 30%, 1ml. of 10% and 1ml. of 4% (w/v) sucrose in solution E]. After centrifugation for 1hr. in a Spinco 40 rotor at 39000 rev./min., the top 3ml. was removed, dialysed against 0.01M-phosphate buffer, pH 6.5, in the cold and stored frozen at -16°. The extracellular fluid was similarly dialysed and stored for chromatography on DEAE-cellulose.

Chromatography of ^{14}C -labelled myeloma proteins. Whatman DE11 DEAE-cellulose was washed and treated as described by Peterson & Sober (1956). DEAE-cellulose (5g.) was equilibrated with 0.01M-phosphate buffer, pH 6.5, and packed to give a column approx. 35cm. $\times$ 0.8cm. The extracellular or intracellular protein samples containing about 10^5 counts/min. in myeloma protein and 20mg. of added non-radioactive myeloma protein were applied. A 100ml. volume of 0.01M-phosphate buffer, pH 6.5, was passed through the column before linear-gradient elution was commenced. A slow gradient, consisting of 250ml. each of 0.01M- and of 0.05M-potassium phosphate buffer, pH 6.5, in two Erlenmeyer flasks, was used to give maximum spreading of the heterogeneous myeloma proteins. The flow rate was controlled at 1ml./min. with a pump. Fractions (4ml.) were collected and their extinction values at 280m μ measured. The radioactivity of total protein and G-myeloma protein precipitable with specific antisera was determined as described below.

Determination of radioactivity of protein. Bovine serum albumin (0.5mg.) was added to 1ml. samples of each fraction and the total protein precipitated with 5% (w/v) trichloroacetic acid. The precipitates were filtered on Oxoid membranes, washed successively with 5% trichloroacetic acid, ethanol-ether (1:1, v/v) and ether, and counted in a Packard Tri-Carb scintillation Counter.

Precipitation of myeloma protein with specific antiserum. Antiserum specific for 5563 myeloma protein was prepared by immunizing rabbits with the pure G-myeloma protein in Freund's adjuvant (Askonas, 1961). The NaCl concentration of selected column fractions was adjusted to iso-osmoticity with 1.5M-NaCl and 0.2ml. samples were measured out. Their immunoglobulin content was calculated on the basis of extinction measurements at 280m μ . Purified myeloma protein was added to a final concentration

of 0.25 mg./sample and a slight excess of antiserum to myeloma protein was added. After incubation at 37° for 20 min. and at 4° overnight the antigen-antibody precipitates were washed twice with 0.14 M-NaCl, suspended in 5% (w/v) trichloroacetic acid, filtered on Oxoid membranes and counted as for total proteins.

Nomenclature. In this paper we use the nomenclature recommended by the World Health Organisation (1964). In addition, to facilitate description of the results, the five 6-6s myeloma proteins are referred to as *a*, *b*, *c*, *d* and *e* according to their rate of migration towards the anode on starch-gel electrophoresis at pH 8.9; *a* refers to the slowest-migrating component and *e* to the most negatively charged myeloma protein (Plate 1).

RESULTS

Conditions leading to alteration in electrophoretic charge properties of G-myeloma protein molecules

Incubation of sera from mice carrying 5563 plasmacytoma. Serum, freshly collected from tumour mice in the cold, shows on starch-gel electrophoresis at pH 8.9 the two slowest-migrating myeloma protein components *a* and *b* at a far higher concentration than the two more negatively charged components *c* and *d* (Plate 1, 1). Incubation of serum from tumour-bearing mice overnight at 37° results in a pronounced shift in the relative concentration of the myeloma protein components, resulting in a decrease in the slowest-migrating and an increase in the more negatively charged protein bands (Plate 1, 3). Overnight incubation at 15° induces little observable change (Plate 1, 2). In addition, we have observed that storage of tumour serum for several years with intermittent freezing and thawing also produces a shift in relative concentration of the bands (Plate 1, 5).

Since serum contains various enzymes it seems feasible that an enzymic reaction is responsible for removing positively charged groups or unmasking carboxyl groups. Serum is known to contain a Co^{2+} -activatable peptidase (Erdős, Sloane & Wohler, 1964), and in the presence of mM-cobalt sulphate the shift in concentration of myeloma protein towards negatively charged bands is more pronounced; in addition, a new protein band, even more negatively charged, accumulated (Plate 1, 4). Thus serum enzymes can have considerable effect on myeloma protein.

