ELSEVIER

Journal of Chromatography A, 698 (1995) 225–250

JOURNAL OF CHROMATOGRAPHY A

Review

Analysis of immunoglobulins by two-dimensional gel electrophoresis☆

Jean-Daniel Tissot[a,*], François Spertini[b]

[a]*Fondation Centre de Transfusion de la Croix-Rouge Suisse, Rue du Bugon 27, CH-1005 Lausanne, Switzerland*
[b]*Division d'Immunologie et d'Allergie du Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland*

Abstract

This paper reviews the indications and limitations of high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as an aid in the analysis of the clonality of immunoglobulins (Igs) with regard to "standard" techniques such as immunofixation electrophoresis. The 2D technique combines charge separation through isoelectric focusing and mass separation by sodium dodecyl sulfate-PAGE. Therefore, Ig heavy chains of different isotypes are separated from one another as well as from light chains. The 2D patterns of polyclonal, oligoclonal and monoclonal Igs are presented. To illustrate the impact of the technique in a clinical environment, selected situations are presented. Finally, the implications of 2D-PAGE analysis regarding the comprehension of the immune humoral system are discussed.

Contents

* Corresponding author.
☆ Dedicated to Dr. François Clément.

0021-9673/95/$29.00
SSDI 0021-9673(94)00844-2

1. Introduction

Antibody repertoire is as diverse as B lymphocyte populations. The specificity of an immunoglobulin (Ig) for an antigen is defined by complementary determining regions in the variable region of the heavy (H) and light (L) chains [1–8]. Each chain can be divided into a variable domain and constant domains that encode effector functions. Comparison of a large number of different variable domain amino acid sequences revealed three regions of sequence hypervariability (complementary determining regions) which are separated from each other by four intervals of relatively constant sequence (frameworks). Heavy chain variable regions are encoded by an exon formed by the joining of three gene segments, V_H, D_H and J_H [9]. These Ig gene segments are assembled via somatic rearrangement, and numerous non-Ig genes have been implicated as being involved in V(D)J recombination [10]. The recombination-activating proteins RAG-1 and RAG-2 are both essential for this process. The L chain variable domains are produced by two rearranging genes, V_L and J_L. The lymphocyte displays a single combination of H and L chains with a unique set of complementary determining regions, out of millions of possible combinations in the total repertoire of Ig molecules. T Cell- and antigen-dependent stimulations of B cells can lead to somatic hypermutations, resulting in the introduction of non-germline encoded diversity into the H and L chain variable domains [11,12]. In addition, individual members of an expanding clone of B cells making IgM or IgD can switch, in the presence of appropriate cytokines, to the production of another antibody class, i.e. IgG, IgA or IgE [13–16]. Highly complex mechanisms control clonal proliferation from a committed stem cell to an Ig-secreting cell, including Ig gene rearrangements, the programmed expression of differentiation-related genes and oncogenes of the B lineage, the T cell network, and signal-transferring molecules [17–28]. Proliferation and differentiation of progenitor B cells occurs in close contact with elements of the microenvironment such as advential cells, reticular cells, endothelial cells, fibroblasts, and macrophages [29,30].

A monoclonal Ig is produced by a single B cell clone, and is an heterodimeric protein, constituted of two H polypeptide chains of the same class and subclass and of two L polypeptide chains of the same type. Ig overproduction by a single expanded B cell clone is termed monoclonal gammopathy (MG), and can be observed in a wide category of benign or malignant diseases [31–36]. The frequency distribution of H chain classes and subclasses as well as L chain type of monoclonal Igs has been determined in different series of patients with MG [37–41]. In a number of lymphoproliferative diseases, only monoclonal L chains, or more rarely, incomplete monoclonal H chains are detected [42–52].

In patients with MG, the clinical manifestations are usually related to the underlying disease; destruction of normal bone, hypercal-

cemia, bone marrow replacement by the malignant clone, lymphadenopathy, splenomegaly, and the amount of Ig produced [40,44,53]. MGs are also incidentally diagnosed after serum and/or urine protein electrophoresis, and may be observed in patients without evidence of multiple myeloma, amyloidosis, macroglobulinemia or other lymphoproliferative diseases [31,33–36,40]. Such MGs are called "monoclonal gammopathy of undetermined significance" (MGUS) [31]. With the improvements in laboratory techniques for the detection of monoclonal Igs, MGUSs have been observed with increasing frequency [36,54–63]. It is known that the incidence of MGUS increases with age, and that between 11 and 25% of cases eventually convert to malignant B cell disorders [31,36,62–65].

MGs may be also detected because they have "perverse" biological activities leading to unusual clinical manifestations. Monoclonal Igs may interact with several specific antigens such as red blood cell antigens, acid polysaccharides, Fc portions of autologous Igs, lipoproteins, phospholipids, neural antigens, serum proteins or cellular proteins [35,66–69]. Some patients present polyneuropathy, organomegaly, endocrinopathy, MG, and skin changes (POEMS syndrome) [70]. Finally, the monoclonal Ig deposition disease may be observed in patients in whom an excessive amount of monoclonal Ig is deposited in a manner and quantity sufficient to compromise organ function [46–50,71].

Identification of monoclonal Igs requires sensitive, rapid and dependable screening methods. Electrophoresis on cellulose acetate membrane is satisfactory for initial screening. However, high-resolution agarose gel electrophoresis (HRE) is more sensitive for the detection of rare monoclonal proteins [72]. Confirmation of the presence of a MG should be performed using methods such as immunoelectrophoresis (IEP) or with immunofixation electrophoresis (IFE). IEP was described in 1953 [73], and until recently was considered as the method of reference. As a routine procedure, it is time-consuming, and requires a high level of technical expertise. As a consequence of the long diffusion process, small amounts of protein become too dilute to be detected, resulting in low sensitivity. The most important drawback of IEP is the so-called "umbrella effect" [74]. Furthermore, the demonstration of κ and λ monoclonal L chains belonging to IgA or IgM isotypes is often masked by the presence of polyclonal IgG, that, because of faster diffusion, have migrated ahead to an area situated between IgA and IgM. IFE was described by Afonso [75], later modified by Alper and Johnson [76], and was applied to the identification of MG in clinical diagnosis by Cawley et al. [77]. IFE, like IPE, includes two steps, but without diffusion process. In the first step, protein fractions are separated by agarose gel electrophoresis; in the second step, they are revealed by overlaying of antiserum specific of the different Ig H and L chains. The technique is considered as the test of choice in the routine diagnosis of MG, because of its simplicity, speed, sensitivity [78–93]. Other advantages of the method are easy interpretation and the absence of diffusion. On the other hand, the enhanced sensitivity of IFE also has yielded a more frequent detection of low concentration MG, and what is more important of multiple γ-globulin bands or subtle bands of restricted heterogeneity [94,95]. In such cases, further assessment of the clonality of Igs depends on additional techniques such as immunoblotting, immuno-isoelectric focusing [60,96–100] or high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

The 2D procedures, described by O'Farrell [101], combines charge separation through isoelectric focusing and mass separations by sodium dodecyl sulfate (SDS)-PAGE. Reduced and denatured proteins are therefore separated according to two unrelated biochemical characteristics. After silver staining, several thousand polypeptide spots can be detected, and analyzed on a single 2D gel. H Chains of different isotypes can be identified according to their restricted positions in the gel. Monoclonal H and L chains can be differentiated from polyclonal H and L chains according to their particular 2D patterns. The usefulness of 2D-PAGE as an aid in the analysis of the clonality of Igs in different clinical situa-

tions was established by a large number of studies [102–128].

2. Experimental approaches

2.1. Patients' samples

Blood samples were collected from healthy blood donors, as well as from patients with various clinical conditions. Samples were analyzed according to the following criteria: (i) known MG (multiple myeloma, Waldenström's macroglobulinemia, MGUS, amyloidosis, biclonal gammopathies); (ii) samples showing ambiguities with IFE analysis; (iii) samples with expected clonal abnormalities (allogeneic bone marrow transplantation, immunodeficiencies, developing infants) with or without IFE ambiguities. Clinical data and other biological information, like bone marrow examinations were obtained to correlate IFE and 2D-PAGE patterns with patients' diagnosis.

