Immunological studies of an atypical (myeloma) immunoglobulin

S. G. O. JOHANSSON AND H. BENNICH

Reprinted from
Immunology
Vol. 13, No. 4, October 1967

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD AND EDINBURGH

Immunology, 1967, **13**, 381.

Immunological Studies of an Atypical (Myeloma) Immunoglobulin

S. G. O. JOHANSSON AND H. BENNICH

The Blood Center, University Hospital, and Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

(*Received 10th January* 1967)

Summary. An 8S myeloma component, isolated from serum of a patient with myelomatosis is described, which appears to have no antigenic determinants in common with human α-, δ-, γ- or μ-polypeptide chains as revealed by immunoelectrophoresis and Ouchterlony gel diffusion analysis.

The myeloma protein migrates in the fast γ-region on electrophoresis at pH 8·6 and has an elution volume on Sephadex G-200 similar to that of 6·5S IgA.

The isolated myeloma component has an approximate molecular weight of 200,000 and a total carbohydrate content of 10·7 per cent.

Reduction with β-mercaptoethanol and acid dissociation yields light polypeptide chains of Type L and a carbohydrate-rich component, in the ratio of 1:4.

Antisera specific to determinants on the heavy chains of the myeloma protein showed no reaction with the immunoglobulins A, D, G or M. Instead unique determinants were found on the heavy polypeptide chains.

INTRODUCTION

Immunoglobulins are classified* as IgA, IgG, IgM or IgD (Rowe and Fahey, 1965b) according to the antigenic determinants carried by their heavy polypeptide chains (Cohen and Porter, 1964a). Furthermore, subclasses are known of IgA (Kunkel and Prendergast, 1966; Terry and Roberts, 1966; Vaerman and Heremans, 1966) of IgG (Grey and Kunkel, 1964; Terry and Fahey, 1964) and of IgM (Deutsch and Mackenzie, 1964; Harboe, Deverill and Godal, 1965).

Highly elevated concentrations of an immunoglobulin, usually conspicuous in electrophoretic patterns of the serum and associated with light chains of distinct antigenic type and electrophoretic mobility (Edelman and Poulik, 1961; Cohen and Porter, 1964b) are often seen in plasmocytic and lymphocytic neoplastic disorders, and sometimes in apparently healthy persons.

These proteins (M-components) usually have one or more antigenic determinants in common with one of the immunoglobulin classes. Therefore, the observation of an M-component lacking antigenic determinants in common with α-, δ-, γ- or μ-chains is particularly interesting (see review: Franklin, Feinstein and Fudenberg, 1966).

* Nomenclature according to *Bull. Wld Hlth Org.*, **30**, 447, 1964.

The present paper describes an atypical myeloma component of Type L isolated from the serum of a patient with myelomatosis and Bence Jones proteinuria; the atypical serum protein could not be identified as an immunoglobulin A, D, G or M.

MATERIALS AND METHODS

Case report

The patient N.D., a 50-year-old farmer, was admitted to the Department of Internal Medicine, University Hospital, Uppsala in July 1965 because of pain in the chest and lower back. Physical examination revealed nothing unusual. Laboratory investigations showed: haemoglobin, 10·2 g/100 ml, blood leucocytes 16,000/mm^3 of which 61 per cent were immature plasma cells. The sedimentation rate was 132 mm/hr and the serum creatinine level, 1·1 mg/100 ml. The urine contained protein and Bence Jones protein was demonstrated. Total serum proteins amounted to 9·5 g/100 ml. A typical M-component (4·5 g/100 ml) was observed in the fast γ-region by paper electrophoresis. The bone marrow smear showed numerous immature plasma cells resembling those in the peripheral blood. X-ray examination of skull, spine and pelvis was normal. Aspiration biopsy of the liver showed a diffuse interstitial infiltration of immature plasma cells.

Serum, urine and saliva

Samples of serum, ACD-plasma (Acedex®, Pharmacia), urine and saliva, collected July 1965 to October 1966, were stored at $-20°$ unless otherwise stated.

Samples of urine (20–25 ml) were filtered through Whatman filter paper and concentrated to 0·2–0·5 ml by ultrafiltration in collodion membranes (Membranfiltergesellschaft, Göttingen, Germany).

Isolation of myeloma protein

Two millilitres of serum were applied on a column (2 × 50 cm) of ethanolysed cellulose (Munktell 400, Grycksbo, Sweden) equilibrated with 0·1 M barbital buffer of pH 8·6 (Porath, 1956). Electrophoresis was carried out at 6 V/cm for 48 hours at 6–8°; protein peaks were located by ultraviolet absorption and the myeloma fraction was concentrated by ultrafiltration (Preparation A).

One volume of freshly collected plasma was diluted with 1 volume of 0·15 M sodium chloride and precipitated at 24–26° with sodium sulphate (18 g/100 ml) as described by Kekwick (1940). The yield of myeloma protein ND after three precipitations was approximately 2 g/100 ml plasma, and on immunoelectrophoresis the fraction contained IgA, IgG and fibrinogen, and occasionally albumin, in addition to the myeloma component. Samples of the precipitate containing about 400 mg protein in 10 ml were applied to a column of Sephadex G-150, 40–50 μ (dry state) 3·2 × 94 cm in 0·1 M Tris–HCl–0·2 M NaCl, pH 7·7, containing 0·002 M $EDTANa_2$ and 0·02 per cent NaN_3. Recycling (Porath and Bennich, 1962) carried out at 20° with a constant flow rate of 2 cm/hr separated most of the IgG from the myeloma protein peak (Preparation B).

Gel filtration on Sephadex G-200

Samples of myeloma serum ND were separated on Sephadex G-200 (Pharmacia) equilibrated with 0·1 M Tris–HCl–0·2 M NaCl, pH 7·7 containing 0·002 M $EDTANa_2$ and 0·02 per cent NaN_3. Serum (9 ml) diluted three times with 0·15 M NaCl was applied

to a column 3·2×93·5 cm. The flow rate was 1·6 cm/hr at 20°. The distribution of the immunoglobulins and the myeloma component was estimated by the SRD method (see below). For comparison a mixture of purified myeloma protein (Preparation B), 6·5S IgA and IgG were separated on the G-200 column under the same experimental conditions.

Ultracentrifugal analysis

Sedimentation velocity measurements were made in a Model E Spinco Ultracentrifuge operated at 59,780 rev/min with 12-mm double sector cells. The measurements were made at 20° and sedimentation coefficients were evaluated by extrapolation to zero protein concentration and corrected to the viscosity and density of water at 20° (Svedberg and Pedersen, 1940). The molecular weight was calculated from measurements at different protein concentrations by the Archibald method as described by Ehrenberg (1957). All measurements refer to Preparation B in equilibrium with 0·1 M Tris–HCl–0·2 M NaCl, pH 7·68. A preparation of 6·5S myeloma IgA, which had the same elution volume on Sephadex G-200 (see 'Results') as myeloma protein ND was analysed in the same way.

Carbohydrate analysis

Hexose was determined by the orcinol–sulphuric acid method as described by Svennerholm (1956). A freshly prepared solution of D-mannose (Pfanstiehl) was used as standard.

