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Electrophoretic characteristics of monoclonal immunoglobulin G of different subclasses

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Abstract

Monoclonal IgG are commonly observed in various B cell disorders, of which multiple myeloma is the most clinically relevant. In a series of serum samples, we identified by immunofixation 73 monoclonal IgG, including 63 IgG_1, 4 IgG_2, 5 IgG_3, and 1 IgG_4. The light chains were of κ type in 45 cases, and of λ type in 28 cases. These monoclonal IgG were further characterized by high resolution two-dimensional polyacrylamide gel electrophoresis (2-DE) in various isoelectric focusing conditions, as well as by 3-DE (2-DE of the proteins extracted from agarose after serum protein agarose electrophoresis). After 2-DE, 38 out of 73 monoclonal γ chains (52%) were visualized using immobilized pH 3–10 gradients for isoelectric focusing. In 6 cases (8%), γ chains were only detected using alkaline immobilized pH 6–11 gradients. In 3 cases (4%), 3-DE revealed monoclonal γ chains hidden by polyclonal γ chains. Finally, in 26 cases (36%), no monoclonal γ chains were clearly visualized. Sixty-one monoclonal light chains (84%) were detected using immobilized pH 3–10 gradients, whereas 12 (16%) were not. Monoclonal γ chains and light chains were highly heterogeneous in terms of p*I* and M_r. However, a statistically significant correlation ($P<0.05$) was observed between the position of the monoclonal IgG in agarose gel and the p*I* of their heavy and light chains ($R=0.733$, multiple linear regression). Because of the extreme diversity of their heavy and light chains, it appears that a classification of monoclonal IgG based only on their electrophoretic properties is not possible.

Keywords: Monoclonal gammopathy; Immunoglobulin G; Antibodies

1. Introduction

Monoclonal gammopathy (MG) is related to the presence in the blood of monoclonal immunoglobulins (Igs), also referred by the term M proteins, and is produced by a single B cell clone. Each monoclonal Ig is constituted of two identical heavy chains of the same isotype and of two light chains of the same type [1], resulting from unique recombination of numerous V_{heavy} and V_{light} chain genes with diversity and joining gene segments, as well as from somatic hypermutations occurring during the immune response [2,3]. Serum protein analysis should be done to detect MG, when multiple myeloma, Waldenström's macroglobulinemia, primary amyloidosis or a related B cell disorder is suspected [4]. It is also indicated for patients with unexplained anemia, back pain, weakness or fatigue, osteolytic lesions or fractures, osteopenia, hypercalcemia, renal insufficiency, presence of proteinuria (particularly Bence Jones proteinuria), diffuse failure of reabsorption in the proximal renal tubule resulting in glycosuria, generalized aminoaciduria, and hypophos-

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phatemia (acquired Fanconi's syndrome), or history of recurrent bacterial infections [5–7]. In addition, serum protein analysis may be of relevance in patients presenting unexplained peripheral neuropathy, carpal tunnel syndrome, nephrotic syndrome, renal insufficiency, cardiomyopathy, refractory heart failure, orthostatic hypotension, acquired von Willebrand's disease, or malabsoprtion, because of the possible association of all these conditions with MG [8–10]. After serum protein agarose electrophoresis (SPE), a monoclonal Ig appears as a discrete band on the electrophoretic strip, or as a tall spike on the densitometer tracing [11,12]. Its identification is quite easy, using either immunofixation (IF) or immunosubstraction with capillary electrophoresis [12,13]. The strategies to detect as well as to identify MG in the clinical laboratory have been recently reviewed [11,13,14]. Other, but not routinely used electrophoretic techniques, have been employed to further characterize monoclonal Igs, including isoelectric focusing (IEF), or high resolution two-dimensional polyacrylamide gel electrophoresis (2-DE). The interest of 2-DE as well as its limitations in the analysis of MG, either in plasma/serum or urine samples, have been described by different groups of investigators [15–28]. The recent observation of 3 patients presenting a unique syndrome of recurrent panniculitis with an IgG paraprotein and depletion of the early components of the classical pathway of complement in association with unusual features, such as abnormal behavior on gel filtration chromatography as well as a heavy chain of high p*I* [29] prompted us to study a series of monoclonal IgG of different subclasses. For that purpose, we used various electrophoretic techniques, including SPE, IF, 2-DE with various IEF conditions, and a combination of SPE and 2-DE, tentatively named three-dimensional electrophoresis (3-DE).

2. Materials and methods

2.1. Serum samples

Between January 1, 1996 and June 30, 1999, serum samples from 73 patients unambiguously diagnosed with monoclonal IgG using either IF or immunosubstraction were collected and stored at −20°C at the Division d'Immunologie et d'Allergie of the Centre Hospitalier Universitaire Vaudois. Aliquots of these samples were kindly made available for this study.

2.2. SPE, IF, and IgG subclass typing

SPE and IF were performed using commercially available kits and following the manufacturer's instructions (Silenus Laboratories, Hawthorn, Australia). Sheep monospecific antisera anti-human γ, α, μ, κ, or λ chains were from the same manufacturer. Serum samples were diluted with barbital buffer pH 8.6, 0.05 *M* for reference high resolution SPE (1:2), and for IF (1:10). Five μl of diluted samples were applied on the gel using a sample « template ». The migration was performed under a direct current of 100 V in an electrophoresis tank from Helena Laboratories (Beaumont, Texas, USA) containing Tris barbital solution. After 30 min, 80 μl of protein fixative or monospecific antisera were applied on the reference or immunofixation slots, respectively. The gels were then incubated in a humid chamber for 30 min at 45°C, washed with 0.15 *M* NaCl, dried, stained with Amidoblack, and destained using 5% v/v acetic acid solution. Typing of IgG subclasses was performed by IF, using kits from another manufacturer (The Binding Site, Birmingham, UK) and sheep monospecific antisera raised against human γ_1, γ_2, γ_3, and γ_4 chains, respectively.