2.2. Immunofixation electrophoresis

This method was performed by using the Paragon gel system of Beckman Instruments (Brea, CA, USA), following the indications of the manufacturer. Serum samples were diluted (barbital buffer pH 8.6, 0.05 *M*) 1:2 for reference electrophoresis, and 1:10 for immunofixation [89]. Six aliquots of 3–5 μl were applied on the 1% agarose gel using a "sample template". The samples were electrophoresed for 30 min under 100 V. After drying, 80 μl of the protein fixative (5% acetic acid and 5% 5-sulfosalicylic acid) were applied to the reference protein migration. Similarly, monospecific antisera (anti-γ, anti-α, anti-μ, anti-κ and anti-λ) were applied to the other five migrations. The preparations were then incubated for 30 min at 45°C, washed extensively, stained, destained and dried.

When low-concentration MG was suspected, as well as when apparently isolated monoclonal L chain was found, the test was repeated either with lower dilution of the serum sample or with specific anti-δ and anti-ϵ H chain antisera.

2.3. Purification of immunoglobulins, cold agglutinins and cryoglobulins

IgA, IgG, IgM and IgD were purified from serum samples by affinity chromatography as described elsewhere [117,123]. Cold agglutinins (CAs) were partially purified from serum samples by cold absorption on and warm elution from group O newborn or adult red blood cells [110,116]. CAs were isolated from blood samples coagulated at 37°C. The serum was left for 96 h at 4°C [127,128]. After centrifugation, the precipitate was washed with phosphate–NaCl buffer at 4°C (three times).

2.4. Two-dimensional polyacrylamide gel electrophoresis

2.4.1. Sample preparation

Plasma/serum sample preparation was described by Hochstrasser et al. [129]. Briefly, 5 μl of plasma/serum were mixed with 10 μl of a denaturing and reducing solution ("solution D"; SDS 1 g, DTE (dithioerythritol) 0.232 g, water 10 ml), and heated at 95°C for 5 min. After a short cooling time, 485 μl of the "sample solution E" (100 μg of dithiothreitol, 400 μg of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 5.4 g urea, 500 μl of pH 3–5–10 ampholytes and 6.5 ml of water) were added. For routine analysis purposes, 30 μl of the final diluted sample (0.3 μl plasma/serum equivalent) were loaded onto the first-dimension gels.

Purified Igs, dialyzed against water, were either diluted or concentrated to obtained a protein concentration of 1 mg/ml. A 50-μl volume was mixed and heated with 20 μl of "solution D". Then 430 μl of "solution E" were added. A 50-μl volume of the final diluted sample (5 μg Ig equivalent) was analyzed by 2D-PAGE.

A 50-μl volume of water dialyzed cold agglutinins (Ig concentrations generally less than 0.05 g/l) were prepared as described above, except that 10 μl of "solution D" and 440 μl of "solution E" were added. A 60-μl volume of the final diluted samples was loaded onto the first-dimension gels.

Cryoprecipitates were directly dissolved in "solution D". A 10-μl volume (25–50 μg of protein) was mixed with 100 μl of "solution E". A 50-μl volume of the final diluted sample (12.5–25 μg of protein) was loaded on the first-dimension gels before isoelectric focusing.

2.4.2. Isoelectric focusing (first dimension)

Until recently, isoelectric focusing was performed with carrier ampholytes but was replaced by immobilized pH gradients, to achieve better resolution of basic proteins.

2.4.2.1. *Carrier ampholytes*

Isoelectric focusing was performed using pH 3.5–10 carrier ampholytes gradients (CAGs). The equipment was from Bio-Rad (Glattbrugg, Switzerland). The anolyte contained phosphoric acid (6 m*M*) whereas the catholyte contained sodium hydroxide (20 m*M*). Samples were loaded onto the basic side of the capillary gels. The later were prepared as described in details by Hochstrasser et al. [129]. Diacrylylpiperazine was used as the cross-linking agent [130,131]. Isoelectric focusing was performed at room temperature with a constant voltage of 200 V for 2 h, followed by 500 V for 5 h, and then 1000 V for 11 h.

2.4.2.2. *Immobilized pH gradients*

Isoelectric focusing using immobilized pH gradients (IPGs) was performed as described by Görg et al. [132], and later modified by Bjellqvist et al. [133]. The equipment, including Immobiline Dry-Strip pH 3–10 NL, 18 cm, was from Pharmacia-LKB (Uppsala, Sweden). All samples were routinely loaded onto the cathodic end of the rehydrated IPG strips, covered with low-viscosity paraffin oil. Isoelectric focusing was performed at 10°C. The voltage was progressively increased from 200 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 kV h. Before the second-dimensional run, IPG strips were equilibrated within the strip tray for 15 min with a solution (100 ml) of Tris–HCl buffer (0.05 *M*, pH 6.8) containing 6 *M* urea, 30% (w/v) glycerol, 2% (w/v) SDS and 2% (w/v) dithioerythritol to resolubilize the peptides. SH groups were subsequently blocked by equilibration (5 min) with the same solution containing iodoacetamide (2.5%, w/v) instead of dithioerythritol and traces of bromophenol blue [133].

2.4.3. SDS-PAGE (second dimension)

The vertical second dimension was performed on 180 × 160 × 1.5 mm 9–16% polyacrylamide gradient gels, also using diacrylylpiperazine as cross-linking agent [130,131]. SDS was not incorporated into the gels, and stacking gels were not employed. CAG capillary gels, rinsed with 150 μl of a transfer solution containing 8 ml of 0.5 g/l bromophenol blue solution, 40 ml of 100 g/l of SDS solution, 20 ml of 0.5 *M* Tris–HCl buffer, pH 6.8, and 72 ml of water, were loaded onto the slab gels. They were not sealed with any agarose. IPG strips were cut to size with second-dimensional gels, and sealed onto slab gels with the transfer solution containing 0.5% agarose. The gels were run at 40 mA/gel constant current and maintained at a temperature between 8 and 12°C.

2.4.4. Silver staining and spot identification

The silver staining protocols, based on those described by Oakley et al. [134] and Rabilloud [135], have been described elsewhere [129,133]. Briefly, at the end of the run, the gels were washed in water, then soaked in ethanol–acetic acid–water (40:10:50) for 1 h and ethanol–acetic acid–water (5:5:90) overnight. After a water wash, the gels were soaked (30 min) in glutaraldehyde (1%) buffered with sodium acetate (0.5 *M*) and the glutaraldehyde was removed by water washes. The gels were then soaked in a 1.7 naphthalenenedisulfonic acid solution (0.05%, w/v) for 30 min and rinsed again with water. The gels were stained in a freshly made ammoniacal silver nitrate solution [112] for 30 min and then rinsed with water. The image was developed in a solution containing 0.05% (w/v) citric acid and 0.1% (v/v) formaldehyde in 1 l of water. Development was stopped with an acetic acid–

water (5:95) solution. All incubations were performed on an orbital shaker.

The gels were photographed with higher molecular masses at the top and the acidic side on the left. Polypeptides were identified by comparison with reference protein maps [120,136,137]. p*I* values were determined using the reference values established by Bjellqvist et al. [133]. Apparent M_r values of polypeptide spots were established by comparison of known proteins used as internal standards.

3. Evaluation of results

3.1. The plasma/serum protein map

The human plasma/serum 2D profile has been extensively studied, also during development [120,136–159]. More than 400 spots, representing about 80 polypeptide chains or 55 proteins have been identified so far on plasma/serum protein maps [120,136,137]. Fig. 1 shows the 2D pattern of a normal plasma sample. Polypeptides are rarely detected as isolated spots, but are observed as a series of spots, displaying charge and size microheterogeneities. Some reflect post-synthetic chemical modifications such as N-terminal acetylation, tyrosylation, phosphorylation, deamidation or proteolytic cleavage, while others are due to the addition of both charged and uncharged sugars [149]. Genetic polymorphism is another important factor [120,142,145,147,149,151–153,155,157–159].