Hexosamine was determined by the method of Cessi and Piliego (1960) as described by Johansen, Marshall and Neuberger (1960). Samples were hydrolysed in 6 N HCl in evacuated, sealed Pyrex tubes for 3 hours at 100°. D-Glucosamine–HCl (Pfanstiehl) was used as standard.

Sialic acid was determined by the thiobarbituric acid method as described by Warren (1959). Samples were hydrolysed by sulphuric acid (0·05 N final concentration) for 70 minutes at 100°. *N*-Acetyl-neuraminic acid (Burr) was used as standard.

Fucose was determined as described by Dische and Shettles (1948) using L-fucose as standard.

The carbohydrate content was expressed as percentage by weight of protein, determined spectrophotometrically (see below).

Reduction with β-mercaptoethanol

Purified myeloma protein ND (Preparation B) was reduced by the method of Fleischman, Pain and Porter (1962) using 0·1 M β-mercaptoethanol.

Separation of reduced-alkylated myeloma protein by gel filtration in acid (Fleischman *et al.*, 1962)

One hundred and fifty milligrams of reduced-alkylated myeloma protein in 10 ml of 0·15 M sodium chloride were separated on Sephadex G-100, 40–120 μ, 3·2×92 cm, equilibrated with 1 M HOAc–0·025 M NaCl at 20°. Fractions of 3·0 ml were collected at a constant flow rate of 2·25 cm/hr and analysed for protein and hexose as described above.

Starch gel electrophoresis

Starch gel electrophoresis was run in horizontal trays (Wake and Baldwin, 1961) in buffers containing 8 M urea–0·05 M formic acid–NaOH, pH 3–3·5 (Edelman and Poulik,

1961) or 8 M urea—0·035 M glycine–NaOH, pH 7–8 (Cohen and Porter, 1964b). Samples (40 μl) containing 5–10 mg/ml were applied on Whatman 3MM, 6 × 14 mm inserted in the gel. Electrophoresis was carried out at 3·5 V/cm for 20–22 hours at 20° and gels were stained with 0·02 per cent Nigrosin.

Immunochemical analysis

Immunoelectrophoresis was done according to the micro-modification of Scheidegger (1955) using 1·5 per cent Bacto agar 'Special Agar-Noble' (Difco) in barbital buffer of pH 8·2 ($I = 0{\cdot}05$) at 6 V/cm for 80 minutes at room temperature.

Quantitative estimations of immunoglobulins were performed by a single radial immunodiffusion method (SRD) as described by Mancini, Carbonara and Heremans (1965). Ouchterlony gel diffusion analysis and the SRD method were performed in 0·3 M phosphate buffer of pH 8·0 (Rowe and Fahey, 1965a).

Antisera

All antisera were raised in rabbits with the exception of one horse anti-normal human serum. Antigen (0·5–1 mg in 0·2 ml) mixed with 0·8 ml of complete Freund's adjuvant was injected intramuscularly and repeated after 14 days and subsequently three times at weekly intervals. The animals were bled 10 days after the last injection.

Antisera were made specific as described below, by absorption for 1 hour at 37° and overnight at 4°. All antisera were tested for specificity by immunoelectrophoresis against normal human serum, and by Ouchterlony gel diffusion analysis against purified IgA, IgG and IgM light polypeptide chains from normal pooled IgG and Bence Jones proteins at different concentrations.

Anti-IgA. Antisera were raised against IgA isolated from three different A myeloma sera by gel filtration on Sephadex G-200 and against one IgA preparation from pooled normal sera (Op. No. 3163, kindly provided by Behringwerke, A.G., Marburg/L, Germany). The antisera were absorbed with serum from a healthy individual lacking IgA.

Anti-IgD. Specific rabbit antisera were kindly provided by Dr D. S. Rowe, University of Birmingham, England. Three antisera had been raised against myeloma protein S.J., one against myeloma protein Ni, and one against myeloma protein Mi.

Anti-IgG. Two preparations of Cohn Fraction II (1204 DtV 254 and 1348 DtV 269, kindly supplied by Kabi, Stockholm, Sweden) purified by DEAE cellulose-chromatography were used for immunization of several rabbits. Each antiserum was absorbed with light chains isolated from reduced-alkylated normal IgG and, if necessary, with IgA from two different A myeloma sera and IgM from normal sera, isolated by gel filtration on Sephadex G-200.

Anti-IgM. Two antisera were used, one raised against IgM isolated from pooled normal serum by starch block electrophoresis and gel filtration on Sephadex G-200, and one against IgM isolated in the same way from the serum of a patient with macroglobulinemia (Killander, 1965). The antisera were absorbed with normal IgG (Cohn Fraction II) and with light chains isolated from normal IgG.

Anti-normal human serum (anti-NHS). One rabbit antiserum was raised against pooled normal sera. One rabbit anti-NHS (Op. No. 2) was obtained from Behringwerke A.G., Marburg/L., Germany. One horse anti-NHS (No. 2292) was obtained from the Red Cross Central Laboratory for Blood Transfusion, Amsterdam.

Anti-Bence Jones protein. Antisera were raised against Bence Jones proteins of Type K and Type L (BJ 112 and BJ 120, kindly provided by Dr H. van Eijk, University of Utrecht, Holland and from several myeloma urine samples) and also against Bence Jones protein isolated from the urine of patient N.D. Rabbits were immunized with 0·15 ml Bence Jones protein (2 mg/ml) mixed with an equal volume of complete Freund's adjuvants (Difco) in each footpad. Two injections were given with an interval of 1 week. The animals were bled 3 weeks after the last injection. The antisera were tested for specificity by immunoelectrophoresis and Ouchterlony gel diffusion analysis, and when necessary absorbed with Bence Jones protein Type K or Type L. These experiments were made in collaboration with Dr I. Berggård, Institute of Medical Chemistry, University of Uppsala.

Anti-myeloma protein ND. Antisera were raised in two rabbits against Preparation A and in two rabbits against Preparation B (see above). Before absorption, all antisera produced two faint parallel precipitin lines in the γ-region when tested by immunoelectrophoresis against normal sera. The same results were obtained with normal IgG (Cohn Fraction II). Antisera absorbed with either 0·05 mg/ml normal IgG (Cohn Fraction II) or 0·05 mg/ml pooled normal sera were specific for the myeloma protein ND. Furthermore, absorption with 0·05 mg/ml Fab-fragment isolated from a papain digest (Porter, 1959) of myeloma protein ND (Bennich and Johansson, 1967) rendered the antisera specific for the Fc-fragment of myeloma protein ND.

Other methods

Zone electrophoresis of serum and concentrated urine (see above) was carried out on filter paper and on cellulose acetate sheets (Spinco Microzone). The total protein concentration in serum was calculated from determinations of protein nitrogen by the Kjeldahl method. These analyses were kindly performed by the Department of Clinical Chemistry, University Hospital, Uppsala.

Unconcentrated urine was analysed for Bence Jones protein by heat precipitation as described by Putnam, Easley, Lynn, Ritchie and Phelps (1959).

The concentration of isolated myeloma protein was estimated from the absorbancy at 280 nm 1 cm using an extinction coefficient of 14 for a 1 per cent solution in 0·01 N HCl.

