Gamma Heavy Chain Disease Studied by Two-Dimensional Electrophoresis and Immuno-Blotting Techniques

Pascale Blangarin,1 P. Deviller,2 K. Kindbeiter,1 and J-J. Madjar1,3

We used two-dimensional polyacrylamide gel electrophoresis and immunoblotting techniques to study serum proteins from a patient with a monoclonal gammapathy. Two-dimensional electrophoresis was optimized for serum proteins with two main goals: (a) to allow the resolution of many serum proteins in both directions, with penetration of the maximum number of proteins in the first dimension; and (b) to obtain the best reproducibility from one experiment to another, within the limits of the current technique. These analyses, combined with immunoblotting, permitted us to characterize a gamma heavy chain disease protein of 34 000-Da molecular mass. Moreover, two-dimensional mapping of the patient's serum proteins allowed demonstration of the microheterogeneity of this monoclonal component.

Gamma heavy chain disease was first described by Franklin et al. (1) 20 years ago. Ascribable to lymphoproliferative disorders, it is characterized by the production of gamma immunoglobulin molecules consisting of incomplete heavy chains and devoid of light chains. Currently its diagnosis depends entirely upon laboratory studies, demonstration of incomplete gamma globulin without light chains in the serum being essential to the diagnosis. Such demonstration of heavy chain disease is generally made by immunoelectrophoresis of the patient's serum. This procedure must demonstrate the presence of an abnormal component reacting with antisera to heavy chain and to the corresponding Fc fragment, but not with antisera to kappa and lambda light chains and antisera to the Fab region (2). The abnormal component must be purified for direct immunochemical demonstration of the lack of light chains and to distinguish the pathologocal protein from complete gamma heavy chain.

Here we report our biochemical study of a gamma heavy chain disease by current methods combining immuno-blotting (3) and two-dimensional electrophoresis on polyacrylamide gel (4). These methods allowed the rapid characterization of the abnormal component, an estimation of its molecular mass that distinguished it from complete gamma heavy chains, and demonstration of heterogeneity of the abnormal gamma heavy chain.

Materials and Methods

Products

Tris, dithioerythritol, and diaminobenzidine tetrahydrochloride were from Sigma Chemical Co., St. Louis, MO 63178. Other chemicals, unless otherwise indicated in the text, were from Merck, D-6100 Darmstadt, F.R.G.

Electrophoresis and Immuno-electrophoresis of Serum Proteins

For electrophoresis of serum proteins we used cellulose acetate membrane (Cellogel®; Sebia, 92130 Issy-les-Moulineaux, France) in a Tris–barbital buffer at pH 9.2 (60 mmol of Tris, 1 mmol of barbital, and 50 mmol of sodium barbital per liter). For immuno-electrophoresis (5) we used agarose plates (Immuno France, 94577 Rungis Cedex, France). Electrophoresis was at pH 8.6 for 70 min at 140 V in a 10 mmol/L barbital, 50 mmol/L sodium barbital buffer. Immunodiffusion was done with the antisera of interest for a minimum of 18 h. Plates were washed with distilled water for another 24 h, dried, and stained with Coomassie Brilliant Blue R 250 (5 g/L) in ethanol/acidic acid/water (45/10/45 by vol). The same solvent mixture was used for destaining.

Two-Dimensional Electrophoresis on Polyacrylamide Gel

Sample preparation. Serum specimens were prepared for this procedure by the sodium dodecyl sulfate method of Anderson and Anderson (6), with the following modifications. We added 5 μL of serum to 10 μL of dissociation solution (100 g of sodium dodecyl sulfate and 150 mmol of diethioerythritol per liter) and heated at 95 °C for 5 min. When the mixture had cooled we added 185 μL of urea solution (per liter, 9.2 mol of urea, 0.1 mol of dithioerythritol, and 20 g/L of Triton X-100) and applied 10 μL of the sample to the isoelectric focusing gel on the cathodic side as described below.