2.3. 2-DE

Serum samples were prepared and submitted to 2-DE using protocols described in details elsewhere [27]. IEF using immobilized pH gradients (IPG) was performed as described by Görg et al. [30] with modifications. Immobiline DryStrip, 18 cm, pH 3–10 NL, pH 3–10 L, pH 6–11 L were from Amersham-Pharmacia (Uppsala, Sweden). Samples were loaded onto the cathodic end of the rehydrated IPG strips, that were covered with low viscosity paraffin oil. IEF was performed at 11°C. The voltage was progressively increased from 300 V to 3000 V during the first 3 h, followed by 3500 V for 1 h, and finally increased to 5000 V for a total of 100 kVh. Before the second dimension run, IPG strips were equilibrated within the strip tray for 20 min with 100 ml of

Tris–HCl buffer (0.05 *M*, pH 6.8) containing 0.6 *M* urea, 30% v/v glycerol, 2% w/v SDS and 2% w/v dithioerythritol to solubilize the peptides. SH groups were subsequently blocked by the same solution containing iodoacetamide (2.5% w/v) instead of dithioerythritol and traces of Bromophenol Blue [31]. The vertical second dimension run was performed on 180×160×1.5 mm 9–16% polyacrylamide gradient gels, using diacrylylpiperazine as cross-linking agent. IPG strips were cut to fit with the second dimension gels, and sealed onto slab gels with a solution containing 0.5% (w/v) agarose in Tris/glycine/SDS (50 m*M*/383 m*M*/3 m*M* in 1 l of H_2O). The electrophoresis was run at 40 mA/gel constant current at a temperature between 8 and 12°C, and the gels were stained with standard ammoniacal silver staining protocol [27]. After silver staining, the gels were analyzed with the software Melanie 3 (GeneBio, Genève, Switzerland) after high resolution densitometry reading with Personal Laser Densitometer (Amersham Pharmacia, Dübendorf, Switzerland). The silver-stained gels were presented with the acidic side on the left and the higher M_r on the top. The M_r and the p*I* of the heavy and light chains were calculated by Melanie 3.0 using values of reference proteins identified on the gels, such as albumin, C3b, transferrin, α1-antitrypsin, apolipoprotein A–I, α and/or β chain of hemoglobin. These reference values were taken from the SWISS-2DPAGE database (http://www.expasy.ch/ch2d/). The mean p*I* of the light and heavy chains appearing as multiple spots (*N*=number of spots) was calculated taking into account the volume of each spot determined using Melanie 3.0. The following formula was used:

$$pI = \frac{\sum_1^N [(pI \text{ spot}) \times (\text{volume})]}{\sum_1^N (\text{volume})}$$

The mean M_r of the light and heavy chains presenting size microheterogeneity was calculated using a similar formula:

$$M_r = \frac{\sum_1^N [(M_r \text{ spot}) \times (\text{volume})]}{\sum_1^N (\text{volume})}$$

2.4. 3-DE

3-DE was performed as previously described by Harrison et al. [18,20] and Tissot et al. [32], with modifications. Two agarose gels were simultaneously prepared. The first gel was used for immunofixation analysis (serum diluted 1:10). The second gel was prepared for SPE, and was loaded in all 6 lanes with the same serum sample (1:2). After electrophoresis, the second gel was maintained wet, without fixation. The band of interest was identified on the first gel for positioning on the SPE gel. A 4 mm-wide agarose strip containing the desired protein band was scraped from the plastic backing material with a spatula, and placed into a column including a membrane of cellulose acetate 0.45 μm. After centrifugation (2 min at 10 000 g), 10 μl of the solution containing the protein were collected. Twenty μl of a denaturing and reducing solution (2 g of SDS, 0.464 g of dithioerythritol, and 10 ml of H_2O) were added. The mixture was heated at 95°C. After a short cooling time, 200 μl of the ''sample solution'' (0.1 g of DTT, 400 mg CHAPS, 5.4 g urea, 500 μl of pH 3–10 ampholytes, and 6.5 ml of H_2O) were added, and 60 μl of the final homogenized sample were loaded onto the first dimension gel for IEF.

3. Results

3.1. Immunoelectrophoresis

Using IF, the isotype of the monoclonal Ig as well as the type of the light chains were easily identified as bands which were stained with their specific antisera (Fig. 1). The subclasses of the monoclonal IgG were also determined by IF, using antibodies specific for IgG_1, IgG_2, IgG_3 and IgG_4 (Fig. 2). Table 1 summarized the immunoelectrophoretic characteristics of the 73 monoclonal IgG studied. Most were IgG_1 (86.3%), whereas κ light chains were the most frequently observed (61.6%).

The electrophoretic mobility of the monoclonal IgG determined by SPE was variable (Fig. 3). Using the value of the length of migration from the sample application point, we calculated an arbitrary index (*G*/*A*) by dividing the value obtained for the monoclonal component (*G*) by that for albumin (*A*). The

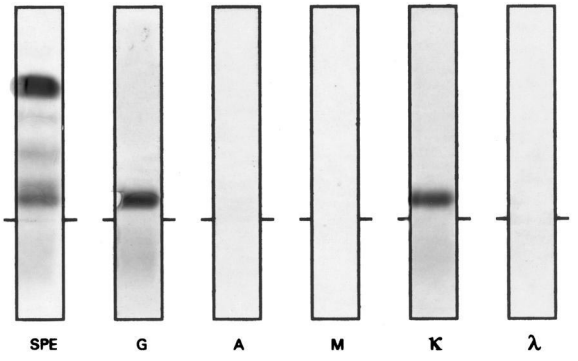

Fig. 1. Serum protein electrophoresis and immunofixation of a sample containing monoclonal IgG κ. SPE, serum protein electrophoresis; G, A, M, κ, λ, immunofixation with specific anti-γ, α, μ, κ, λ antisera.

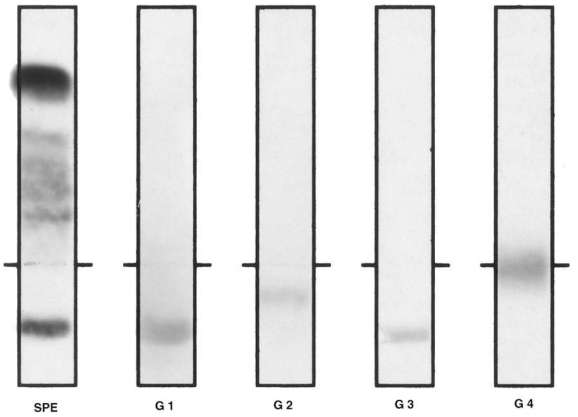

Fig. 2. Immunofixation of samples containing monoclonal IgG of different subclasses. SPE, serum protein electrophoresis; G1, G2, G3, G4, immunofixation with specific anti-γ_1, γ_2, γ_3, and γ_4 antisera.

values of the *G*/*A* index were normally distributed. The mean was 26.61±14.26 (SD). The difference was statistically not significant between the *G*/*A* index of IgG κ (*N*=45; 25.17±15.45) and that of IgG λ (*N*=28; 28.92±12.03) (*P*=0.278, *t*-test).

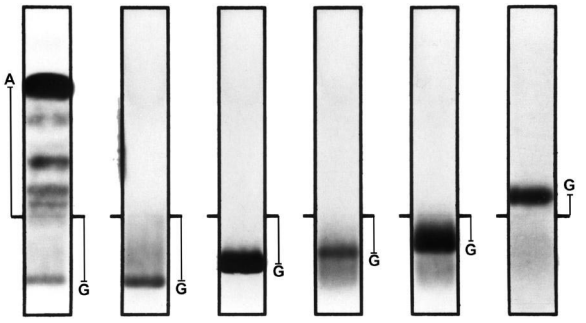

Fig. 3. *G*/*A* Index. Using the value of the length of migration from the sample application point, we calculated an arbitrary index (*G*/*A*) by dividing the value obtained for the monoclonal component (*G*) by that obtained for albumin (*A*).