Incubation of purified myeloma proteins under various conditions. The changes in charge properties of the 5563 myeloma protein caused by incubation of myeloma serum are mimicked when purified myeloma protein is incubated with sterile normal mouse serum (not illustrated). The purified myeloma protein was prepared by slow gradient elution from DEAE-cellulose (see the Materials and Methods section). A chromatographic fraction was obtained containing mainly the slowest electro-

phoretic protein *a* and a little *b* myeloma protein (see Plate 1, 8). This fraction was used in further experiments on the conditions that promote heterogeneity.

Incubation of purified myeloma protein with carboxypeptidase A or B led to partial disappearance of myeloma protein *a* and appearance of a third band *c*, as well as further degradation of the molecule resulting in a new protein band migrating with a mobility slower than the original myeloma protein (Plate 1, 6 and 7). Shorter-term incubations up to 4 hr. showed very little change. The change could not be specifically attributed to either carboxypeptidase A- or B-like action. Detailed structural analysis would be required to elucidate the alteration in charge properties.

It was then found that addition of enzymes is not essential for conversion of myeloma protein *a* into the more negatively charged components. This change takes place very slowly even in the absence of added enzymes although contamination of the purified myeloma protein with serum enzymes cannot be excluded. Changes in the starch-gel pattern are found after overnight incubation of the purified myeloma protein in tris buffer, pH 8.2, at 37° (Plate 1, 10). After similar treatment at pH 6.8 no definite change was visible. Involvement of SH groups in the rearrangement has been excluded since the presence of iodoacetate during incubation did not inhibit the changes in electrophoretic mobility.

The results suggest that G-myeloma globulin *a* can become heterogeneous under a variety of conditions; it is converted both *in vivo* and *in vitro* into more negatively charged molecules. This alteration in charge properties is gradual, incubations for many hours being necessary to obtain the

EXPLANATION OF PLATE 1

Analyses of myeloma protein molecules by starch-gel electrophoresis. Electrophoresis was carried out for 6 hr. at pH 8.9 (see the Materials and Methods section). Equal volumes (15 μL) of the protein solutions were loaded on to pieces of Whatman 3MM filter paper (0.4 cm. $\times$ 0.8 cm.), which were put into the gel. Arrows indicate the point of sample application. 1, Fresh ascitic fluid collected in the cold from mouse with ascitic form of 5563 tumour. 2, As 1, incubated overnight at 15° under toluene. 3, As 1, incubated overnight at 37° under a layer of toluene. 4, As 3, except that CoSO_4 was present at a concentration of 1 mM. 5, Serum from mouse bearing 5563 tumour, diluted fourfold with 0.9% NaCl and stored frozen for 4 years with occasional freezing and thawing. 6, As 8, treated with carboxypeptidase B overnight. 7, As 8, treated with carboxypeptidase A overnight. 8, 0.5% myeloma protein, purified by chromatography on DEAE-cellulose with gradient elution with 0.01–0.05 M-phosphate buffer, pH 6.5. 9, As 8. 10, As 9, incubated overnight at 37° in 0.1 M-tris buffer, pH 8.2.

PLATE I

EXPLANATION OF PLATE 2

Starch-gel electrophoretic pattern of selected fractions obtained by chromatography of radioactive, newly-synthesized, myeloma protein with carrier myeloma protein on DEAE-cellulose. Tubes were selected from the gradient illustrated in Fig. 1. The material from each tube was freeze-dried and dissolved in 0.1 ml. of water before application on the starch gel. 1, Carrier myeloma protein; 2, tube no. 66; 3, tube no. 69; 4, tube no. 74; 5, tube no. 90.

Fig. 1. Chromatography of radioactive, newly synthesized, 5563 myeloma protein with carrier myeloma protein on DEAE-cellulose. Tumour cells were incubated in the presence of ^{14}C -labelled amino acids as described in the Materials and Methods section. Extracellular fluid and 20mg. of myeloma protein purified from serum were chromatographed on DEAE-cellulose (5g.), with linear-gradient elution (0.01–0.05 M-potassium phosphate buffer, pH 6.5). Fractions (4ml.) were collected. ●—●, Extinction of fractions at 280 μ ; ○—○, radioactivity precipitable with 5% (w/v) trichloroacetic acid; □, radioactivity precipitable with specific antiserum to 5563 myeloma protein.

striking changes. It is not yet clear whether this molecular change is due to enzyme action or to non-enzymic loss of labile groupings from the molecule.