First dimension. Glass tubes, 140 mm long and 1.5 mm i.d., were used for the first dimension. Acrylamide gels 120 mm long were cast automatically, in groups of 11, with a specially designed apparatus (to be described elsewhere) with 4 mL of the acrylamide solution (per liter, acrylamide: methylenebisacrylamide 40 and 2.4 g, 9.2 mol of urea, 20 g of Triton X-100, and 20 g of 'Pharmalytes 2D™-3-10 ampholytes from Pharmacia Fine Chemicals AB, S-751 04 Uppsala 1, Sweden). After polymerization, eight gel tubes were placed in a home-made cylindrical electrophoresis apparatus made of two 500-mL tanks (7).

Any unpolymerized acrylamide at the top of each gel was aspirated with a syringe before samples were applied. The electrophoresis tanks were filled completely, 0.1 mol/L NaOH on the upper and 25 mmol/L H3PO4 in the lower. No overlay solution was used on top of the samples. For electrofocusing we used a microprocessor-controlled power supply (Sebia, 92130 Issy-les-Moulineaux, France) with the following variable limits: 1.0 W per eight gels, 750 V, and 10 000 V/h. After electrofocusing was completed, the gel rods were gently extruded into a buffer containing Tris HCl (62.5 mmol/L, pH 6.8) plus 20 g of sodium dodecyl sulfate and 20 μg of bromphenol blue per liter. They were immediately layered on top of the slab gel without equilibration as described below. The pH gradients were systematically measured in each experiment in the following way: two gel tubes were frozen at ~30 °C; immediately after the first dimension step, one of them having been focused with the...
serum protein sample, the other with the sample solution without serum proteins. After thawing, the two gels were extruded onto a dry glass plate and cut into 1-cm pieces. The pH gradient was determined by measuring the pH of each piece of gel after a 1-h incubation in 1 mL of a 10 mmol/L KCl solution.

Second dimension. Slabs for the second dimension were 130 × 130 mm squares, 1 mm thick. They were polymerized in between glass plates, with the temperature controlled by use of circulating water at 20 °C. Both this system and the electrophoresis apparatus for holding the slabs have been previously described (8). It is now commercially available (Sebia SM2). Gels were of 115 g/L homogeneous acrylamide (from a stock solution of 300 g/L acrylamide:methylenebisacrylamide) in the Laemmli buffer system (9). No stacking gel was used for the second dimension. Gel rods were layered on top of the slabs and maintained without sealing between the upper edges of the glass plates (8). Before we added the upper buffer, we covered the gel rods with 100 μL of a pH 6.8 buffer containing, per liter, 62.5 mmol of Tris HCl, 25 g of sodium dodecyl sulfate, 200 g of glycerol, and 0.5 μL of bromphenol blue. For migration, in the second dimension we used the same constant temperature as for polymerization of acrylamide. A two-step automatic program was used with the microprocessor-controlled power supply: first step, 0.5 W per slab, 10 min; second step, 7.5 W per slab, 600 V/h. This second-dimension run was complete in 2.5 h.

Silver staining of proteins. This was done essentially as described by Oakley et al. (10), with some modifications. We used distilled, de-ionized water and vinyl gloves throughout. After the second-dimension run, gels were placed for 10 min in individual glass trays, each with 200 mL of a fixative solution (10 mol/L formaldehyde/methanol/water, 7:30:63 by vol). This solution was discarded and gels were extensively washed for three hours in water, then incubated for 15 min in a 5 mg/L dithioerythritol solution (11). Gels were rinsed with water and incubated for 15 min in the ammonical silver solution of Oakley et al. (10). After being rinsed three times (5 min each) in water, the gels were developed individually in the citric acid/formaldehyde solution (10) for 4 to 6 min. After a quick rinse in water, they were fixed for 10 min in dilute (10) acetic acid and stored in a mixture containing, per liter, 0.5 g of citric acid and 0.5 mL of a methylene solution (12.9 mol/L).

Results

Serum Protein Electrophoresis on Cellulose Acetate Membrane

Figure 1 shows the electrophoretogram obtained under non-denaturing conditions for serum proteins for the patient (P) and a control (T). An additional component appears in the P serum between the beta-2 band and the gamma area, representing 5.6% of the total serum proteins (66 g/L). In parallel, there is a significant decrease in the gamma proteins, to 9.6%. This additional band found in the patient's serum, together with the decrease in total immunoglobulins, suggests a gammapathy, whose nature we further investigated.