3.2. γ Chain patterns (2-DE, 3-DE)

After 2-DE, polyclonal γ chains from human sera appeared as a non-spotted band in the 40 000–60 000 Da basic area of the gel (Fig. 4 A). Among the 73 samples studied, we detected monoclonal γ chains in only 38 (52%) using « standard » IEF conditions (nonlinear IPG, pH 3–10) (Table 2). These monoclonal γ chains presented charge microheterogeneity in 33 cases, appearing as four or more distinct spots, separated by 0.1 to 0.3 p*I* unit (Fig. 4 B). In 5 cases (3 γ_3, 1 γ_2, and 1 γ_1 chains), they also presented size microheterogeneity (Fig. 5); the variation of the apparent M_r was 7300±3700 Da. In 35 cases (48%), no individualizable spots were observed in the γ chain area of the gels. However, in 14 out of these 35 cases, the presence of a M component was suspected by the presence of additional sets of spots, located in the 95 000–120 000 Da area (Fig. 4C). These spots corresponded most likely to dimers of the monoclonal γ chains. The dimers were also detected in particular samples, in the presence of γ chain monomers in the 40 000–60 000 Da region of the 2-DE gels (Figs. 4B, 5A, 7B).

In 21 out of 35 cases, no spots suggesting the

Table 1
Immunoelectrophoretic characterization of the monoclonal IgG

Light chain type	IgG_1	IgG_2	IgG_3	IgG_4	Total
κ	39 (53.4%)	2 (2.7%)	3 (4.1%)	1 (1.4%)	45 (61.6%)
λ	24 (32.9%)	2 (2.7%)	2 (2.7%)	0	28 (38.4%)
Total	63 (86.3%)	4 (5.5%)	5 (6.8%)	1 (1.4%)	73 (100%)

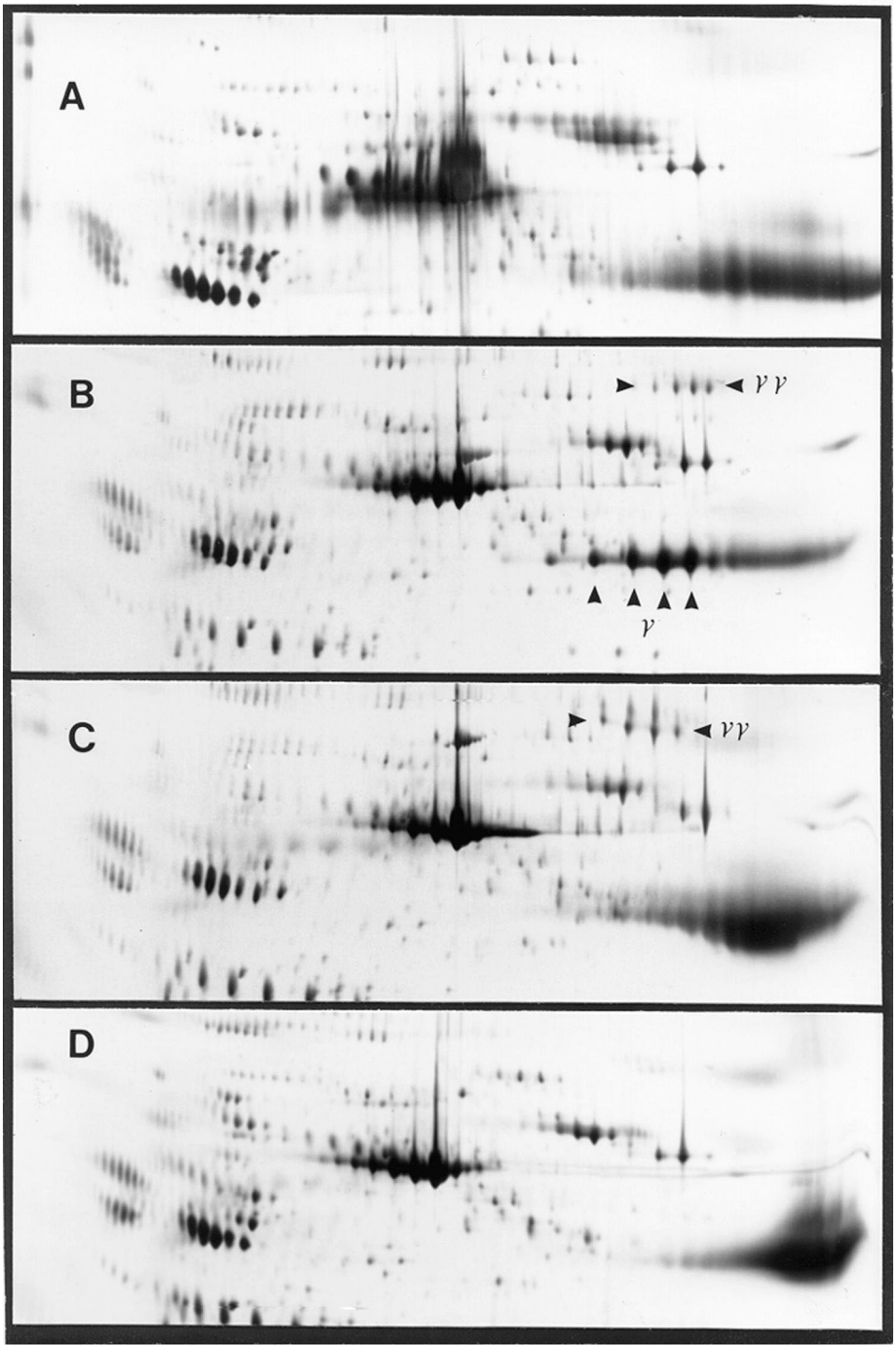

Fig. 4. Details of 2-DE gels corresponding to M_r 40 000–130 000 Da and p*I* 4.4–9.0. (A) Normal serum sample; (B–D) serum samples containing monoclonal IgG. An apparent monoclonal γ_2 chain with charge microheterogeneity is only visible in panel B, whereas γ–γ dimers are observed on panel B and C. Note the absence of significant modification in panel D (monoclonal γ chain not visible). First dimension: immobilized pH 3–10 nonlinear gradient.