In contrast to cell or tissue samples, 2D plasma/serum protein patterns are largely independent of the carrier ampholytes and 2D-PAGE protocols [120,133]. Thus, plasma/serum protein maps obtained in different laboratories can be easily evaluated relative to previously published data. By comparison with "reference" normal plasma/serum protein maps, distinct protein modifications can be observed on 2D-PAGE pictures of pathological samples, allowing the diagnose of diseases solely on the basis of the protein profile (reviewed in Ref. [120]).

3.2. Polyclonal and monoclonal immunoglobulins

The map locations of IgG γ-H chains and of κ- and λ-L chains were described by Anderson and Anderson [138]. Based on the analysis of monoclonal Igs of different isotypes, the position of IgA α chains and IgM μ chains were identified by Thorsrud et al. [102] and Latner et al. [103,104]. The latter authors also described the position of IgD δ chain, whereas IgE ϵ chain was identified by Jellum and Thorsrud [106].

3.2.1. Polyclonal heavy chains

Since proteins are separated under denaturing and reducing conditions, H chains are well separated from L chains. In addition, H chains of different isotypes are detected on non-overlapping areas of the gels (Figs. 1–3). Polyclonal H chains are observed as "fuzzy" zones, without distinct individualizable spots. Polyclonal μ chains are noticed above the large albumin spot and at the acidic side of transferrin, in an area of the gel corresponding to p*I* of 5.6 to 6.4. In patients with normal IgM levels, μ chains are not well resolved (Fig. 1). They are clearly detectable in patients with high IgM serum levels (Fig. 2). Along with full length μ chains, μ-chain fragments (μ-S fragments [107,123,149]) as well as an IgM-associated peptide can also be noticed (Fig. 2). The IgM-associated peptide is an unknown protein, as shown by N-terminal microsequencing (APPGVRLVGGLH) [128]. Polyclonal α chains are resolved at an apparent M_r lower than albumin, just above the spots of antithrombin III. The p*I* of α chains is usually between 4.9 and 6.1. In plasma, γ chains are found under the β chain of fibrinogen (absent in serum), at the basic side of the gels, and in an area corresponding to p*I* of 6.2 to more than 10. Polyclonal δ chains, as well as ϵ chains are not detectable.

3.2.2. Polyclonal light chains

Polyclonal L chains appear as clustered non-discrete spots, and form cloudy zones with unevenly distributed densities (Fig. 1). The appar-

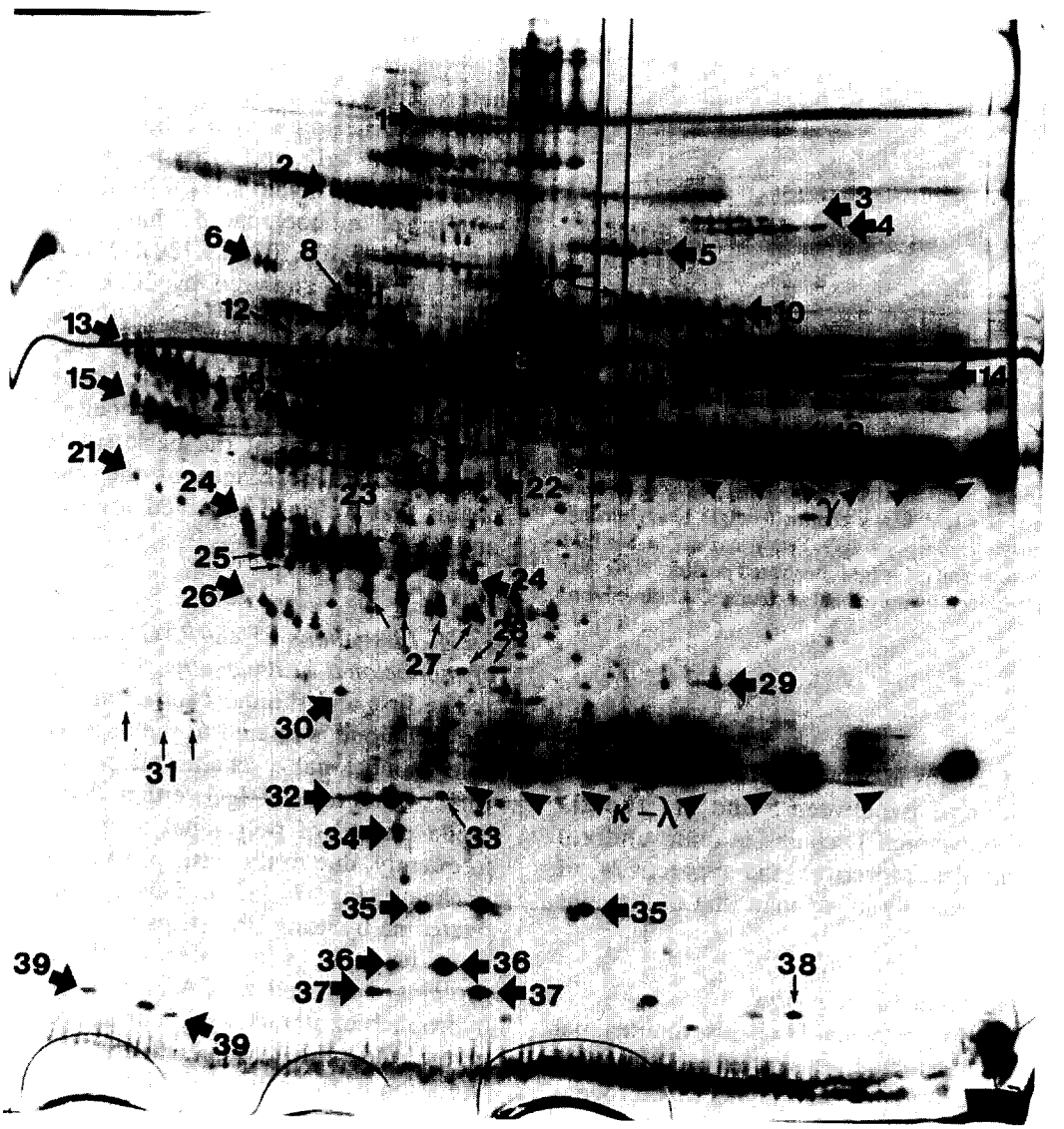

Fig. 1. The normal plasma map. Polypeptides (0.3 μl of plasma) were separated by pH 3–5–10 carrier ampholytes gradient, followed by gradient 9–16% PAGE in presence of SDS. The ammoniacal silver stained gel was photographed with the higher molecular mass at the top and the acidic side on the left. 1 = α2-Macroglobulin; 2 = ceruloplasmin; 3 = Glu-plasminogen; 4 = Lys-plasminogen; 5 = complement factor B; 6 = complement C1s; 7 = protransferrin; 8 = prothrombin; 9 = α1-B-glycoprotein; 10 = transferrin; 11 = hemopexin; 12 = α2-antiplasmin; 13 = α1-antichymotrypsin; 14 = fibrinogen α chain; 15 = α2-HS-glycoprotein; 16 = α1-antitrypsin; 17 = antithrombin III; 18 = fibrinogen β chain; 19 = extend fibrinogen γ chain; 20 = Gc-globulin (the vitamin D-binding protein); 21 = lysin-rich glycoprotein; 22 = fibrinogen γ chain; 23 = apo A-IV; 24 = haptoglobin β chain; 25 = Zn-α-glycoprotein; 26 = apo J; 27 = cleaved haptoglobin β chain; 28 = apo E (phenotype E 3/4); 29 = γ chain of complement C4; 30 = α1-microglobulin; 31 = apo D; 32 = apo A-I; 33 = proapo A-I; 34 = retinol binding protein; 35 = haptoglobin α1 chain; 36 = transthyretin (prealbumin); 37 = haptoglobin α2 chain; 38 = hemoglobin β chain; 39 = apos C-II and C-III; a = albumin; μ = polyclonal heavy chains of IgM; α = polyclonal heavy chains of IgA; γ = polyclonal heavy chains of IgG; κ–λ = polyclonal Ig light chains. First dimension: CAG.