RESULTS

STUDIES ON MYELOMA SERUM ND

The M-component appears as a sharp band in the γ-region on cellulose acetate electrophoresis (Fig. 1); a weaker cathodal band corresponded to Bence Jones protein. On Sephadex G-200 IgG is significantly retarded relative to the myeloma protein (Fig. 2). On immunoelectrophoresis of the myeloma serum using a polyvalent immunoglobulin antiserum two distinct precipitin lines were obtained, which could not be detected in normal serum (Fig. 3a). One discrete line was located in the slow γ-region and one in the fast γ-region. IgG appeared to be present in lowered concentrations, whereas IgA and IgM could not be detected by immunoelectrophoretic analysis. The actual concentrations of the different immunoglobulins in serum ND estimated by the SRD method are given in Table 1.

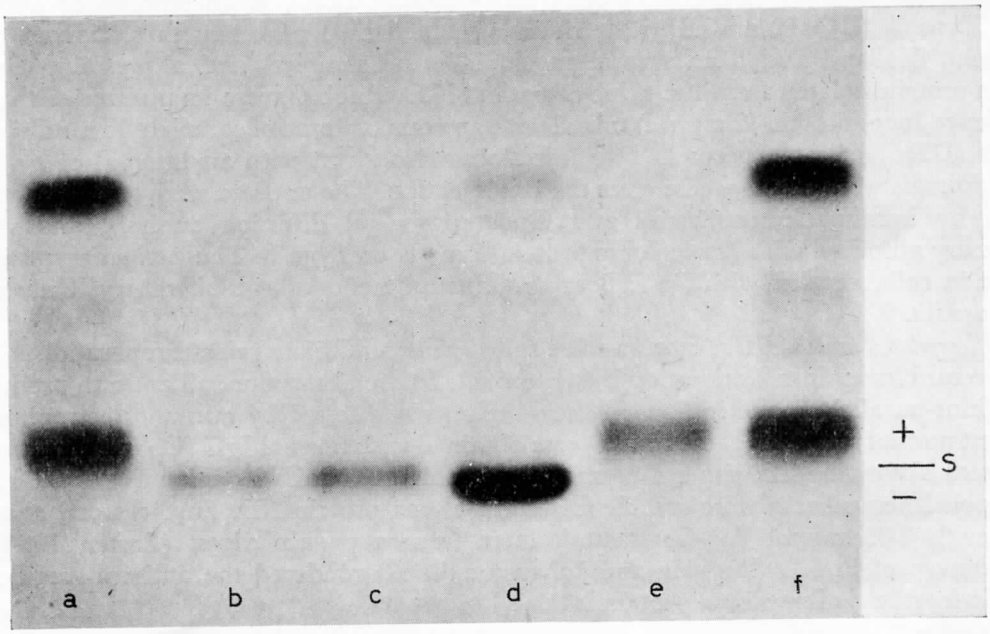

FIG. 1. Electrophoresis on cellulose acetate sheet (Microzone, Spinco) pH 8·6, of myeloma serum ND (a and f); light chains from myeloma protein ND-Preparation B, 15 mg/ml (b); Bence Jones protein ND, 15 mg/ml (c); concentrated urine ND (d); myeloma protein ND– Preparation B, 17 mg/ml (e).

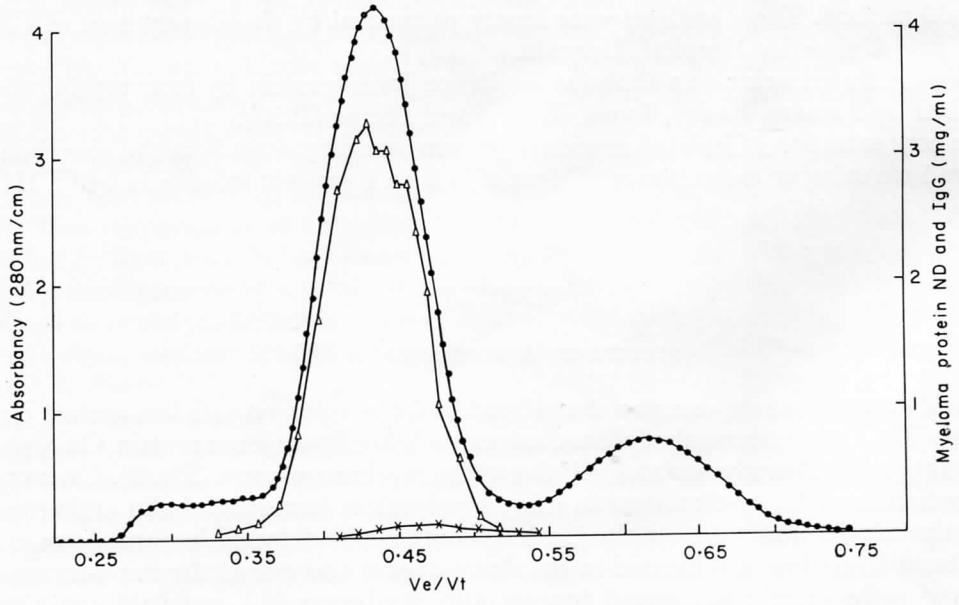

FIG. 2. Separation of myeloma serum ND on a 3·2 × 93·5 cm column of Sephadex G-200 equilibrated with a Tris-buffer of pH 7·7. The distribution of myeloma protein ND (△) and IgG (×) was determined by single radial immunodiffusion (Mancini *et al.*, 1965). ●, Absorbancy.