Table 2
Characteristics of the monoclonal γ chains after 2-DE using non-linear pH 3–10 immobilized pH gradient for isoelectric focusing

Electrophoretic patterns	IgG_1	IgG_2	IgG_3	IgG_4	Total (%)
Charge microheterogeneity	28	3	2	0	33 (45.2)
Charge and size microheterogeneities	2	1	2	0	5 (6.8)
Not detected	33	0	1	1	35 (48)
Total	63	4	5	1	73 (100)

presence of monoclonal γ chains were observed on the electrophoretograms (Fig. 4D). Due to the absence of spots, we tested different IEF conditions. Fig. 6 A-E shows a representative example of the resolution of a monoclonal γ chain. With pH 3–10 nonlinear IPG strips (Fig. 6A), the spots were observed on the right ''alkaline'' side of the gels. By contrast, when pH 6–11 alkaline gradients were used (with or without adding alkaline ampholytes in the sample buffer), they were located on the left ''acidic'' side of the gels (Fig. 6B, C). When pH 3–10 linear IPG strips were tested, the γ chain spots were again observed on the right ''alkaline'' side of the gels (Fig. 6D). When 3-DE was performed (Fig. 6E), the position of the spots in the 2-D gels was only related to the IEF conditions used for 2-DE. When these different IEF conditions were applied to the 35 cases without detectable monoclonal γ chain

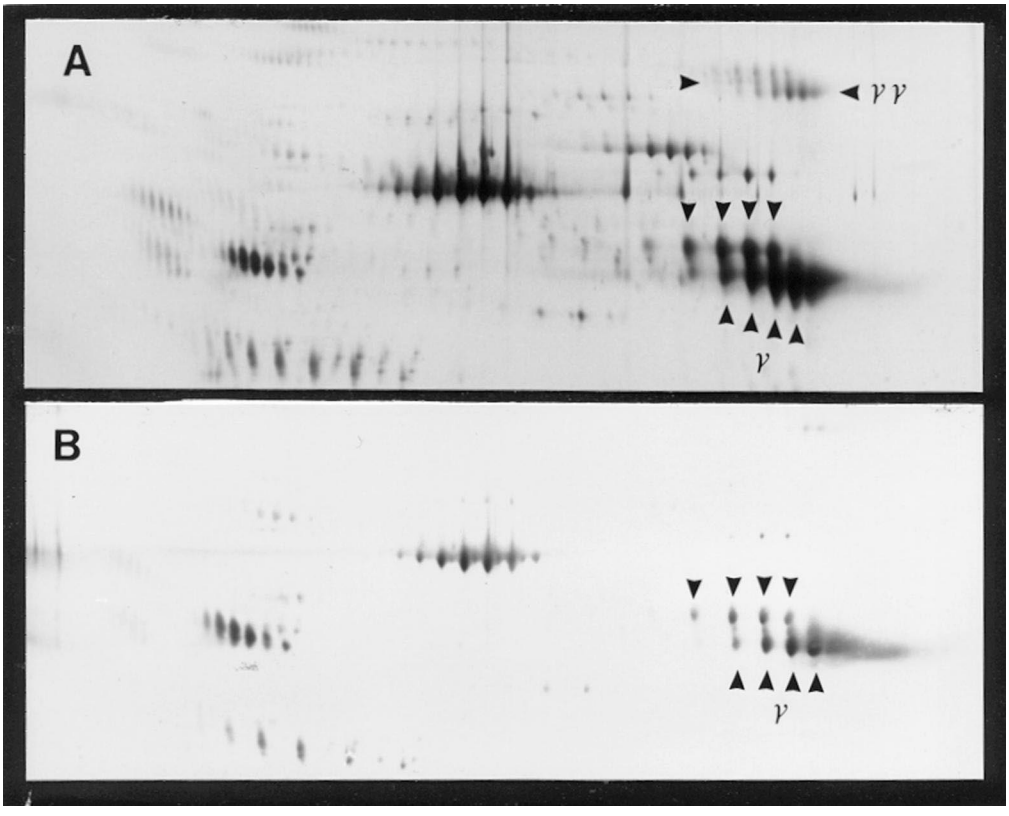

Fig. 5. Details of 2-DE gels corresponding to M_r 40 000–130 000 Da and p*I* 4.4–9.0. (A) sample containing a monoclonal IgG_1; (B) same sample, diluted 1:10. A monoclonal γ chain presenting charge and size microheterogeneities is detected. γ-γ dimers are detected in panel A. First dimension: immobilized pH 3–10 nonlinear gradient.