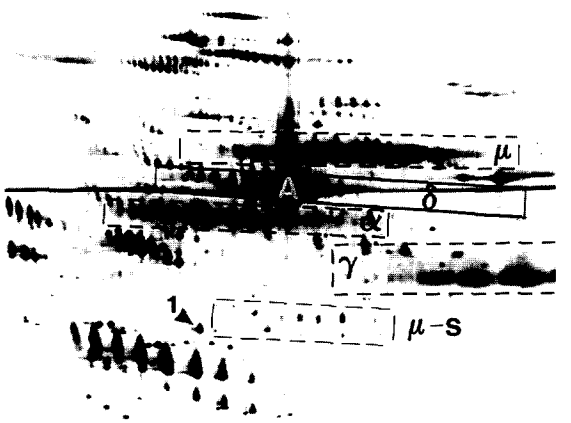

Fig. 2. Immunoglobulin heavy chain areas. Patient with high IgM serum levels (21 g/l). The insets show the restricted and non-overlapping areas in which monoclonal heavy chains can be observed. μ = IgM heavy chains; δ = IgD heavy chains; α = IgA heavy chains; γ = IgG heavy chains; μ-S = IgM heavy chain fragments; 1 = IgM-associated peptide. Note that transferrin is hidden by polyclonal μ chains. First dimension: CAG.

ent M_r of L chains is between 21 000 and 27 000, whereas their p*I* is between 5 and 11. The 2D pattern of polyclonal L chains is quite uniform between samples. Moreover, the apparent M_r of κ-L chain is typically lower than that of λ chains [107].

3.2.3. Monoclonal heavy chains

In contrast to polyclonal H chains, monoclonal H chains are detected as sets of well-resolved spots characterized by charge microheterogeneity (Figs. 3–6). Microheterogeneity of monoclonal Ig is observed by isoelectric focusing and immunoblot analysis, and has been primarily attributed to post-translational sialylation and possibly to acetylation, deamidation and glycosylation of the H chain [95,160–162]. Monoclonal α , γ and μ chains are usually located at the same position than the corresponding polyclonal H chains. There are, however, examples of aberrant H chain patterns, most frequently detected in patients with H chain disease [107]. An "abnormal" monoclonal γ3 chain is shown in Fig. 4C. In contrast to the "usual" monoclonal γ chains detected at 2D-PAGE analysis (Figs. 3C, 4B), this particular H chain has a larger apparent M_r with size heterogenity as well. It is possible that this pattern corresponds to the IgG3 subclass [106]. Monoclonal IgD are infrequently observed in patients with myeloma [37–40], but the 2D pattern of monoclonal δ chain has been described [103,104,107,113,120,121,123]. In our series of MGs analyzed by 2D-PAGE, we observed four monoclonal IgDs. On plasma/serum 2D maps, monoclonal δ chains are also characterized by charge microheterogeneity, and to a lesser degree by size heterogeneity. They are detected within or at the basic side of albumin (Fig. 5). The p*I* ranges of the monoclonal δ chains that we have observed were between 5.2 and 8.

3.2.4. Limitations of 2D-PAGE in the analysis of monoclonal heavy chains

The first and the most important limitation of the technique is related to the absence of detectable monoclonal γ chains on protein maps in 75% of monoclonal IgG, either due to an "umbrella effect" of polyclonal γ chains, or, more frequently, due to the basic p*I* of the monoclonal γ chains (Fig. 7) [123]. This problem was not overcome by using IPG strips for the first dimension, in spite of a better resolution of basic proteins compared to CAG. It is possible that hydrophobic interactions occur between the IPG matrix and some basic γ chains leading to precipitation of the peptides of interest. The second restraint is related to the difficulties to recognize basic monoclonal μ chain spots (p*I* 6.1–6.8) when they are resolved among the spots of transferrin (Fig. 6). The third limitation is due to the wide p*I* range of monoclonal δ chains. We observed a monoclonal δ chain that migrated at the same position as albumin, and was therefore not detectable on the serum protein map.

Finally, as monoclonal H chains exhibit major charge and to a lesser degree size microheterogeneities, multiple clonal Igs of the same isotypes cannot be detected individually,