Characterization of newly synthesized myeloma protein

Ascitic tumour cells were incubated at 37° with ^{14}C -labelled amino acids and non-radioactive carrier myeloma protein was added separately to both the extracellular fluid and the intracellular soluble proteins extracted by disrupting the cells with deoxycholate. The samples were dialysed against 0.01 M-phosphate buffer, pH 6.5, and chromatographed on columns of DEAE-cellulose. Radioactivity of protein was determined in each gradient fraction as described in the Materials and Methods section. The elution pattern of extracellular fluid plus carrier myeloma proteins is shown in Fig. 1. The radioactivity of the newly formed myeloma protein is present in a symmetrical peak, suggesting homogeneity compared with the extinction pattern at 280 μ of the added heterogeneous carrier myeloma protein prepared from serum. The peak of radioactive protein does not coincide with the main peak of the carrier myeloma protein, which corresponds to the component migrating slowest on starch-gel electrophoresis at pH 8.9, as shown by the starch-gel pattern of four single chromatographic fractions chosen across the gradient (Plate 2). Chromatography of the intracellular extract gave identical results.

It was ascertained that the radioactive protein in

the symmetrical peak represented myeloma protein by adding antiserum specific for 5563 myeloma protein to samples of the gradient fractions. The columns (Fig. 1) show that all of the radioactivity was precipitable with the specific antiserum. The results thus show that the newly formed G-myeloma protein is homogeneous as opposed to the heterogeneous myeloma protein isolated from serum, and that it has slightly different chromatographic properties on DEAE-cellulose at pH 6.5, being eluted slightly ahead of the serum myeloma protein.

Alteration in vitro of the chromatographic properties of newly formed myeloma protein

Since it had been observed that the myeloma protein component migrating slowest at pH 8.9 became more negatively charged on incubation with serum, a portion of the radioactively labelled extracellular fluid was incubated overnight at 37° with sterile normal mouse serum; non-radioactive carrier myeloma protein was then added and the material chromatographed on DEAE-cellulose as before. A shift in chromatographic and charge properties of the newly formed radioactive myeloma protein resulted (Fig. 2). The radioactive protein now follows the elution pattern of the carrier myeloma protein more closely. The gradient fraction representing the peak of radioactivity and extinction at 280 μ contained the myeloma protein component migrating slowest on starch-gel electrophoresis at pH 8.9; the chromatography conditions were thus strictly comparable with those illustrated by Fig. 1 and Plate 2.

Fig. 2. Chromatography of ^{14}C -labelled myeloma protein after incubation with normal sterile mouse serum overnight at 37° . Carrier myeloma protein was added after the incubation. The chromatographic conditions were the same as those described in Fig. 1. A portion of the same ^{14}C -labelled extracellular fluid and purified G-myeloma protein were used as for chromatography illustrated by Fig. 1. ●—●, Extinction at $280\text{m}\mu$; ○—○, radioactivity precipitable with 5% (w/v) trichloroacetic acid.

DISCUSSION

The present results indicate that plasma cell tumour 5563 forms and secretes a single molecular species of γG -immunoglobulin. The heterogeneity of the G-myeloma globulin seen in the serum of mice carrying this tumour line is due to subsequent changes in chromatographic and electrophoretic charge properties of the newly formed molecules.

We have noted two types of changes leading to the conversion of the newly formed molecules into the heterogeneous myeloma protein components present in the serum of the tumour mice. One type shows up on chromatography on DEAE-cellulose at pH 6.5 (see Fig. 1), in which system newly formed radioactive myeloma protein is eluted slightly ahead of the carrier myeloma protein isolated from serum; this first reaction changes the chromatographic properties of the newly formed myeloma protein so that all of it is eluted with the carrier myeloma protein (see Fig. 2).