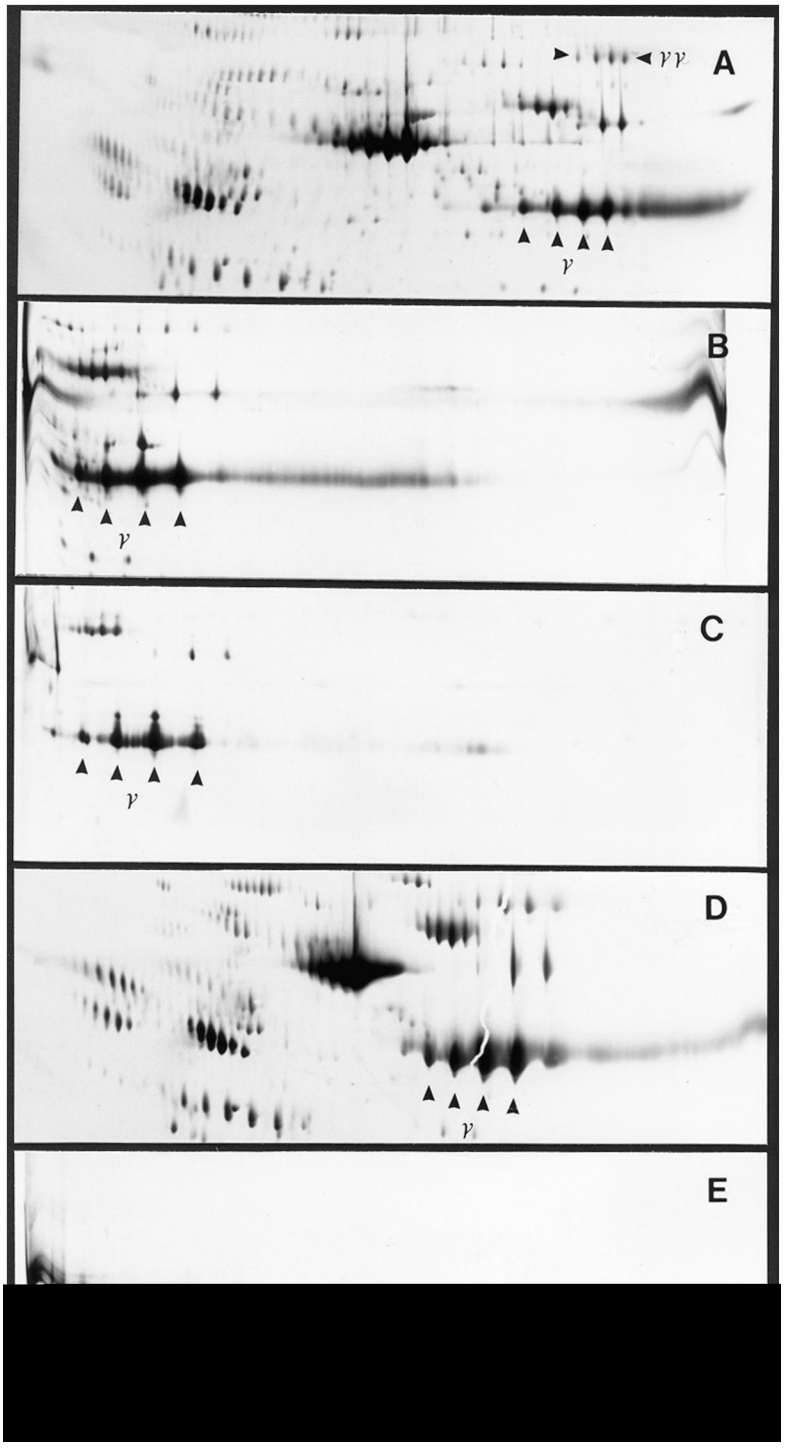

Fig. 6. Details of 2-DE gels corresponding to M_r 40 000–130 000 Da, in different isoelectric focusing conditions. 2-DE of a serum containing monoclonal IgG_2. (A) immobilized pH 3–10 nonlinear gradient; (B) pH 6–11 linear gradient in the presence of pH 9–11 ampholytes; (C) pH 6–11 linear gradient in the absence of pH 9–11 ampholytes; (D) immobilized pH 3–10 linear gradient; (E) immobilized pH 3–10 nonlinear gradient, proteins extracted from the agarose after serum protein electrophoresis (3-DE, see material and methods).

in ‘‘standard’’ IEF conditions, a monoclonal component was detected in 6 cases, and only by using alkaline pH gradients, as examplified in Fig. 7. With the exception of 3 cases, in which the monoclonal γ chains were hidden by polyclonal γ chains (data not shown), 3-DE did not allow a better resolution of ‘‘non-visible’’ monoclonal γ chains, despite that the method was able to separate monoclonal IgG from other plasma proteins, and permitted to identify their different chains (Fig. 6E, 8D).

3.3. Light chain patterns (2-DE, 3-DE)

Polyclonal κ and λ light chains from normal human sera appeared as a wide band in the 25 000–28 000 Da part of the gel map (Fig. 8A). In contrast to polyclonal light chains, monoclonal light chains appeared as prominent spots in 61 out of 73 cases (84%) (Fig. 8B–D). In 12 cases (16%), they were not detected, even when different IEF conditions such as pH 6–11 IPG or 3-DE were used. Table 3 summarized the characteristics of the monoclonal light chains according to their type. Charge microheterogeneity was frequently observed with (8 cases) or without (40 cases) size microheterogeneity. The 2-DE patterns of the monoclonal light chains were not related to the type of the light chains (Chi-square, $P = 0.072$), albeit charge and size microheterogeneities were only observed within κ light chains.

3.4. pI, Mr and G/A index

The p*I* of the monoclonal light chains was determined in 61 samples (39 κ and 22 λ). The range

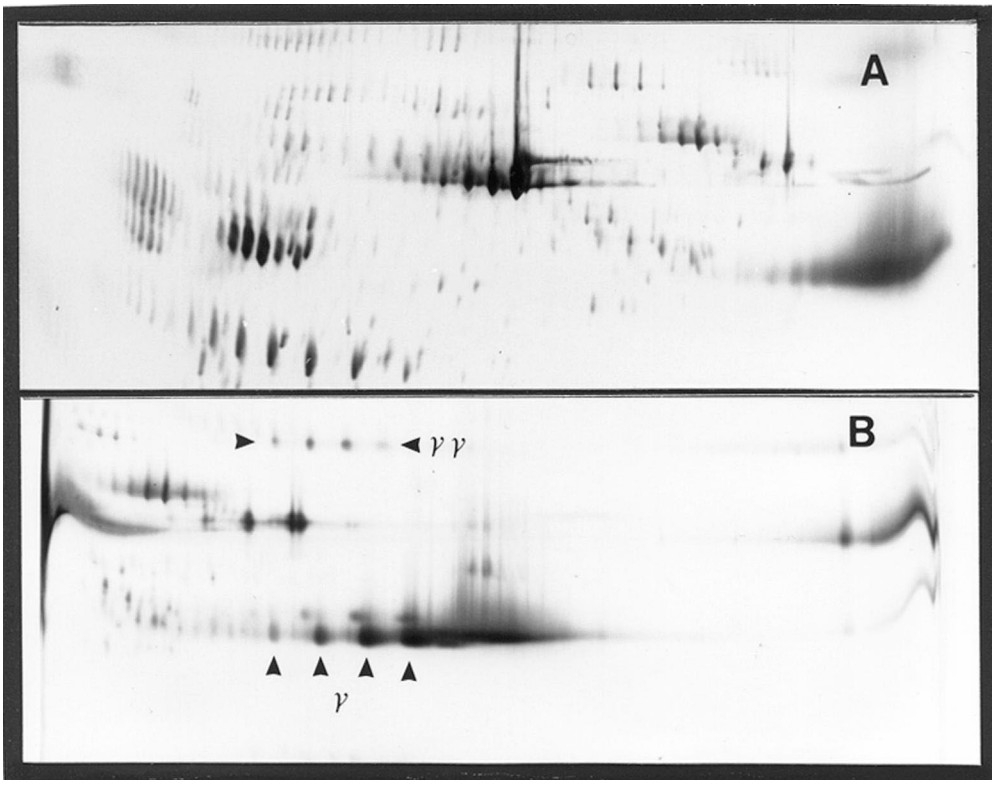

Fig. 7. Details of 2-DE gels corresponding to M_r 40 000–130 000 Da, in two different isoelectric focusing conditions. 2-DE of a serum containing monoclonal IgG_1. (A) immobilized pH 3–10 nonlinear gradient; (B) pH 6–11 linear gradient in the presence of pH 9–11 ampholytes. The monoclonal γ chain was not detected using « standard » conditions (panel A), but was clearly detected using alkaline conditions (panel B).