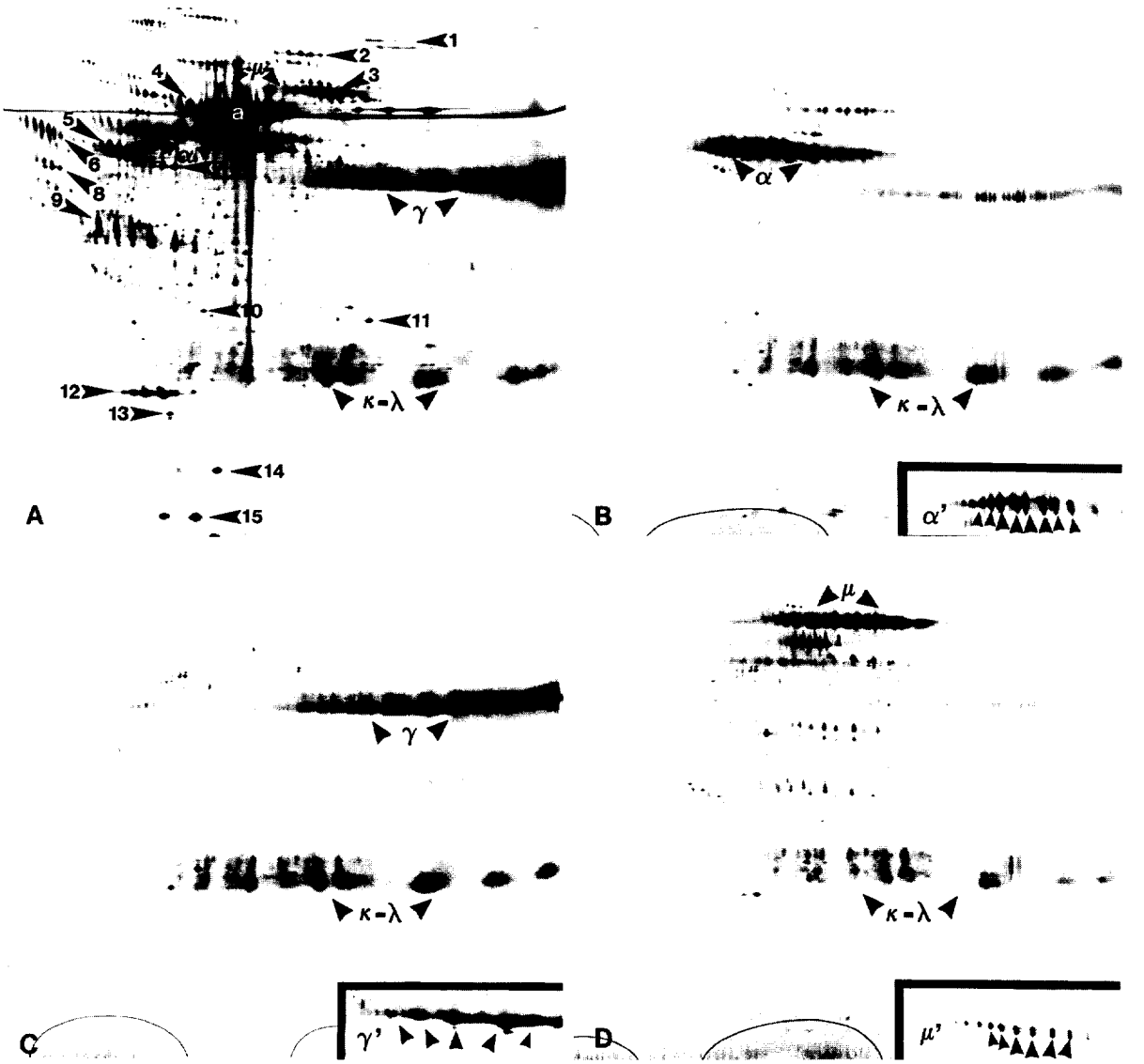

Fig. 3. Two-dimensional patterns of purified polyclonal and monoclonal Igs of different isotypes. (A) Normal serum, (B) purified IgA, (C) purified IgG and (D) purified IgM. Insets (B–D) show purified monoclonal heavy chains. 1 = Plasminogen; 2 = factor B; 3 = transferrin; 4 = hemopexin; 5 = α1-antitrypsin; 6 = α1-antichymotrypsin; 7 = Gc-globulin; 8 = α2-HS-glycoprotein; 9 = haptoglobin β chain; 10 = apo E (phenotype E 3/3); 11 = γ chain of complement C4; 12 = apo A-I; 13, retinol binding protein; 14 = haptoglobin α1 chain; 15 = transthyretin; a = albumin; μ = polyclonal heavy chains of IgM; α = polyclonal heavy chains of IgA; γ = polyclonal heavy chains of IgG; κ–λ = polyclonal Ig light chains; α' = monoclonal α chain; γ' = monoclonal γ chain; μ' = monoclonal μ chain. Charge microheterogeneity of monoclonal heavy chains is highlighted by arrows. First dimension: CAG. (From Tissot et al. [123]).

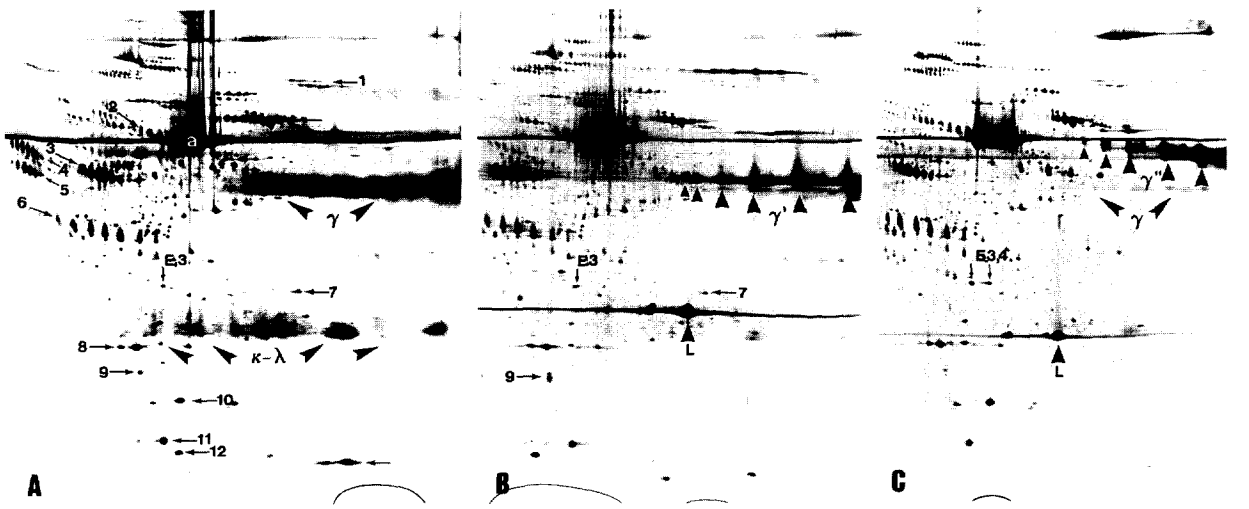

Fig. 4. Pattern variations of monoclonal IgG. (A) Normal serum, (B) serum from a patient with monoclonal IgG and (C) serum from a patient with a monoclonal IgG3 that activated complement. 1 = Glu- and Lys-plasminogen; 2 = hemopexin; 3 = α1-antitrypsin; 4 = α1-antichymotrypsin; 5 = α2-HS-glycoprotein; 6 = haptoglobin β chain; 7 = γ chain of complement C4; 8 = apo A-I; 9 = retinol binding protein; 10 = haptoglobin α1 chain; 11 = transthyretin (prealbumin); 12 = haptoglobin α2 chain; a = albumin; E3 = apo E (phenotype E3/3); E3,4 = apo E (phenotype E3/4); γ = polyclonal heavy chains of IgG; κ–λ = polyclonal Ig light chains; γ' = "typical" monoclonal γ chain; γ'' = aberrant monoclonal γ chain, characterized by a higher apparent M_r, as well as by charge and size microheterogeneity. Note the absence of the γ chain of complement C4 in (C). First dimension: CAG.

due to superposition of several sets of spots in a restricted area of the 2D gel [122].

3.2.5. The spectrum of light chain patterns and correlations with immunofixation electrophoresis

The analysis of L chain patterns provides invaluable information on the apparent diversity of Igs, as illustrated by several examples shown in Fig. 8.

The study of plasma/serum samples from patients with hypergammaglobulinemia, but without detectable monoclonal or restricted Ig bands at IFE, reveals different L chain patterns when compared to the normal Ig L chain pattern. In some patients, L chains appear as a wide spread of numerous ill-defined spots (Fig. 8A, B) or as many small but well-defined spots (Fig. 8F, G). A combination of the two patterns is also observed (Fig. 8C–E).

L Chain pattern from patients with oligoclonal Igs in their serum (appearing as faint bands at IFE, as exemplified for the case shown in Fig. 9) are detected as a limited number of well distinguishable spots (Fig. 8H–M). Oligoclonality can be also observed in patients without IFE abnormalities (developing infants, in some patients treated by allogeneic bone marrow transplantation, as well as in some patients presenting immunodeficiencies or with acute infections [111,117,120,122–125]).

In about two thirds of patients with MG, monoclonal L chains are detected as a dominant and well-defined spot (Fig. 8N, O). In one third of the patients, examined monoclonal L chains disseminate in more than one spot (Fig. 7). Microheterogeneity of L chain patterns has already been reported in the literature [95,114]. In a study of 41 patients with MG, Harrison [114] observed monoclonal L chains exhibiting significant microheterogeneity in 75.6% of cases, contrasting with our observation of 29% [123,125]. Most likely, this discrepancy is related to the amount of monoclonal Ig in plasma/serum sam-