Immunodetection of Immunoglobulins

Immunodetection was done by two different methods. First of all, we used conventional immuno-electrophoresis on agarose plates (5). Analysis with a polyvalent antiserum to normal human serum shows an additional precipitation line closer to the trough than the polyclonal IgG precipitation line (Figure 2a). Moreover, the same specific precipitation line was observed for the patient's proteins with the anti-gamma and anti-Fc antibodies, but not with the anti-Fab, anti-alpha, anti-mu, anti-lambda, anti-kappa, or anti-delta (Figure 2, a and b).

The positive reaction given by this monoclonal protein with anti-gamma and anti-Fc indicates that there are antigenic determinants in common with IgG immunoglobulins. On the other hand, because there was no immunoreaction involving either kappa and lambda light chains or Fab fragments, this monoclonal protein could evidence a gamma heavy chain disease (1, 2). If this was the case, the gamma heavy chain should show a significant reduction in size. To clarify this point, we did one-dimensional sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions and transferred the proteins to a nitrocellulose sheet for immunodetection (3, 12). The results are shown in Figure 3. For both control and patient, anti-gamma, anti-Fab, and anti-Fc (all three directed against the

Electrotransfer and Immunological Detection of Proteins on the Nitrocellulose Sheet

Here we used the general method of Towbin et al. (3) as modified by Burnette (12). Transfer was carried out overnight in a "Trans blot Electrophoretic Transfer Cell" (Bio-Rad Laboratories, Richmond, CA 94804) at 60 V and a constant controlled temperature of 20 °C. Nitrocellulose sheets were of the 0.45-μm type (from Schleicher and Schüll, D-3354 Dassel, F.R.G.). After the electrophoretic transfer was complete, the proteins were fixed and stained for 10 min in a Ponceau S/trichloroacetic acid solution (from Sebia). The nitrocellulose sheet was then washed in several changes of distilled water. If the initial gel was monodimensional, protein bands appeared pink and, guided by their visibility, we could cut the blot into individual tracks. First antibodies were rabbit immunoglobulins directed against different classes or fragments of human immunoglobulins (Dako, DK-2000 Copenhagen F, Denmark). Second antibody was a peroxidase-linked anti-rabbit IgG (H+L) Fab obtained from Biosys, 60200 Compiegne, France. The substrate was made by mixing, immediately before use, 50 mg of diaminobenzidine tetrahydrochloride in 100 mL of 0.1 mol/L Tris HCl, pH 7.6, with 1 mL of a 10 mL/L dilution of concentrated H2O2 (33.2 mol/L).

Fig. 1. Electrophoretogram of serum proteins electrophoresed on a conventional cellulose acetate membrane under non-denaturing conditions.

Migration was from left to right, for 32 min at 200 V at room temperature. T and P are control and patient's proteins, respectively. The arrow indicates the position of the additional band.
corresponding fraction of human IgG) react with a 52 000-Da component, corresponding to the normal gamma heavy chains. Another component, of 29 000-Da apparent molecular mass, reacts with the anti-Fab for both control and patient. These bands probably correspond to the immunoglobulin light chains. Finally, a third component is found at 34 000-Da for the patient only, which reacts with anti-γ and anti-Fc, but not with anti-Fab. Other controls were done (not shown in Figure 3) showing no immunological reaction of the 34 000-Da component with anti-α, anti-μ, anti-δ, anti-κ, and anti-λ, confirming the immunoelectrophoretic characterization shown in Figure 2.

These results confirm the biological diagnosis of a gamma heavy chain disease expressed as a monoclonal component and identified as a 34 000-Da peptide under reductive denaturing conditions.