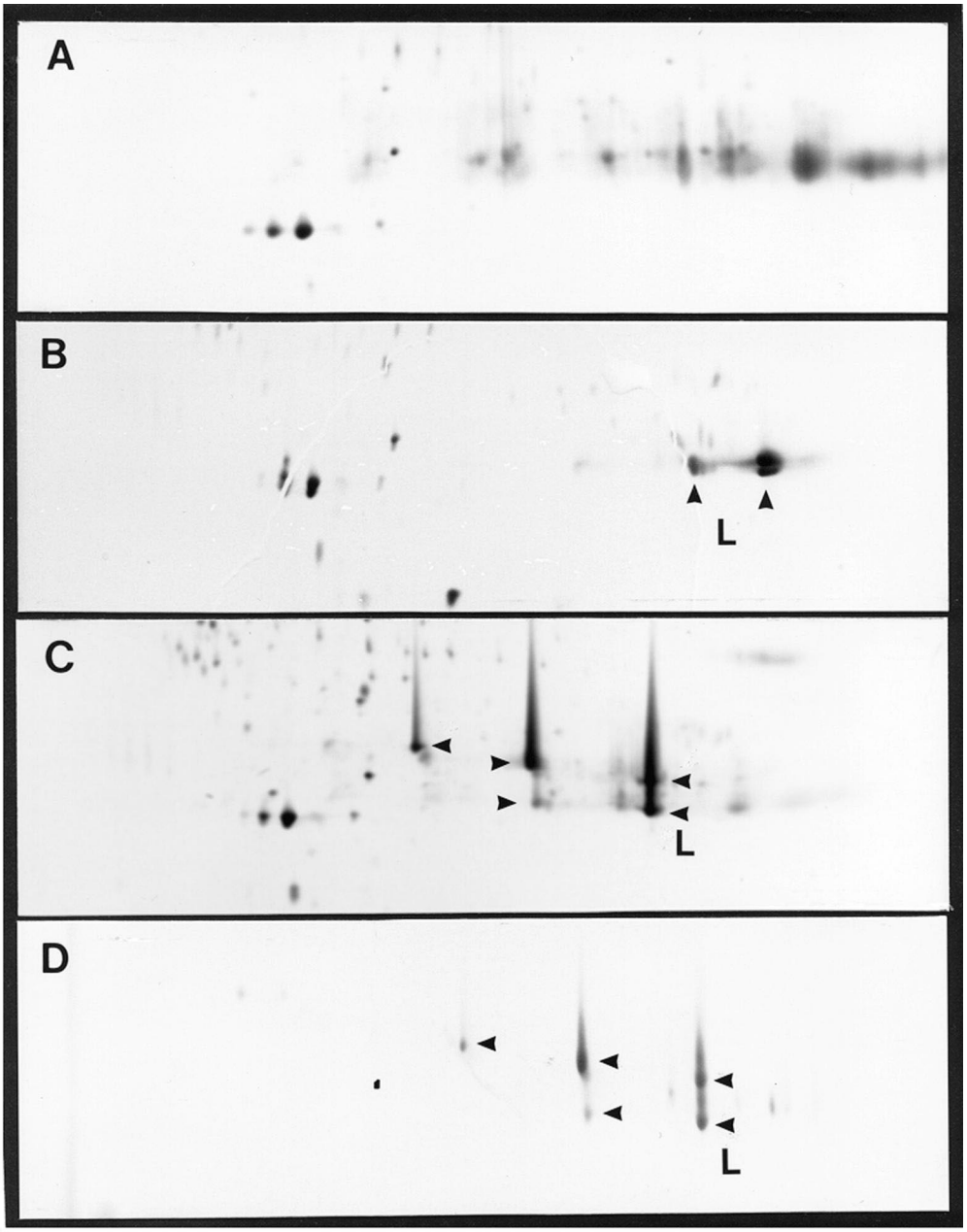

Fig. 8. Details of 2-DE gels corresponding to M_r 20 000–35 000 Da and p*I* 4.4–9.0. (A) normal serum sample; (B,C) serum samples containing monoclonal IgG; (D) proteins extracted from the agarose after serum protein electrophoresis of the serum depicted in panel C (3-DE, see Material and methods). A monoclonal λ chain with charge microheterogeneity is depicted in panel B, whereas a κ chain exhibiting charge and size microheterogeneities is presented in panels C and D. L, spots corresponding to the monoclonal light chains. First dimension: immobilized pH 3–10 nonlinear gradient.

Table 3
Characteristics of the monoclonal light chains after 2-DE, using non-linear pH 3–10 immobilized pH gradient for isoelectric focusing

Electrophoretic patterns	κ	λ	Total (%)
Single spot	6	7	13 (17.8)
Charge microheterogeneity	25	15	40 (54.8)
Charge and size microheterogeneities	8	0	8 (11)
Not detected	6	6	12 (16.4)
Total	45	28	73 (100)

of the p*I* varied from 4.98 to 7.23. The mean p*I* of the κ light chains was 6.39±0.63 whereas that of λ light chains was 6.27±0.65. There was no statistically significant difference between the p*I* of the two types of light chains ($P=0.456$, *t*-test. The median p*I* values of κ or λ light chains were significantly different ($P<0.001$, Kruskal–Wallis one-way analysis of variance on ranks) from the median p*I* of γ chains (Fig. 9).

The p*I* of the monoclonal γ chains was determined in 44 samples (38 with γ chains detected in ''standard'' IEF conditions, and six detected using pH 6–11 IPG). It ranged between 6.03 to 8.97, with a median of 7.01. As expected, the median p*I* (7.75) of the γ chains detected only using pH 6–11 IPG was significantly ($P<0.001$, Mann-Whitney rank sum test) higher than that (6.99) of the γ chains detected using pH 3-10 IPG (Fig. 10).

The apparent M_r was determined in the same series of samples. There was no statistically significant difference between the M_r of the 2 types of light chains ($P=0.115$, *t*-test), despite that the mean M_r of κ chains (25 514±1170) was slightly lower to that of λ chains (26 140±2178). As expected, the apparent M_r of γ chains (54 983±2510) was significantly

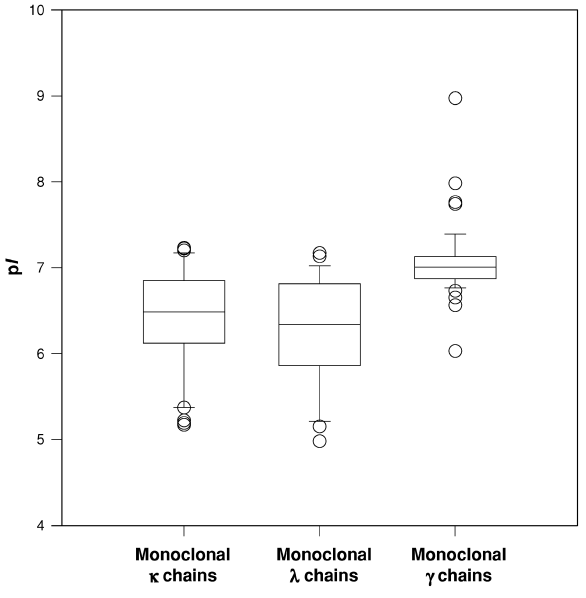

Fig. 9. p*I* of monoclonal κ, λ and γ chains. The box plots display the 25th, 50th, and 75th percentiles. The capped bars indicate the 10th and 90th percentiles. Circles mark all data outside the 10th and 90th percentiles. The median p*I* of γ chains was significantly higher than those of the light chains ($P<0.001$, Kruskal–Wallis one-way analysis of variance on ranks). The difference between the mean p*I* of κ and the p*I* of λ light-chains was statistically not different ($P=0.456$, *t*-test).