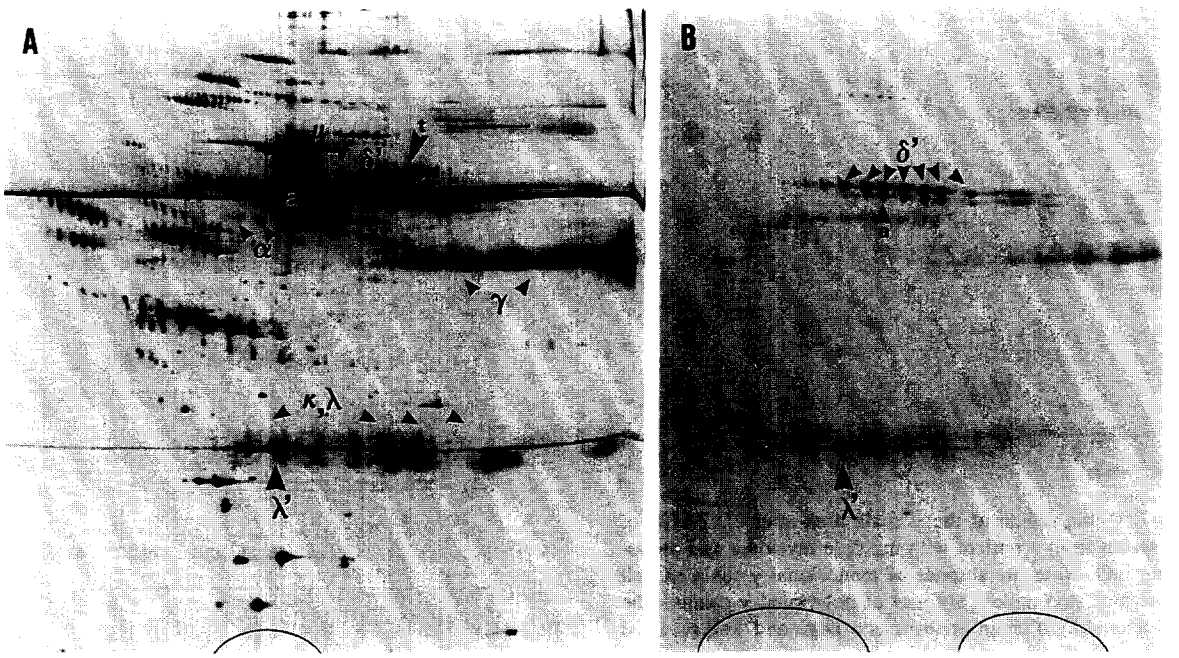

Fig. 5. 2D-PAGE pattern of a monoclonal IgD-λ. (A) Serum and (B) purified IgD (anti-λ Sepharose). a = Albumin; μ = polyclonal heavy chains of IgM; α = polyclonal heavy chains of IgA; γ = polyclonal heavy chains of IgG; κ,λ = polyclonal Ig light chains; δ' = monoclonal δ chain monoclonal; λ' = monoclonal λ chain; t = transferrin. Note that in serum, polyclonal Igs are decreased, and that the acidic part of the monoclonal δ chain is hidden by albumin. First dimension: CAG. (From Tissot et al. [123]).

ples, to the different amounts of samples that were loaded onto the first-dimension gels, as well as to differences in silver staining procedures.

Fig. 6. 2D-PAGE pattern of a basic monoclonal μ chain. Serum sample from a patient with Waldenström's macroglobulinemia. The monoclonal μ chain spots are observed among the spots of transferrin, and can be easily missed. μ = Monoclonal μ chain; t = transferrin. First dimension: IPG.

3.2.6. Semi-quantitative determination of immunoglobulin concentrations

Serial dilutions of protein-A purified monoclonal IgG fractions revealed that 2D-PAGE can be used to semi-quantitatively determine the amount of a monoclonal Ig in a sample (Fig. 10). After 2D-PAGE of 0.3 μl of serum, L chain spots larger than the main spot of apolipoprotein (apo) A–I correspond to Ig concentrations greater than 2 g/l, whereas spots smaller than proapo A–I correspond to Ig concentrations lower than 0.2 g/l [117].

3.2.7. Limitations of 2D-PAGE in the analysis of the clonality of light chains

2D-PAGE analysis of L chain patterns does not permit definitive conclusions regarding clonality on a single-case basis, since monoclonal L chains do not always appear as single spots.

Fig. 7. Detail region of the γ chain area after 2D-PAGE of the serum from a patient with multiple myeloma and monoclonal IgG. Note the absence of monoclonal γ chain as well as the almost complete absence of polyclonal γ chains. The monoclonal light chain appears as a large and well resolved spot (large arrow), accompanied by a smaller and more acid spot (small arrow). First dimension: CAG.

Furthermore, also due to L chain microheterogeneity, semi-quantitative determinations of prominent clonal Igs according to the size of the L chain spots underestimate the concentration of several monoclonal Igs. In addition, the L chain type cannot be accurately determined only according to size differences between κ and λ chains. However, based on the SDS molecular mass discriminant values, Tracy et al. [107] correctly assigned the L chain type in more than 75% of the cases.

Several monoclonal L chains are not detected on the protein map because of their basic p*I* by using CAG [123]. However, the use of IPG

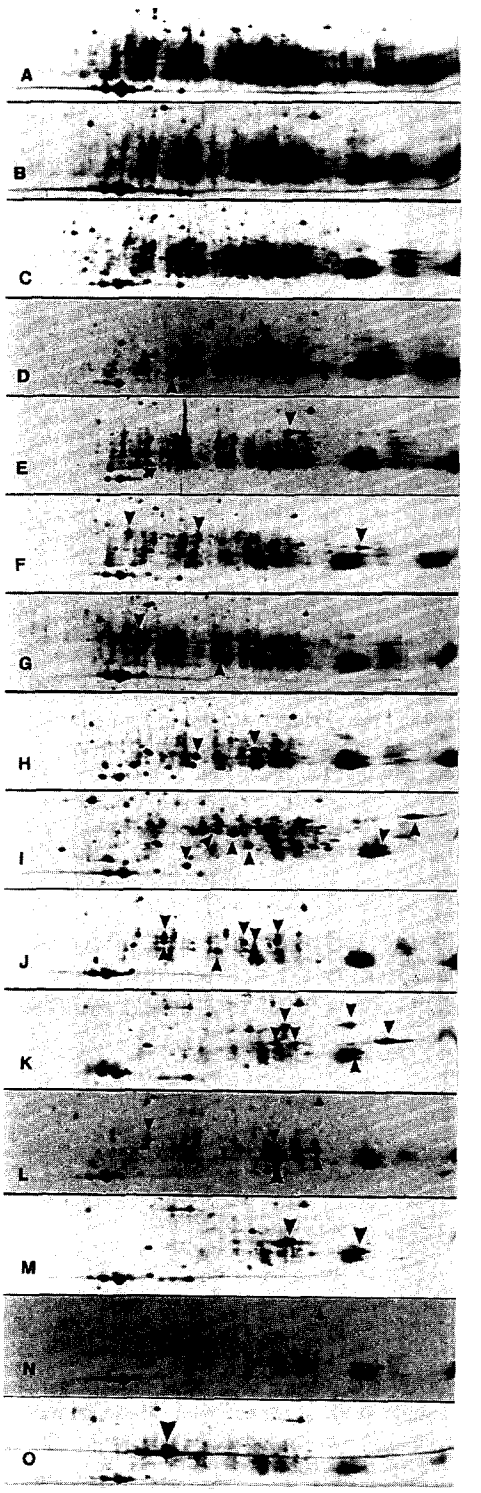

Fig. 8. The spectrum (from polyclonal to monoclonal) of Ig light chain patterns. (A–G) Hypergammaglobulinemia (IFE patterns unremarkable), (H–M) oligoclonal gammopathy (restricted, but multiple clonal Ig bands at IFE analysis) and (N and O) monoclonal gammopathy (single monoclonal Ig band at IFE analysis). The most prominent Ig light chain spots are indicated by arrows. First dimension: CAG (adapted from Tissot et al. [123]).

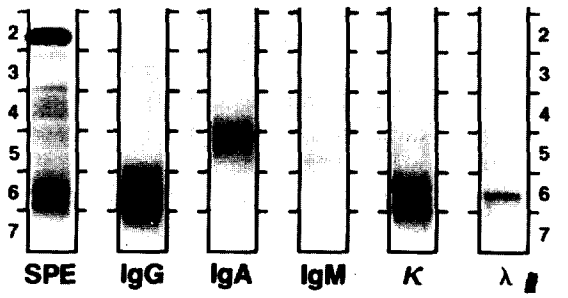

Fig. 9. Immunofixation electrophoresis showing multiple restricted clonal IgG-κ and IgG-λ bands. SPE = serum protein electrophoresis.

clearly yields better resolution of basic L chains. Fig. 11 illustrates the resolution of basic light chains with IPG and shows an example of a monoclonal L chain spot that was not detected with CAG.