Two-Dimensional Analysis and Immunodetection of Serum Protein

Microheterogeneity of deleted heavy chain has already been described before (for a review, see 2). Further to investigate this point and also to locate the position of this gamma monoclonal component, we did two-dimensional electrophoretic analysis of serum proteins for both control and patient. Figure 4 shows, for the P proteins, a row of at least four spots in the 34 000-Da region of the gel and with an apparent pi between 5.5 and 6.5. Moreover, the more acidic the different forms, the higher the molecular masses appear to be. On the control gel these four spots are completely absent (compare Figure 4, A and B). To ascertain if all these four components and no more, represented the abnormal γ heavy chain, we did electrophoresis and immuno-detection of proteins, first separated by two-dimensional electrophoresis, on a nitrocellulose sheet. The results are shown in Figure 5 for the P proteins. With anti-γ first antibody, all of the four suspected spots reacted, as also did the full row of spots corresponding to the normal gamma heavy chains. Moreover, two other groups of proteins were detected with this antibody. The first group was made up of tiny spots, one to the right and two under the major, most basic component (indicated by the lower arrows in Figure 5); these latter two were of lower molecular mass not found with the control proteins (not shown). The second group is visible to the upper left of the gel; it is found also with the control proteins (not shown). The first group probably represents degradation products of the abnormal gamma heavy chain, at least for the two components of lower molecular mass and in very minor amounts; the second group is probably ascribable to nonspecific binding of the first antibody to major components such as albumin or α1-antitrypsin. When we changed the electrophoretic conditions used for the first dimension, the four major spots were resolved into many more components as shown in the insert of Figure 5 (the legend of which gives the details of the electrophoretic conditions used for this particular experiment).

Fig. 2. Results of immunoelectrophoresis of serum proteins on an agarose plate
Patterns obtained with the patient's serum (P) are compared with those given by a normal serum (N). A, the reactions given on using a polyvalent antiserum to normal human serum (HS) and monoclonal antisera to γ, Fab, and Fc fragments directed against the corresponding IgG fraction. B, the reactions given on using monoclonal antisera to α, μ, λ, κ, δ, and γ components. Arrows indicate the precipitating line given by the abnormal component.

Fig. 3. Immunodetection of proteins after electrotransfer on a nitrocellulose sheet
The initial nondimensional sodium dodecyl sulfate gel was of 10% acrylamide (starting from a 30%0.8% acrylamide: methylenebisacrylamide stock solution) in the Laemmli system (9). Serum to be analyzed was diluted 1000-fold in the sample buffer (see text) and heated for 5 min at 95°C. After cooling, 10 μL of samples were layered on the gel. Migration was at 200 V for 2 h in the temperature-controlled apparatus (8). After transfer and staining with Ponceau S, as described in Methods, the different lanes containing T and P serum proteins were cut and incubated with first antibody of interest: lanes 1 and 2, anti-γ for T and P, respectively; lanes 3 and 4, anti-Fab for T and P, respectively; lanes 5 and 6, anti-Fc for T and P, respectively. On each side are the molecular-mass markers after transfer and staining with Ponceau S. Markers are from Pharmacia Fine Chemicals.

Fig. 4. Two-dimensional analysis of serum proteins
The acidic side of the first-dimension gel is to the left. T and P are serum proteins from control and patient, respectively. Arrows indicate the extra spots in the 34 000-Da region of the gel.

The pH gradient obtained in the first dimension is shown under the control gel. A, pH curve obtained with the sample buffer alone; B, pH curve obtained with the sample buffer containing the serum proteins as described in Methods.
Discussion

Since the first description of gamma heavy chain disease by Franklin et al. in 1964 (1), approximately 60 cases have been reported (for reviews see 2, 13). To our knowledge, this is the first report of two-dimensional analysis of serum proteins for such monoclonal gammopathy.

Other monoclonal gammopathies have already been investigated (6, 14-18). Tracy et al. (14, 17, 18) optimized the "Iso-Dalt" system for such a study. They localized on the serum protein map the heavy and light chains with use of both myeloma serum and "electroblotting" techniques (14).