The second type of change leads to the heterogeneity of G-myeloma globulin as it is found in serum; where starch-gel electrophoresis at pH 8.9 reveals five myeloma protein bands, each of sedimentation coefficient 6.6s, but differing in electrophoretic mobility. Both the initial change in chromatographic properties and the subsequent conversion into heterogeneous myeloma proteins with increasing negative charge can be reproduced by incubation *in vitro* of purified myeloma protein or the newly formed radioactively labelled myeloma protein in the presence of serum (see Plate 1 and Fig. 2); conversion into multiple components also takes place at a lower rate in the absence of serum.

Kölsch (1966) in a preliminary report describes a similar phenomenon when dealing with another mouse tumour line, MOPC-21, forming G-myeloma globulins. The molecular basis for these changes is not yet clear. The heterogeneity of the myeloma protein is most likely caused by loss of labile groupings from the molecule, with resulting changes in charge properties. Examples of electrophoretic and chromatographic heterogeneity of proteins have been reported involving labile amide groups (e.g. sperm-whale myoglobin; Edmundson & Hirs, 1962; Edmundson, 1965) and differences in carbohydrate content (e.g. ribonuclease A and B; Plummer & Hirs, 1964). Further work is required to establish whether similar phenomena are responsible for conversion of the G-myeloma globulin into heterogeneous components.

The conversion of the newly-synthesized myeloma protein into band *a* would seem to occur by a different process from that which subsequently gives rise to the multiple protein bands. The initial conversion is complete whereas the secondary changes leading to the electrophoretic heterogeneity occur slowly and incompletely. Myeloma protein band *a* does not disappear completely. It is compatible with the available evidence that the initial change in the newly synthesized myeloma protein could be the formation of blocked *N*-terminal amino acids. Pyrrolidonecarboxylic acid has been found at the *N*-terminus of heavy chains from rabbit γG -immunoglobulin (Wilkinson, Press & Porter, 1966) and pathological human G-myeloma globulin (Press, Piggott & Porter, 1966). The cyclization of *N*-terminal glutamyl or glutaminyl residues would

be a feasible reaction to explain the conversion of the newly synthesized myeloma protein into band α . This proposal is amenable to further experimentation.

Originally, it was reported that myeloma protein extracted from the microsomal pellet of 5563 tumour cells showed the same heterogeneity as the myeloma protein in the serum (Askonas, 1961). The extraction procedure involved incubation at 37° of a large amount of microsomal pellet and concentration of the material at room temperature. At the time we were unaware of the changes in charge properties of the myeloma protein that occur unless the tumour extracts and sera are handled in the cold.

The loss of labile groupings from γ G-immunoglobulin molecules may occur more generally and contribute to the observed electrophoretic heterogeneity of specific antibodies within the γ G-class of immunoglobulins. Most of the G-myeloma globulins in mouse and man show this striking banding on starch-gel electrophoresis. With 5563 G-myeloma globulin the electrophoretic heterogeneity of the starting material is reflected in heterogeneity of both the light and heavy chains if starch-gel electrophoresis is carried out in urea at pH 8.6; at acid pH there are no charge differences (B. A. Askonas, unpublished work). On papain digestion, both the Fab and Fc fragments have heterogeneous charge properties (Askonas & Fahey, 1961), so that alteration in charge properties takes place in various parts of the γ G-molecule. Reisfeld & Small (1966) have described banding of heavy chains as well as light chains isolated from specific antibody. It is possible that this is due in part to the sort of phenomenon we have described, although the heterogeneity of cell clones forming specific antibody must still be an important factor in the production of heterogeneous antibody.

The finding that the neoplastic plasma cell produces a single molecular species of γ G-immunoglobulin has an important bearing on the 'one cell-one antibody' hypothesis. The studies on antibody production by single cells have shown that most antibody-forming cells are phenotypically restricted to producing molecules with a single antibody specificity (see review by Nossal & Mäkelä, 1962). Similarly, individual cells appear to form only one class of light chains (κ or λ), one class of heavy chain

(α , γ or μ) or one heavy-chain allotype (Pernis & Chiappino, 1964; Cebra, Colberg & Dray, 1966). These observed limitations do not prove that a given plasma cell synthesizes only a single molecular species of immunoglobulin. However, the present results and their implication with regard to the origin of the heterogeneity of γ G-immunoglobulins greatly strengthen the idea that a single plasma cell, whatever the genetic potential of its precursor cell, is generally restricted to the expression of one structural gene of light-chain type and one of heavy-chain type.

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