Finally, modifications unrelated to Ig L chains can be observed in the L region. Such modifications may be observed in patients with severe inflammation and very high C reactive protein levels. This protein is detected as two or three

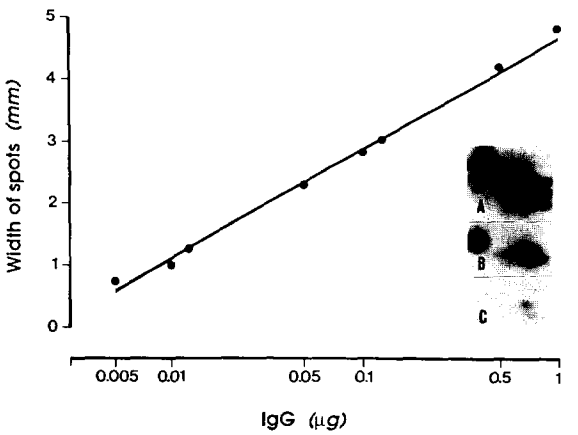

Fig. 10. Semi-quantitative determination of Ig concentration according to the width of Ig light chain spots. Series of electrophoretograms of different protein-A purified monoclonal IgG were performed and revealed a good correlation ($r > 0.095$, semi-logarithmic plot) between the amounts of Igs loaded onto first-dimension gels and the width of the light chain spots. Each point represents the mean of five different light chain spots. Insets show details of electropherograms of three different light chain spots corresponding to (A) 1 μg; (B) 0.1 μg; (C) 0.01 μg of purified IgG (per spot). First dimension: CAG. (From Tissot et al. [117]).

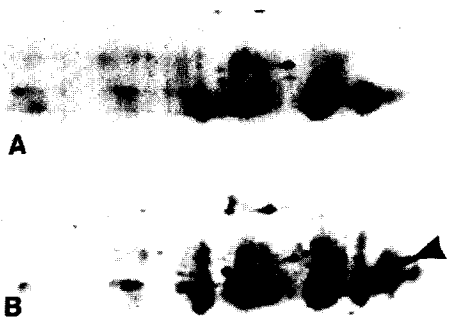

Fig. 11. Details of the basic part of the Ig light chain area. (A) Normal serum and, (B) serum sample from a patient with Waldenström's macroglobulinemia and monoclonal IgM. The monoclonal light chain (arrowed) was not detected by 2D-PAGE analysis using CAG, but was clearly observed with IPG. First dimension: IPG.

spots, above apo A–I, in an area corresponding to p*I* 5.1–5.3 (Fig. 12).

3.3. Clinical applications

To illustrate the potential impact of 2D-PAGE analysis in a clinical environment, several selected situations are presented.

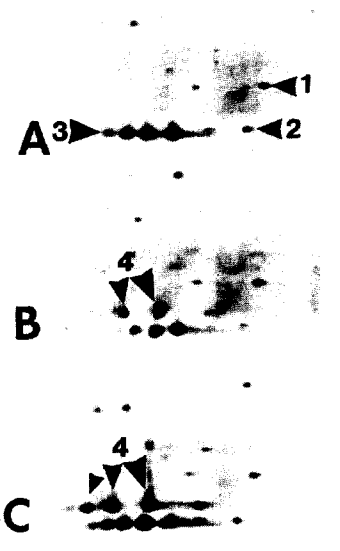

Fig. 12. Details of the acidic part of the Ig light chain area. (A) Normal serum, (B, C) serum samples from patients with severe inflammation. 1 = Amyloid P serum protein; 2 = proapo A-I; 3 = apo A-I; 4 = C reactive protein. First dimension: IPG.

3.3.1. Cold agglutinins

CAs are defined as autoantibodies capable of agglutinating erythrocytes at temperatures below 37°C. The possible clinical importance of CAs is best evaluated in the laboratory by determination of their thermal amplitude, defined by the highest temperature at which the antibody is active [163,164]. Hemolytic anemia related to CAs may be observed in a wide variety of diseases. Monoclonal CAs, generally belonging to the IgM isotype are observed in patients with lymphoproliferative disorder or suffering from the "idiopathic" chronic cold agglutinin disease (CCAD) [165]. Polyclonal CAs, also belonging to the IgM isotype, may be found in patients with various infectious diseases, but more particularly in patients with Epstein–Barr virus or *mycoplasma pneumoniae* infection. We have previously shown that the determination of the clonality of CAs could be a useful aid in the precocious differentiation of the CCAD and "parainfectious" CA-associated hemolytic anemia [110,116,120]. These preliminary observations are confirmed in a series of 25 consecutive patients with hemolytic anemia and CAs (Table 1). The 2D-PAGE analyses of three selected cases are shown in Fig. 13. All three patients presented increased IgM serum levels and unremarkable IFE analysis. The electropherograms of the purified CAs display polyclonal IgM in two patients (Fig. 13A', B'), and monoclonal IgM in the third patient (Fig. 13C').

Table 1
Clinical findings, specificity and clonality of cold agglutinins in a series of 25 consecutive patients

Patient	Clinical diagnosis	Specificity[a]	Clonality of the CA[b]
J.T	CCAD[c]	Anti-I	Monoclonal
C.C	Epstein–Barr virus infection	Anti-i	Polyclonal
B.G.	Lymphoma	Anti-I	Monoclonal
E.B.	Lymphoma	Anti-I	Monoclonal
T.E.	Angioimmunoblastic lymphadenopathy	Anti-I	Monoclonal
A.J.M.	Mycoplasma pneumoniae	Anti-I	Polyclonal
C.G.	Mycoplasma pneumoniae	Anti-I	Polyclonal
L.M.	Waldenström's macroglobulinemia	Anti-I	Monoclonal
P.J.P.	Type II cryoglobulinemia	Anti-I	Monoclonal
G.B.	Chronic lymphocytic leukemia	Anti-i	Monoclonal
B.A.	Chronic hepatitis C	Non-specific[d]	Polyclonal
H.L.	CCAD	Anti-I	Monoclonal
S.M	CCAD	Anti-I	Monoclonal
S.L.	CCAD	Anti-I	Monoclonal
B.X.	CCAD	Anti-I	Monoclonal
K.E.	Lymphoma	Non-specific	Monoclonal
B.Y.	CCAD	Anti-I	Monoclonal
C.J.	CCAD	Anti-I	Monoclonal
M.J.	Lymphoma	Anti-I	Monoclonal
L.M.	CCAG	Anti-I	Monoclonal
B.P.	Epstein–Barr virus infection	Anti-i	Polyclonal
V.M.	Lymphoma	Non-specific	Monoclonal
P.D.	CCAD	Anti-I	Monoclonal
C.L.	CCAD	Anti-I	Monoclonal
P.M.-L.	CCAD	Anti-I	Monoclonal

[a] Activity of the autoantibody determined with adult red cells (group O; antigen I) and with cord blood red cells (group O; antigen i).
[b] 2D-PAGE analysis.
[c] CCAD = Chronic cold agglutinin disease.
[d] Reactivity identical either with adult or cord red blood cells.