The electrophoretic conditions described in the present report, including sample preparation and silver staining of proteins, were optimized for fast, reproducible analysis of serum proteins in our two-dimensional gel system. Different commercially available ampholytes were used in different combination and with or without different amounts of lysine and arginine, as described previously (14, 16, 18). The influence of anolyte and catholyte concentrations, for a given ampholyte, has also been investigated for the pH gradient shape as well as for the resulting relative position of the different protein spots. Such a systematic study will be published elsewhere. Nevertheless, conditions used here represent only an acceptable compromise for separating serum proteins with a wide range of both pl and molecular mass. For a given protein, better resolution can be obtained by varying the electrophoretic conditions, mainly in the first dimension as shown by the example given in Figure 5. Moreover, if, in our hands, the conditions used here for sample preparation allow a better penetration of high-M, proteins and γ heavy chains in the first-dimension gel, some of them are still streaking and imperfectly focused. In our case we focused our attention mainly on the reproducibility of the method by standardizing as precisely as possible all the different conditions throughout the whole technique. In particular, this was done very precisely for the different electrical variables for both dimensions, as well as for the second dimension where slab gels are cast and run at a constant controlled temperature, whatever their number from one to eight, in the same apparatus (5). In the present state of the technique, two main problems remain unsolved, hindering reproducibility from one experiment to another in different laboratories: the quality and reproducibility of given carrier ampholytes and the passage from the first to the second dimension.

For the gamma heavy chain disease reported here, the diagnosis was suspected from results of electrophoresis on cellulose acetate membranes and immunoelectrophoresis on agarose plates. It was confirmed by one-dimensional sodium dodecyl sulfate/polyacrylamide electrophoresis with electrotransfer and immunodetection on nitrocellulose sheet of the monoclonal component. The latter method allowed us to confirm the immunocharacterization as well as to estimate the molecular mass of the abnormal gamma heavy chain. We did not determine the IgG subclass in the present study. Two-dimensional gel electrophoresis, followed by electrotransfer and immunodetection of proteins, allowed us to localize the abnormal component on the two-dimensional map and to demonstrate the microheterogeneity of the protein.

This microheterogeneity can be ascribed to two main reasons, as already emphasized by Seligman et al. (2).

First, it could result from a "limited proteolytic cleavage following the synthesis of an internally deleted heavy chain" (2). This hypothesis is supported by the results of our two-dimensional gel procedure, which show not only a difference in charge but also small differences in the molecular masses of the different forms. If this is the case, the more acidic component would represent, or be close to, the initial deleted heavy chain and, becoming shorter, the different proteolytic products appear more basic.

The microheterogeneity might also be due to the "high carbohydrate content of most heavy chain disease proteins" (2). To test this hypothesis we unsuccessfully tried to reduce the heavy chain disease protein to a single spot by treatment with neuraminidase (EC 3.2.1.18). Different conditions and concentrations were used. The drastic resulting changes of the two-dimensional map confirmed that neuraminidase was active on many glycosylated proteins (not shown). This failure may be due to steric considerations, as already emphasized (14, 16), but also to the "presence of unusual carbohydrate moieties located at the terminal end" (2) of such deleted heavy chains. The small differences we observed in the molecular masses of the different forms does not exclude the possibility of the heterogeneity being due to glycation, because the carbohydrate moieties might alter the electrophoretic mobility in the presence of sodium dodecyl sulfate, even without any important change in molecular mass.

By allowing the molecular mass determination of the monoclonal component, one-dimensional electrophoresis on sodium dodecyl sulfate/polyacrylamide gel, followed by immunocharacterization of the transferred proteins, confirmed the biological diagnosis of the gamma heavy chain disease. Two-dimensional gel electrophoresis alone indicates, in a very visible way, the presence of serum polypeptides of highly suspicious behavior and allows determination of their molecular mass. Electrotransfer of the proteins separated in this way fully confirmed both the microheterogeneity and the immunocharacterization of this component.
Nevertheless, conventional methods of electrophoresis or immunoelectrophoresis remain of first interest for the daily routine analysis of serum proteins.

We thank R. Grantham for critically reading the manuscript. This work was done in the Laboratoire de Biologie Moléculaire et Cellulaire and supported by Université Claude Bernard-Lyon I, UER de Biologie Humaine, and a grant from Ministère de l'Industrie et de la Recherche (contract GBM no. 50 0 209/07). P. Blangar is the recipient of an A.N.R.T./Sebia fellowship (convention CIFRE no. 34/83). K. Kindbeiter is financially supported by a grant from ANVAR (aide à l'innovation no. A 83 05 057 V 044 0). The microprocessor-controlled power supply was loaned by Sebia, 92190 Issy-les-Moulineaux, France.

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