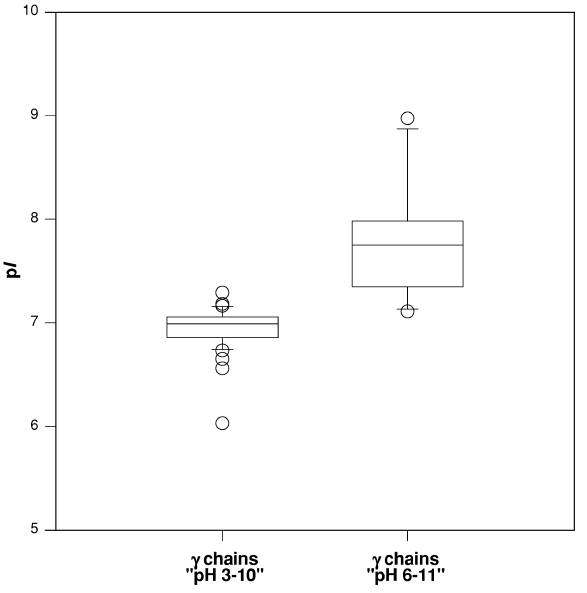

Fig. 10. Measured p*I* of ''visible'' monoclonal γ chains in pH 3–10 immobilized gradients and in pH 6–11 immobilized gradients. The box plots display the 25th, 50th, 75th percentiles. The capped bars indicate the 10th and 90th percentiles. Circles mark all data outside the 10th and 90th percentiles. The difference between the 2 groups was statistically significant ($P<0.001$, Mann–Whitney rank sum test).

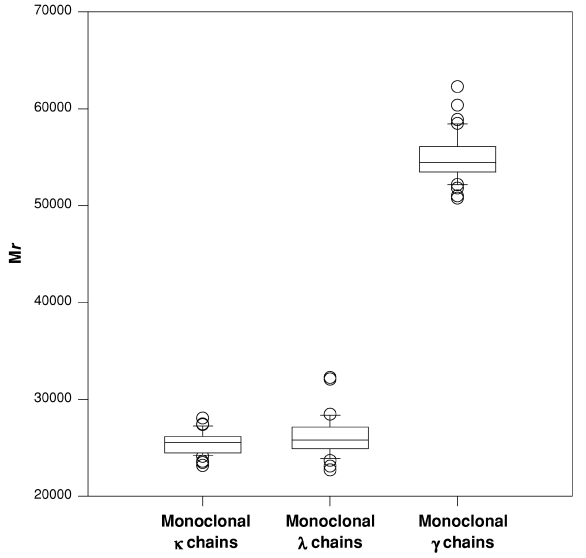

Fig. 11. M_r of monoclonal κ, λ and γ chains. The box plots display the 25th, 50th, 75th percentiles. The capped bars indicate the 10th and 90th percentiles. Circles mark all data outside the 10th and 90th percentiles. M_r of γ chains was statistically significantly higher than those of the light chains ($P<0.001$, Kruskal–Wallis one-way analysis of variance on ranks). The difference between the mean M_r of κ and λ light chains was statistically not different ($P=0.115$, t-test).

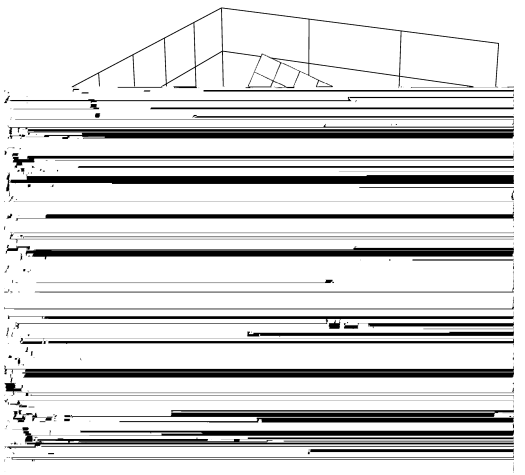

Fig. 12. Three dimensional scatter and mesh showing the relations between the G/A index, and the measured pI of the monoclonal heavy chains and that of the monoclonal light chains. G/A index $= -200.827 + (19.707 \times$ pI heavy chains$) + (13.053 \times$ pI light chains) ($R=0.733$).

higher than those of the light chains ($P<0.001$, Kruskal–Wallis one-way analysis of variance on ranks, Fig. 11).

The G/A index, as well as the pI of the monoclonal light and heavy chains were available together in 38 out of 73 cases. A statistically significant correlation was observed between these 3 parameters (Fig. 12). Multiple linear regression analysis showed that the dependent variable was the G/A index, and that it can be predicted from a linear combination of the independent variables that were the pI of the light and heavy chains ($P<0.05$). Using the equation derived from the multiple linear regression analysis [G/A index $= -200.827 + (19.707 \times$ pI heavy chains$) + (13.053 \times$ pI light chains) ($R=0.733$)], we calculated the pI of the ''non-visible'' γ chains, taking into account the G/A index and the measured pI of their light chains ($N=23$). The median value of the calculated pI (7.66) was statistically different from that of the measured pI (7.01) ($N=44$) (Mann–Whitney rank sum test, $P<0.001$, Fig. 13). The

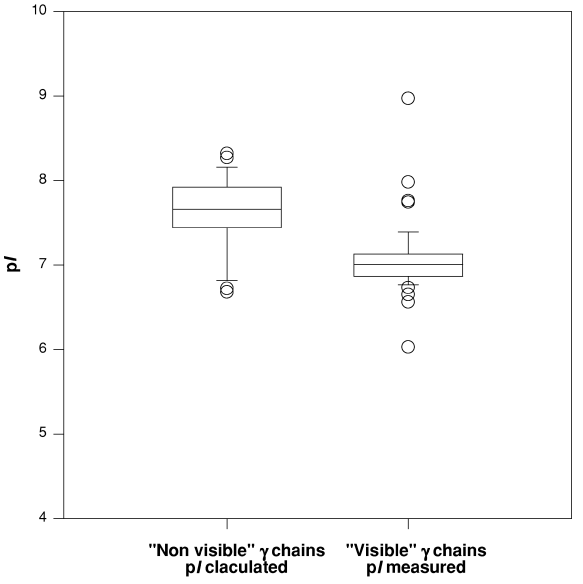

Fig. 13. Calculated pI of ''non-visible'' and measured pI of ''visible'' monoclonal γ chains after 2-DE. The box plots display the 25th, 50th, 75th percentiles. The capped bars indicate the 10th and 90th percentiles. Circles mark all data outside the 10th and 90th percentiles. The difference between the 2 groups was statistically significant ($P<0.001$, Mann–Whitney rank sum test).