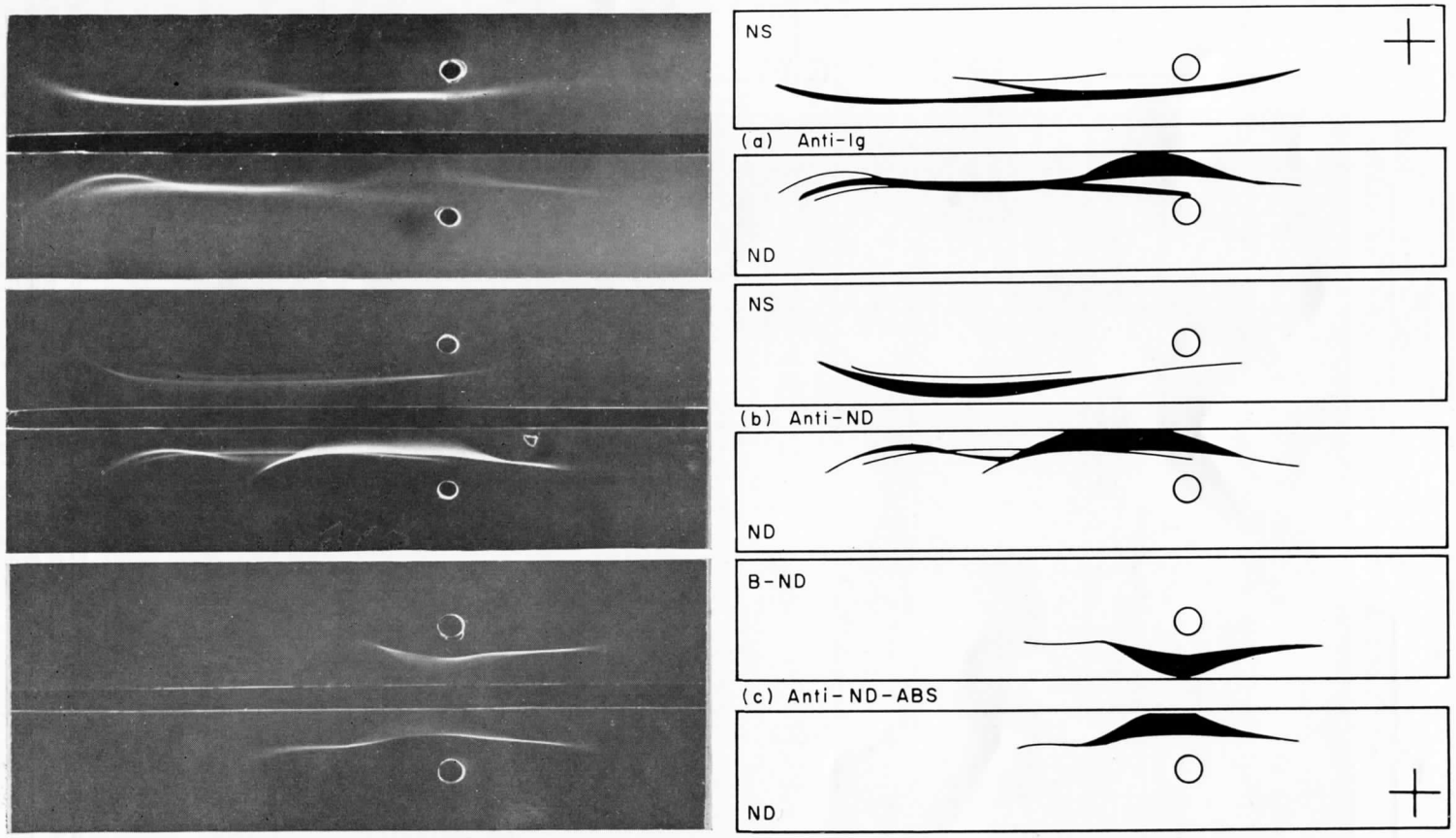

FIG. 3. Immunoelectrophoretic analysis in agar, pH 8·2, of normal human serum (NS); myeloma serum (ND); Preparation B of myeloma protein ND(B-ND), using: (a) a polyvalent immunoglobulin antiserum (anti-Ig); (b) an unabsorbed antiserum to Preparation A of myeloma protein ND (anti-ND); (c) an antiserum to Preparation B of myeloma protein ND, absorbed with normal human IgG, Cohn Fraction II and autologous Fab-fragment in excess (anti-ND-ABS).

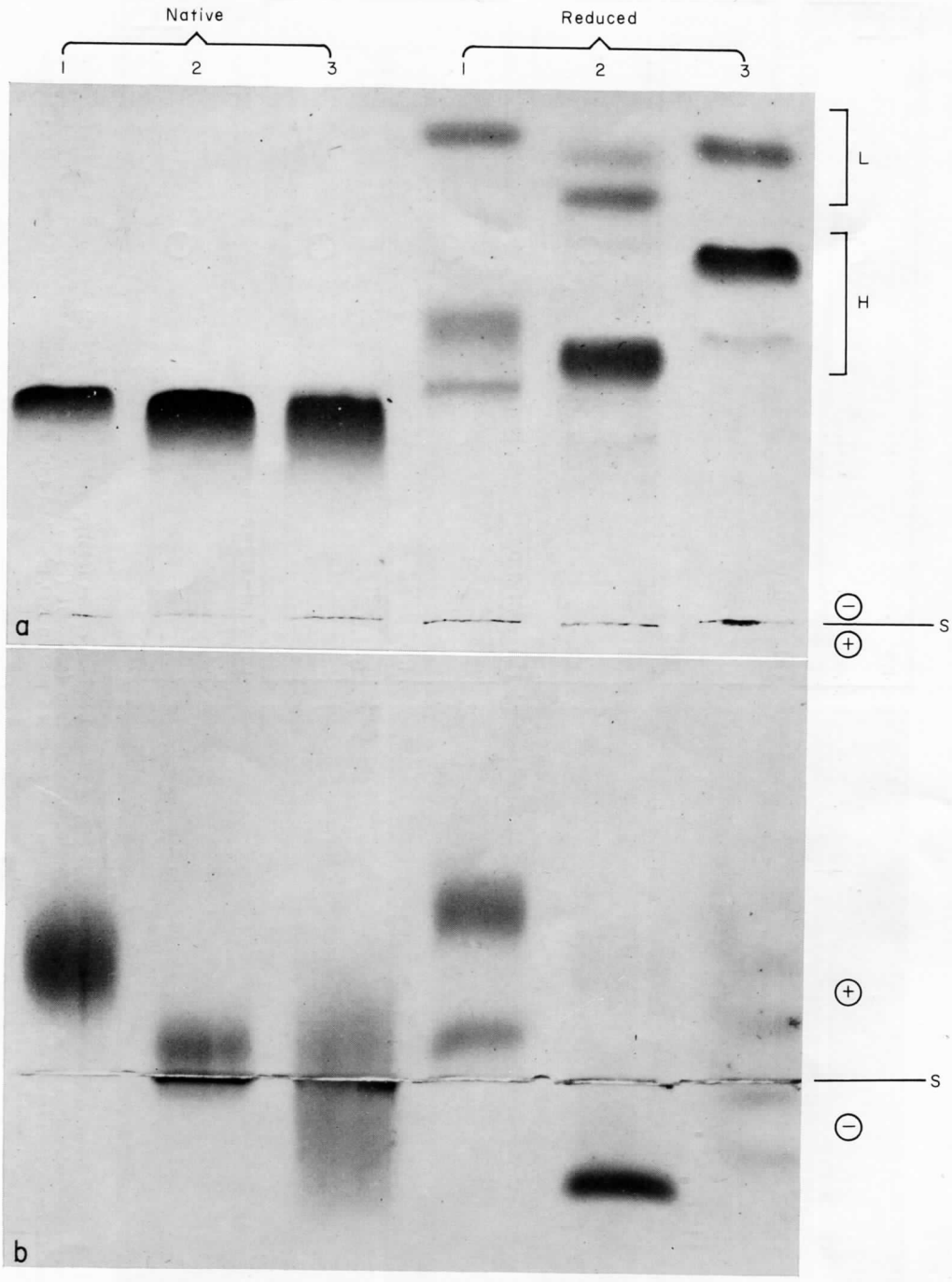

FIG. 7. Electrophoresis in 8 M urea at pH 3·5 (a) and pH 7 (b), of native and reduced-alkylated samples of 6·5S IgA (1); myeloma protein ND–Preparation B (2); IgG (3), GL_{B5} myeloma (pH 3·5) and Cohn Fraction II (pH 7). Heavy chains (H) and light chains (L). Light chain ND shows strong tendency for aggregation depending upon the experimental conditions for reduction (Bennich and Johansson, 1967).

The fast γ-component reacted with antisera specific to Bence Jones protein of Type L but not Type K. Both antisera reacted with the IgG present in the myeloma serum.*

Antisera made specific for IgA, IgD, IgG and IgM failed to detect the component in the fast γ-region and also failed to reveal any myeloma component of the A, D, G or M classes.*

Antisera to myeloma protein (Preparation A) produced a heavy precipitin line in the fast γ-region as well as a line corresponding to IgG (Fig. 3b). A precipitin line was seen

TABLE 1

CONCENTRATIONS OF IMMUNOGLOBULINS AND MYELOMA PROTEIN ND DETERMINED BY THE SRD METHOD (VALUES ARE EXPRESSED IN mg/ml)

	IgA	IgD	IgG	IgM	Myeloma protein ND
Myeloma serum ND*	0·12	<0·01	3·90	<0·01	38·0
Myeloma serum ND†	0·07	<0·01	3·30	0·05	41·5
Preparation A	0·01	<0·01	1·26	<0·01	16·2
Preparation B	0·03	<0·01	0·40	<0·01	17·0
Saliva ND‡	0·02	<0·01	0·04	<0·01	0·31
Saliva ND§	0·03	<0·01	0·05	<0·01	0·43
Normal sera¶	1·58	0·11	13·23	0·84	<0·01

* 15th February 1966.
† 2nd October 1966.
‡ 8th July 1966.
§ 14th July 1966.
¶ Average values for ninety-seven healthy individuals (Johansson, to be published).

also in the slow γ-region, which occupied the same position as the Bence Jones protein present in serum ND. This line crossed the IgG line, which in turn crossed over the heavy precipitin line present in the fast γ-region. Antisera to protein ND absorbed with normal IgG or normal sera gave a visible reaction only with the component migrating in the γ-region, which could also be detected after additional absorption of antisera with autologous Fab-fragments (0·3 mg/ml) as shown in Fig. 3(c).

STUDIES ON ISOLATED MYELOMA PROTEIN ND

The myeloma protein ND was isolated by column electrophoresis (Preparation A) as shown in Fig. 4 and by gel filtration (Preparation B) of a sodium sulphate precipitate (Fig. 5). The concentrations of immunoglobulins A, D, G and M in Preparation A and B are summarized in Table 1, and the chemical and ultracentrifugal analyses of Preparation B appear in Table 2.

Gel filtration on Sephadex G-200 revealed that Preparation B was eluted with the same volume as the myeloma protein present in serum (Fig. 6) and that its position was indistinguishable from that of 6·5S IgA (Fig. 6).

After reduction and alkylation the protein contains two components on starch gel electrophoresis (Fig. 7) or gel filtration in acid (Fig. 8). The first fraction is rich in carbohydrate and on starch gel electrophoresis at acid pH migrates significantly faster than the parent

* These observations were kindly confirmed by Dr D. S. Rowe, University of Birmingham and by Dr M. W. Turner, Department of Immunology, Institute of Child Health, University of London.

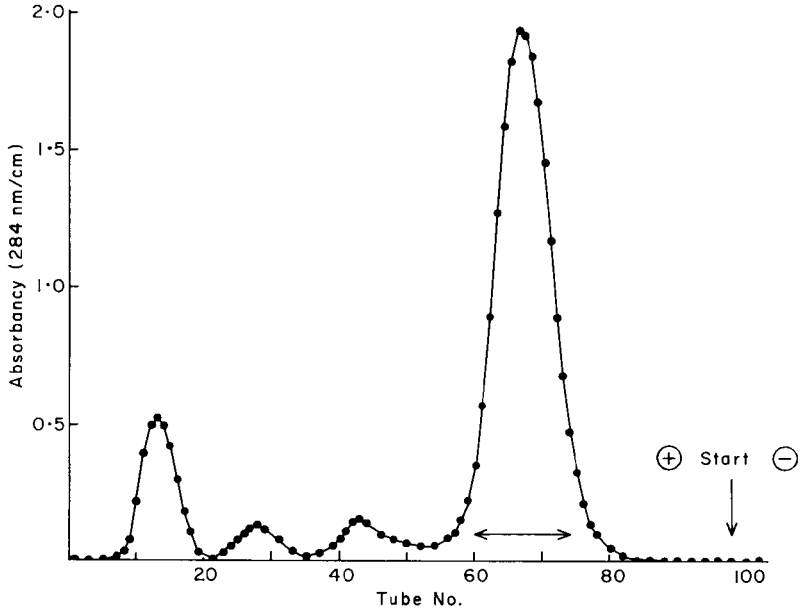

FIG. 4. Separation of myeloma serum ND by electrophoresis on a 2 × 50 cm column of Munktell cellulose 400 equilibrated with 0·1 M barbital buffer of pH 8·6, at 6 V/cm for 48 hours. The cross-bar indicates fractions collected and referred to as myeloma protein ND–Preparation A.

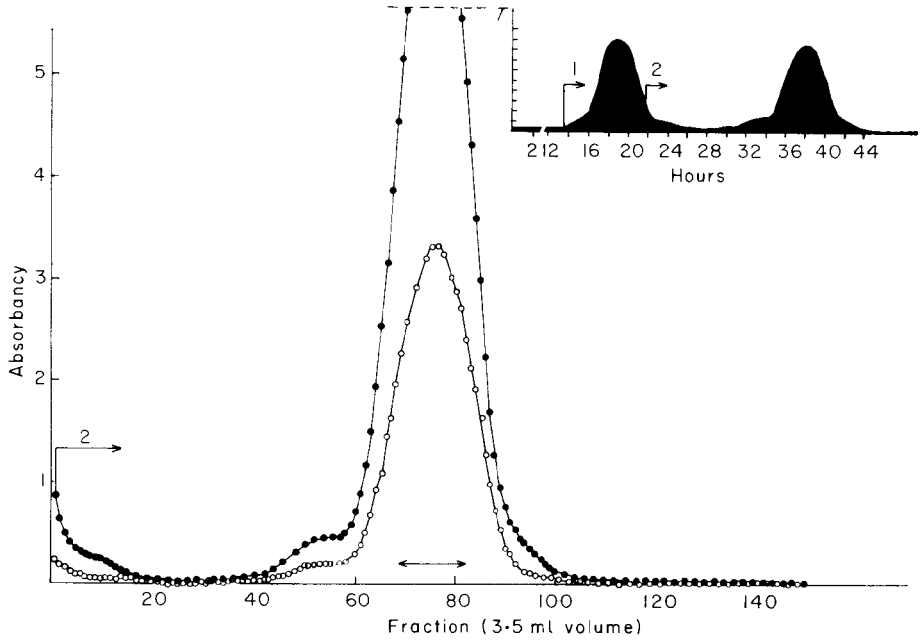

FIG. 5. Separation of a sodium sulphate precipitate of myeloma plasma ND on a 3·2 × 94 cm column of Sephadex G-150 equilibrated with a Tris-buffer of pH 7·7. The sample was re-cycled once as indicated by the inserted figure, which shows the transmission at 254 nm. At position 1 the column system was closed to allow effluent to pass directly into column; at position 2 the system was opened for elution. The cross-bar indicates fractions collected and referred to as myeloma protein ND–Preparation B. Absorbancy 280 nm (●); Hexose, orcinol–sulphuric acid reagent, absorbancy 520 nm (○).

protein and slower than α- and γ-chains. The second component (Fig. 8) contains no carbohydrate and was identified as light chains of Type L (Fig. 9) having a mobility corresponding to the B-1 band in an alkaline buffer system (Cohen and Porter, 1964b) (Fig. 7b).