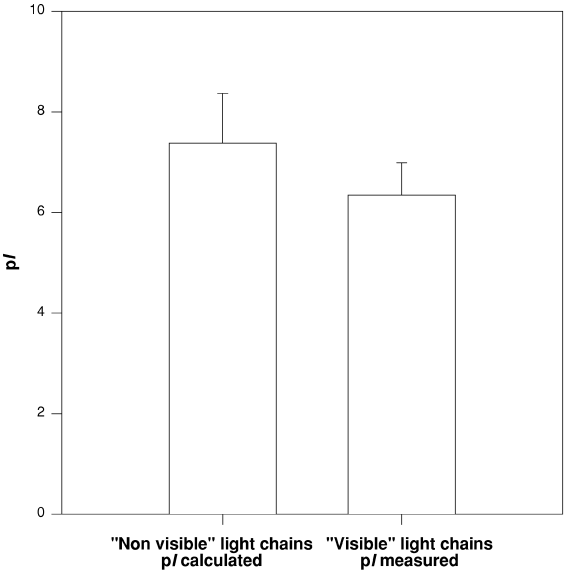

Fig. 14. Calculated p*I* of ''non-visible'' and measured p*I* of ''visible'' monoclonal light chains after 2-DE. The boxes display the means, and the bars indicate the standard deviations. The difference between the 2 groups was statistically significant ($P<0.001$, *t*-test).

same approach was also used to calculate the p*I* of the ''non-visible'' light chains ($N=6$). Their mean value (7.38) was significantly higher ($P<0.001$, *t*-test) than that of the mean value of the ''visible'' light chains (6.35) ($N=61$) (Fig. 14).

4. Discussion

The presence of abnormal substances precipitating in the presence of nitric acid redissolving by heating, and precipitating again by cooling was observed in the urine of patients during the 19th century by H. Bence Jones (reviewed in Ref. [33]). These substances were later identified as Ig light chains and were most frequently associated with multiple myeloma. Using a moving boundary method of electrophoresis, Arne Tiselius demonstrated the homogeneity of serum globulins. In 1937, he also described serum protein fractions corresponding to albumin, α-, β-, and γ-globulins [34]. The first published diagram of a human serum protein electrophoresis was published in 1939 [35,36]. Since that time, various electrophoretic methods have been applied to study the serum of patients suffering from multiple myeloma.

In order to gain insight into the electrophoretic properties of M proteins, we studied a series of 73 sera with monoclonal IgG. In our series, IgG_1 was the most frequent subclass (86.3%), followed by IgG_3 (6.8%), IgG_2 (5.5%) and IgG_4 (1.4%). This distribution was similar to those reported in the literature [37–43]. We observed that the migration of monoclonal IgG in agarose gel was highly heterogeneous and was unrelated to the type of their light chains or the subclasses determined by If. However, the migration of each M component, measured by the *G*/*A* index, was significantly related to the p*I* of their heavy and light chains. On the contrary to Tracy et al. [15,16], we were unable to observe, after 2-DE, a particular behavior of the monoclonal γ chains related to their subclass. In addition, only 38 out of 73 (52%) monoclonal γ chains were detected with this technique using ''standard'' IEF conditions. These results extend our previous observations showing that only 75% of the monoclonal γ chains were resolved by 2-DE when the first dimension was performed using non immobilized pH gradients [27]. At that time, we speculated that an alkaline p*I* of the heavy chains was the main cause of the absence of their resolution on 2-DE gels. This hypothesis was confirmed in this study for only 6 cases (8%), in which the γ chains were detected using pH 6-11 IPG. Interestingly, the mean p*I* of these alkaline γ chains (7.82) was statistically not different ($P=0.362$, *t*-test from that of the calculated p*I* of the non visible γ chains (7.61), indicating that an extreme p*I* was not the reason of the ''non visibility'' of the γ chains.

In 14 samples (19%), we observed no γ chain monomers but γ chain dimers of approximately 100 000 Da. In these cases, the absence of monomeric γ chains was probably an artifact related to the polymerization of monoclonal γ chains during sample preparation or IEF. Such a finding was already reported in the literature [44]. In 12 of our 73 cases (17%), neither γ chain monomers nor γ chain dimers were detected, whatever the IEF conditions used for 2-DE (linear vs. nonlinear IPG strips, presence vs. absence of alkaline ampholytes in the sample buffer, cathodic vs. anodic sample loading), or usage of 3-DE. This latter method, described by Harrison et al. [18], is able to resolve ambiguities by separating

the M component from the polyclonal background, thus eliminating the umbrella effect of the polyclonal Igs. In our series, this umbrella effect was observed in only 3 out of 73 cases (4%). Monoclonal light chains were more frequently detected (61 out of 73 cases, 84%). When they were not visible, their absence appeared unrelated to extreme p*I* values, because their calculated p*I*, albeit higher than that of the visible monoclonal light chains, were always in the range covered by the pH gradients used in this study. Taken together, these results suggest that the solubility of some monoclonal γ and/or light chains in the sample buffers used for IEF was not adequate, leading either to their polymerization or to their precipitation during the sample preparation and/or the first dimension run.

Each monoclonal γ chains detected after 2-DE in our study appeared as a set of spots having different p*I*, as it has been already showed (reviewed in [27]). A further degree of microheterogeneity, involving the charge and the size of the γ chains was observed in 5 cases of our series (3 γ_3, 1 γ_2, and 1 γ_1 chains). Such complex microheterogeneous patterns were reported in the literature in particular cases of μ, γ or δ chains [21,26,27,32,45]. Charge microheterogeneity or charge and size microheterogeneities involved also frequently monoclonal κ and λ light chains studied in plasma [18,20] or urine samples [46–49]. In our series, the analysis of the monoclonal light chains showed that these molecules were significantly more acidic than γ chains and that they were characterized by a wide range of p*I* spending on more than 2 units of pH. The mean p*I* of κ chains was not different from that of λ chains. The mean M_r of κ chains was slightly lower than that of λ chains, but, contrarily to what was reported in a series of 40 samples analyzed by 2-DE by Tracy et al. [16], or in a series of 70 Bence Jones proteins studied by Marshall et al. [47], our results were not statistically different. However, it is to be noted that the apparent M_r of the κ and λ chains was highly variable, and ranged between 22 000 and 32 000 Da, with a mean of 25 000 Da.

Taken together, our results confirm that monoclonal IgG are extremely complex molecules characterized by a highly heterogeneous electrophoretic behavior, depending on the methods used. Because of the extreme diversity of the different γ chains as well as of the κ and λ chains, it appears that a classification of monoclonal IgG based only on their electrophoretic properties is not possible.

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