TABLE 2
ULTRACENTRIFUGAL AND CARBOHYDRATE ANALYSIS

	Myeloma protein ND*	IgA†
Molecular weight‡	196,000	139,000
$S^{\circ}_{20w} \times 10^{13}$‡	7·92	6·53
Hexose	5·53	2·60
Fucose	0·56	0·44
Hexosamine	3·64	3·68
Sialic acid	0·98	0·77
Total carbohydrate§	10·71	7·49

* Preparation B.

† Isolated from A myeloma serum. The elution volume of purified protein on Sephadex G-200 is similar to that of myeloma protein ND (Preparation B) (see Fig. 6).

‡ Extrapolated to zero protein concentration. The partial specific volume was assumed to be $\bar{v}_{20} = 0{\cdot}713$.

§ Average values from three determinations, expressed as per cent by weight of protein as determined spectrophotometically ($E^{1\ \text{per cent}}_{280\,\text{nm}} = 14$).

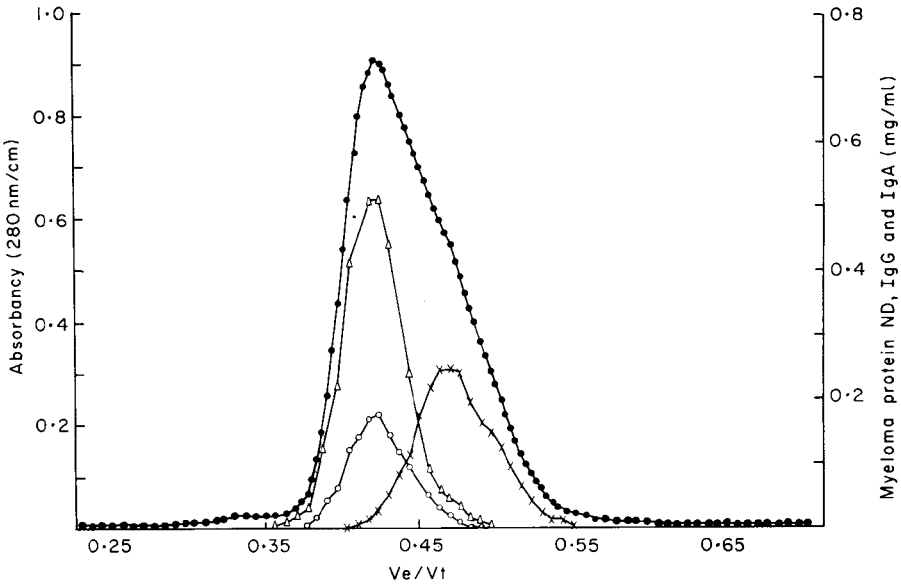

FIG. 6. Separation of a mixture containing myeloma protein–Preparation B, (32·4 mg), 6·5S IgA isolated from an A myeloma serum (11·7 mg) and normal IgG, monomer (16·4 mg) on a 3·2 × 93·5 cm column of Sephadex G-200 equilibrated with a Tris-buffer of pH 7·7. Quantitative determination of protein ND (△), IgA (○) and IgG (×) was performed by single radial immunodiffusion (Mancini *et al.*, 1965). The results should be compared with Fig. 2. ●, Absorbancy.

Electrophoresis on cellulose acetate at pH 8·6 showed that the isolated light chains had the same mobility as the Bence Jones protein from patient ND (Fig. 1).

Immunoelectrophoretic analysis of Preparation B using specific antisera to protein ND revealed a distinct precipitin line in the fast γ-region similar to that observed in the patient

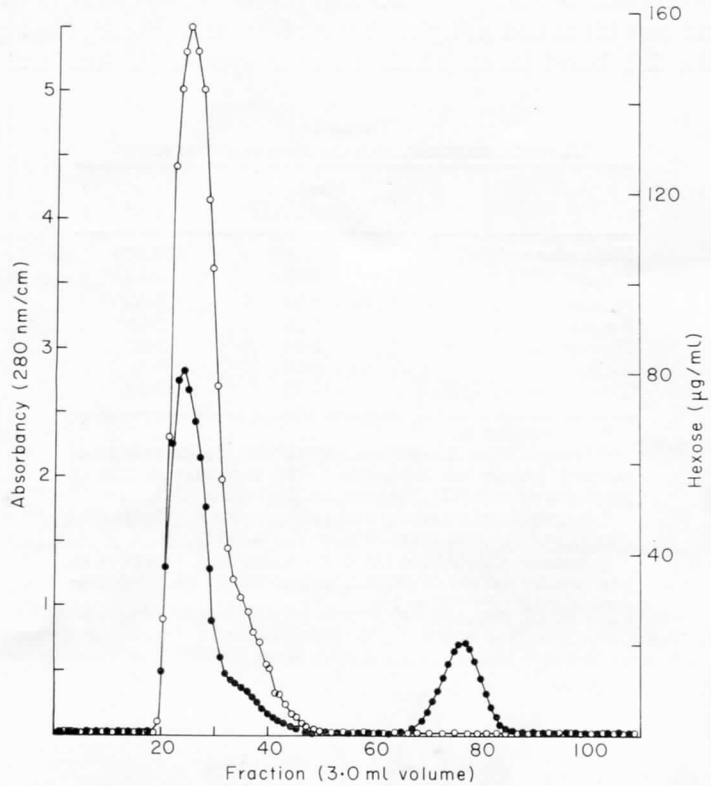

FIG. 8. Separation of reduced and alkylated myeloma protein ND–Preparation B on a 3·2 × 92 cm column of Sephadex G-100 equilibrated with 1M acetic acid–0·025 M sodium chloride. Fractions 19–50 and 65–85 represent 79 and 21 per cent of total protein (280 nm) respectively. ○, Hexose; ●, Absorbancy.

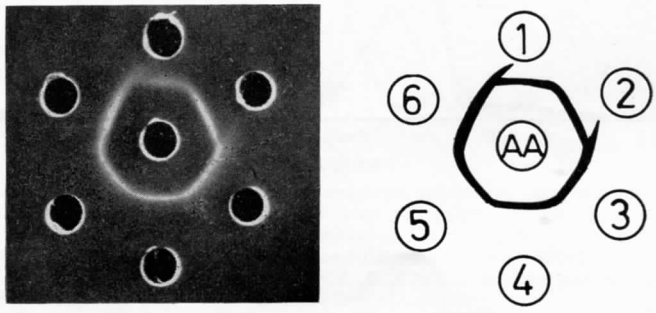

FIG. 9. Gel diffusion analysis of myeloma protein ND, light chain ND, Bence Jones protein ND and three Bence Jones proteins of Type L, using a specific antiserum to a Bence Jones protein of Type L from a patient A.A. (centre well). *Outer wells:* Bence Jones protein of Type L from three patients other than patient A.A., 0·25 mg/ml (1, 5 and 6); myeloma protein ND–Preparation B, 0·5 mg/ml (2); Bence Jones protein ND, 0·1 mg/ml (3); light chain ND, 0·25 mg/ml (4).

serum. Absorption of the antisera with 0·05–10 mg of normal IgG (Cohn Fraction II), 0·05–4 mg of light chains from myeloma protein ND (Preparation B), 0·1–5 mg of Bence Jones protein from patient ND or 0·05–0·3 mg of autologous Fab-fragment did not inhibit the reaction (Fig. 3c). Preparation B did not react with two different rabbit anti-NHS and one horse anti-NHS on immunoelectrophoresis. Similarly, no reaction occurred on gel diffusion with four specific anti-IgA, five specific anti-IgD, four specific anti-IgG and two specific anti-IgM sera.

A comparison of the antigenic determinants of protein ND and immunoglobulins A, D, G and M by Ouchterlony gel diffusion analyses revealed no antigenic determinants common to protein ND-heavy chains and α-, δ-, γ- or μ-chains. Identical results were obtained when antisera to protein ND were absorbed with autologous Fab-fragments (Fig. 10).

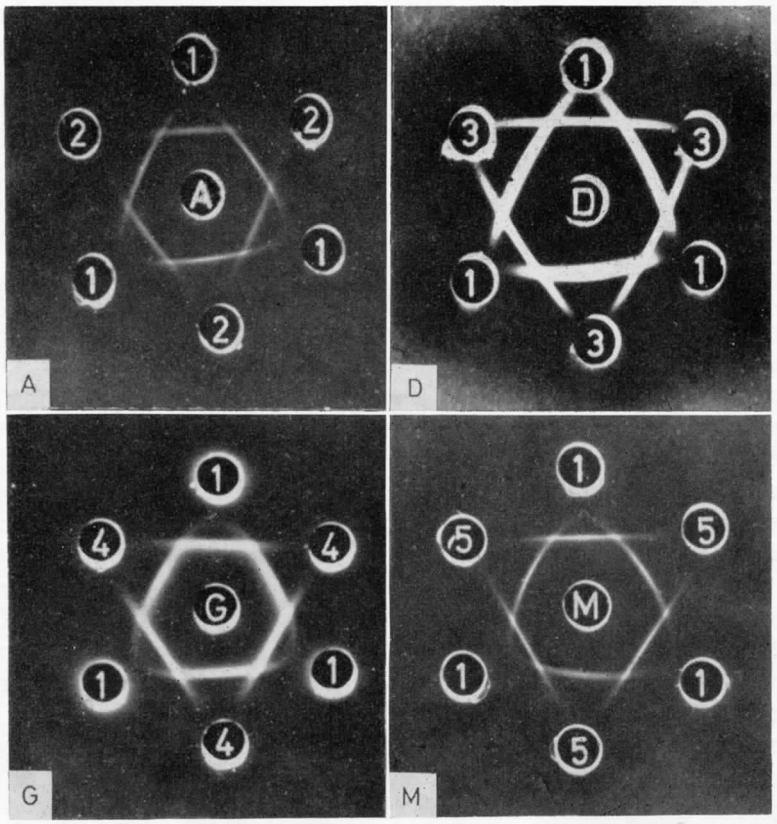

FIG. 10. Gel diffusion analysis of myeloma protein ND and immunoglobulins A, D, G and M. *Outer wells:* Anti-myeloma protein ND, absorbed with 0·2 mg/ml of autologous Fab-fragment (1); anti-IgA, absorbed with 10 mg/ml of IgG, Cohn Fraction II and 0·1 mg/ml of serum from an apparently healthy individual lacking IgA (2); anti-IgD, preparation X8A-SJ, kindly provided by Dr D. S. Rowe (3); anti-IgG, absorbed with 1 mg/ml of light chains from normal IgG, Cohn Fraction II (4); anti-IgM, absorbed with 0·8 mg/ml of normal IgG, Cohn Fraction II (5). *Centre well:* To samples of myeloma protein ND–Preparation B containing 0·2 mg protein/ml was added: IgA isolated from an A myeloma serum, 0·5 mg/ml (A); serum 'Stokes' containing 0·3 mg of IgD per ml (D); IgG, Cohn Fraction II, 0·3 mg/ml (G); IgM isolated from a macroglobulinaemia serum, 0·5 mg/ml (M).

Gel diffusion analysis of G1, G2, G3, G4* myeloma sera, and several A myeloma sera and M macroglobulin sera, using specific antisera to protein ND absorbed with normal IgG failed to reveal any visible reaction.

DISCUSSION

Myeloma proteins usually belong to one or other of the immunoglobulin classes and the occasional finding of a component, which appears atypical in this respect is particularly interesting. Three kinds of atypical myeloma or myeloma-like proteins have been observed (see review by Franklin *et al.*, 1966). One is characterized by the absence of light polypeptide chains and was accordingly assumed to represent a 'heavy chain' disease protein (Franklin, Lowenstein, Meltzer and Bigelow, 1964). The second group consists of myeloma components, which lack class specific antigenic determinants. Usually these proteins have been found to lack heavy polypeptide chains and accordingly are referred to as serum Bence Jones protein (Adner and Laurell, 1966). The presence of light polypeptide chains in the isolated myeloma protein ND and its reaction with an autologous antiserum absorbed with light chains and autologous Fab-fragments, shows that the myeloma component in serum ND belongs to the third group of atypical myeloma proteins. These are characterized by the presence of both heavy and light chains, *and* the absence of recognizable class specific antigenic determinants. Such proteins are of particular importance as they might represent a new subclass or a previously unrecognized class of immunoglobulins; the finding of immunoglobulin D described by Rowe and Fahey (1965b) is a recent example of the latter case.

The present report has described the finding of an atypical myeloma component present in serum from a patient with myelomatosis and Bence Jones proteinuria, which does not react with several specific antisera to immunoglobulins A, D, G and M, but can be detected using antisera to Bence Jones protein of Type L. Comparative studies of immunoglobulins A, D, G and M and the purified protein ND failed to reveal common antigenic determinants. Apparently all previously described class specific antigenic determinants are either absent or not accessible for reaction in immunoglobulin ND. Although the possibility of hidden determinants cannot be excluded as an explanation of our results, there is at present no experimental evidence which favours this hypothesis.

Chemical and physical studies reveal that immunoglobulin ND apparently differs in several respects from what is known at present of immunoglobulin A, D, G and M. Its high molecular weight, taken together with its failure to react with class specific antisera, suggest that the myeloma protein might represent a complex between an immunoglobulin and an unknown compound which shields the antigenic determinants of the immunoglobulin. However, if this were true, the components forming such a complex must be covalently bound and fail to dissociate after reduction or urea treatment at either acid or alkaline pH; isolated ND heavy chains do not react with antisera to α-, δ-, γ- or μ-chains. Physical examination of protein ND by electron-microscopy (Höglund, Bennich, Johansson, to be published) shows that it represents a very compact structure, presumably cylindrical in shape, about 120 Å in diameter and about 40 Å high; such a compact structure might explain why the protein behaves similarly to 6·5S IgA on Sephadex G-200. Further chemical and physical studies including enzymic cleavage of protein ND show

*According to the nomenclature recently (March 1967) recommended by WHO Reference Laboratory for Immunoglobulins.

that it has several unique properties as well as many general features in common with the previously recognized classes of immunoglobulins.

The possibility that the unique antigenic determinants of immunoglobulin ND represent individually specific determinants appears less likely since antisera to the protein retain their activity after absorption with autologous Fab-fragments. If protein ND represents an example of a rare subclass of any of the known immunoglobulins, one would have expected to be able to detect the presence of class specific antigenic determinants unless they are hidden. It seems, therefore, that the myeloma protein ND described in the present report is structurally related to normal immunoglobulins and carries unique antigenic determinants on its heavy polypeptide chains. Analysis of sera from patients and healthy individuals by Ouchterlony gel diffusion and the SRD method using specific antisera to protein ND revealed no visible reactions. We, therefore, conclude that if a normal counterpart to protein ND exists, it must be present in a concentration lower than 1–10 μg/ml serum.

ACKNOWLEDGMENTS

The authors are indebted to Dr C. Högman and Dr J. Porath for encouragement and laboratory facilities; to Dr I. Berggård and Dr M. W. Turner for helpful discussions; and to Mrs Birgitta Andersson, Miss Stina Karlsson and Miss Lena Waxell for skilful assistance with the experiments.

The work was supported in part by grants from the Swedish Medical Research Council, The Swedish Natural Science Research Council, the Medical Faculty at the University of Uppsala and the Magnus Bergwall Foundation, which are gratefully acknowledged.

REFERENCES

ADNER, P. L. and LAURELL, C.-B. (1965). 'A case of myeloma with a pathological protein in the α_1-globulin fraction.' *Scand. J. Haemat.*, **2**, 253.

BENNICH, H. and JOHANSSON, S. G. O. (1967). 'Studies on a new class of human immunoglobulins. II. Chemical and physical properties.' *Nobel Symposium III* (Stockholm, 12–17 June 1967), 'Structure and control of biosynthesis of gamma globulins.' Almqvist and Wiksell (In press).

CESSI, C. and PILIEGO, F. (1960). 'The determination of amino sugars in the presence of amino acids and glucose.' *Biochem. J.*, **77**, 508.

COHEN, S. and PORTER, R. R. (1964a). 'Structure and biological activity of immunoglobulins.' *Advanc. Immunol.*, **4**, 287.

COHEN, S. and PORTER, R. R. (1964b). 'Heterogeneity of the peptide chains of γ-globulin.' *Biochem. J.*, **90**, 278.

DEUTSCH, H. F. and MACKENZIE, M. R. (1964). 'Serological demonstration of antigenic heterogeneity of individual human γ_1-macroglobulins.' *Nature (Lond.)*, **201**, 87.

DISCHE, Z. and SHETTLES, L. B. (1948). 'A specific color reaction of methylpentoses and a spectrophotometric micro method for their determination.' *J. biol. Chem.*, **175**, 595.

EDELMAN, G. M. and POULIK, M. D. (1961). 'Studies on structural units of the γ-globulins.' *J. exp. Med.*, **113**, 861.

EHRENBERG, A. (1957). 'Determination of molecular weights and diffusion coefficients in the ultracentrifuge.' *Acta chem. scand.*, **11**, 1257.

FLEISCHMAN, J. B., PAIN, R. H. and PORTER, R. R. (1962). 'Reduction of γ-globulins.' *Arch. Biochem. Biophys.* Suppl. **1**, 174.

FRANKLIN, E. C., LOWENSTEIN, J., MELTZER, M. and BIGELOW, B. (1964) 'Heavy chain disease. A new disorder of serum γ-globulins.' *Amer. J. Med.*, **37**, 332.

FRANKLIN, E. C., FEINSTEIN, D. and FUDENBERG, H. H. (1966). 'The problem of the untypable M protein.' *Acta med. scand.*, **179**, suppl. 445, 80.

GREY, H. M. and KUNKEL, H. G. (1964). 'H chain subgroups of myeloma proteins and normal 7Sγ-globulin.' *J. exp. Med.*, **120**, 253.

HARBOE, M., DEVERILL, J. and GODAL, H. C. (1965). 'Antigenic heterogeneity of Waldenström type γM-globulins.' *Scand. J. Haemat.*, **2**, 137.

JOHANSEN, P. G., MARSHALL, R. D. and NEUBERGER, A. (1960). 'Carbohydrates in protein. II. The hexose, hexosamine, acetyl and amide-nitrogen content of hen's-egg albumin.' *Biochem. J.*, **77**, 239.

KEKWICK, R. A. (1940). 'The serum proteins in multiple myelomatosis.' *Biochem. J.*, **34**, 1248..

KILLANDER, J. (1965). 'Separation of human immunoglobulins by gel filtration and zone electrophoresis.' *Acta Soc. Med. upsalien.*, **68**, 230.

KUNKEL, H. G. and PRENDERGAST, R. A. (1966). 'Subgroups of γA immune globulins.' *Proc. Soc. exp. Biol. (N.Y.)*, **122**, 910.

MANCINI, G., CARBONARA, A. O. and HEREMANS, J. F. (1965). 'Immunochemical quantitation of antigens by single radial immunodiffusion.' *Immunochemistry*, **2**, 235.

PORATH, J. (1956). 'Methodological studies of zone electrophoresis in vertical column. I. Fractionation in cellulose powder columns of substances of low molecular weight exemplified by amino acids and related compounds.' *Biochim. biophys. Acta (Amst.)*, **22**, 151.

PORATH, J. and BENNICH, H. (1962). 'Recycling chromatography.' *Arch. Biochem. Biophys.*, Suppl. **1**, 152.

PORTER, R. R. (1959). 'The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain.' *Biochem. J.*, **73**, 119.

PUTNAM, F. W., EASLEY, C. W., LYNN, L. T., RITCHIE, A. E. and PHELPS, R. A. (1959). 'The heat precipitation of Bence-Jones proteins. I. Optimum conditions.' *Arch. Biochem. Biophys.*, **83**, 115.

ROWE, D. S. and FAHEY, J. L. (1965a). 'A new class of human immunoglobulins. I. A unique myeloma protein.' *J. exp. Med.*, **121**, 171.

ROWE, D. S. and FAHEY, J. L. (1965b). 'A new class of human immunoglobulins. II. Normal serum IgD.' *J. exp. Med.*, **121**, 185.

SCHEIDEGGER, J. J. (1955). 'Une micro-méthode d'immuno-électrophorèse.' *Int. Arch. Allergy*, **7**, 103.

SVEDBERG, T. and PEDERSEN, K. O. (1940). *The Ultracentrifuge*. Clarendon Press, Oxford.

SVENNERHOLM, L. (1956). 'The quantitative estimation of cerebrosides in nervous tissue.' *J. Neurochem.*, **1**, 42.

TERRY, W. D. and FAHEY, J. L. (1964). 'Subclasses of human γ2-globulin based on differences in the heavy polypeptide chains.' *Science*, **146**, 400.

TERRY, W. D. and ROBERTS, M. S. (1966). 'Antigenic heterogeneity of human immunoglobulin A proteins'. *Science*, **153**, 1008.

VAERMAN, J.-P. and HEREMANS, J. F. (1966). 'Subclasses of human immunoglobulin A based on differences in the alpha polypeptide chains.' *Science*, **153**, 647.

WAKE, R. G. and BALDWIN, R. L. (1961). 'Analysis of casein fractions by zone electrophoresis in concentrated urea.' *Biochim. biophys. Acta (Amst.)*, **47**, 225.

WARREN, L. (1959). 'The thiobarbituric acid assay of sialic acids.' *J. biol. Chem.*, **234**, 1971.