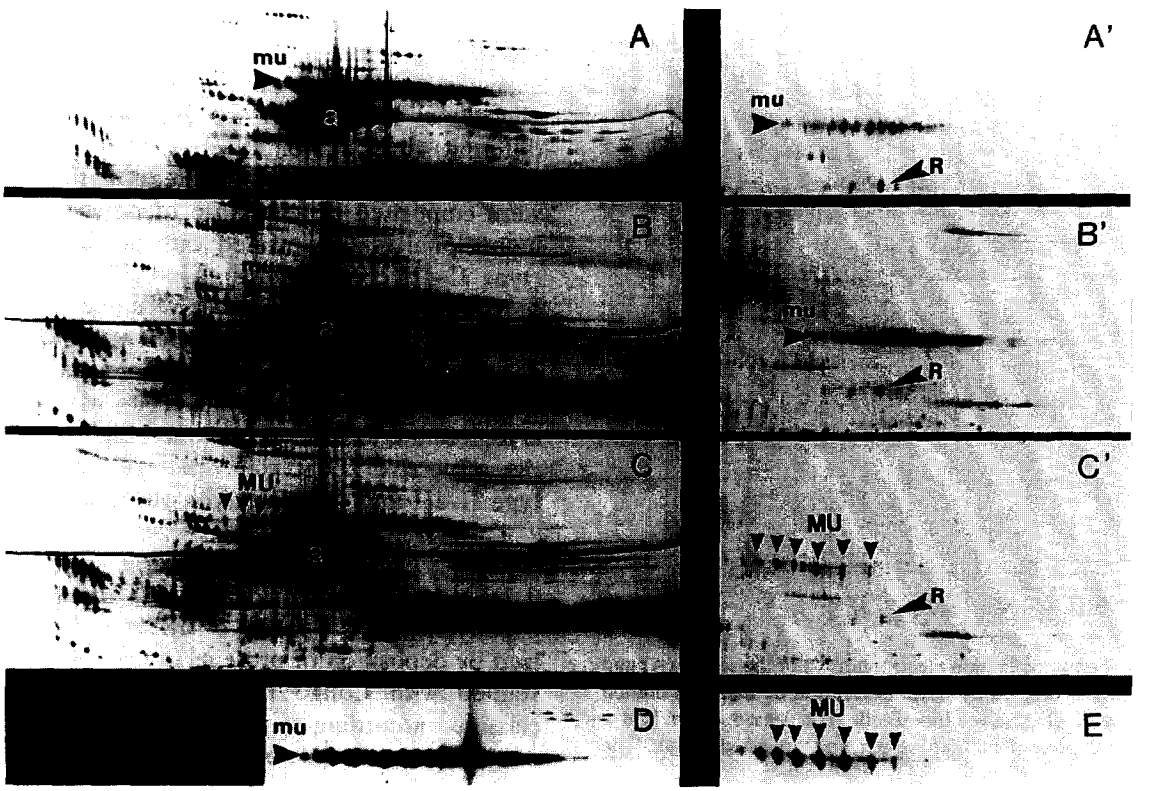

Fig. 13. Details of electrophoretograms of plasma/serum samples (A–C) and their purified cold agglutinins fractions (A'–C'). Purified polyclonal (D) and monoclonal (E) IgM heavy chains are shown as controls. (A, A') Serum and purified CAs from a patient with chronic virus C hepatitis, (B, B') plasma and purified CAs from a patient with *mycoplasma pneumoniae* infection and (C, C') plasma and purified CA from a patient with chronic cold agglutinin disease. a = Albumin; R = reference protein used to highlight the relatively acidic p*I* of the monoclonal μ chain found in patient C; mu = polyclonal μ chains; MU = monoclonal μ chains. The charge microheterogeneity of monoclonal μ chains is highlighted by arrows. First dimension: CAG.

The first (Fig. 13A, A') suffered from mild anemia and chronic virus C hepatitis infection. The second patient (Fig. 13B, B') presented an acute *mycoplasma pneumoniae* infection and severe anemia. Follow up investigations revealed that the CAs observed in the second patient were "transient", whereas the CAs detected in the first patient persisted over the years. To the best of our knowledge, persistent polyclonal CAs have not been reported in the literature. The third patient (Fig. 13C, C') suffered from the CCAD. It is noteworthy that monoclonal IgM was detectable on the plasma 2D map, but was not found at IFE analysis. By contrast, as exemplified for one case in Fig. 14, when monoclonal CAs are detectable at IFE analysis, the 2D technique is not useful for diagnostic purposes.

3.3.2. Cryoglobulins

Cryoglobulins are Igs that precipitate in the cold. Each cryoglobulin has an individual thermal curve with well-defined kinetics of precipitation. Three types of cryoglobulins have been identified [166]. Type I cryoglobulins contain a single monoclonal Ig, whereas type II cryoglobulins are mixture of monoclonal Igs (most frequently IgM) and polyclonal IgG. Type III cryoglobulins are mixture of polyclonal Igs of different isotypes (generally IgG and IgM). Type I cryoglobulins are observed in patients suffering from lymphoproliferative disorders. Type II and

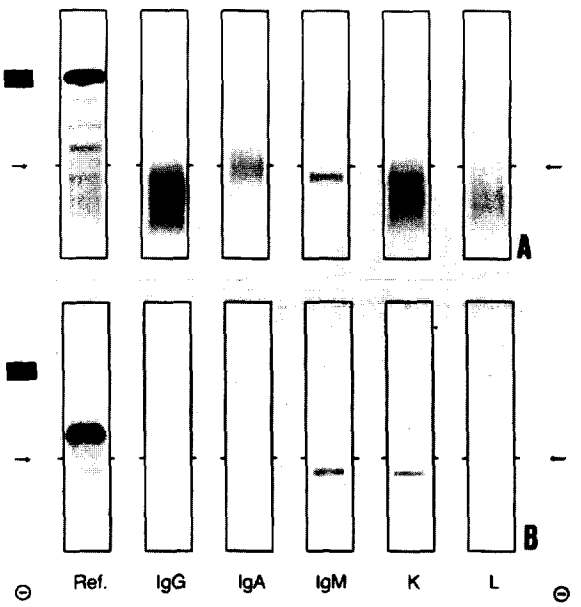

Fig. 14. Immunofixation electrophoresis of the serum (A) and of the CAs isolated by warm absorption on and cold elution from group O adult red blood cells (B). A monoclonal IgM-κ is clearly present in both serum and "purified" CAs. The patient presented malignant B cell lymphoma.

type III cryoglobulins are found in patients with a wide variety of diseases, including infections, autoimmune disorders, lymphoproliferative diseases, and chronic liver diseases. Type II and type III cryoglobulins are called "essential" when they are observed in patients with no apparent primary disease. However, recent data strongly suggest that most of the "essential" cryoglobulins are associated with hepatitis C virus infection [127,167–175]. 2D-PAGE analysis of cryoprecipitates as well as immunoblotting analysis revealed that a number of cryoglobulins present patterns that are intermediate between those of type II and III cryoglobulins [127,128,173]. In addition, the 2D technique provides precise information on the isotypes, as well as on the clonality of Igs, that precipitate at low temperature. In a series of 41 cryoprecipitates that were analyzed in our laboratory, we found 29 samples presenting 2D patterns allowing their identification according to the classification of cryoglobulins proposed by Brouet et al. [166]. We detected twelve cryoprecipitates that showed 2D patterns that differed from those expected [128]. Two samples were mixture of oligoclonal IgM with trace amounts of Igs of different isotype (tentatively named type II–$III_{variant}$ cryoglobulins), whereas ten samples were comprised of a mixture of polyclonal and monoclonal IgM associated with polyclonal IgG (type II–III cryoglobulins). Upon IFE analysis, only the most prominent IgM clone was detected, or inversely, the monoclonal IgM component was hidden by polyclonal IgM, explaining why they appeared as type II or as type III cryoglobulins by this technique. Fig. 15 shows the respective 2D μ chain patterns of type II, type II–III and type III cryoglobulins.

3.3.3. Light chain amyloidosis (AL)

Amyloidosis is a group of diseases, characterized by deposition of proteins in tissues, that are stained with Congo red [46,47,71]. They are classified according to the chemical composition of the precursor proteins. Amyloidosis directly related to Ig L chain deposition is called AL. AL represents the most common form of systemic amyloid deposition disease. It complicates the course of 10–20% of patients with clinical myeloma, but can also be observed without evidence of clinical lymphoproliferative syndrome [47,71,176]. In a large series of patients presenting MG, primary AL was found in 9% of the cases [40]. Approximately 8 to 15% of the patients, whose serum and urine are examined in laboratories experienced in defining MG, did not have a detectable monoclonal L chain in either fluids [47,176]. Our experience in 2D-PAGE analysis of plasma/serum, as well as urine from patients with AL, is summarized in Table 2. Monoclonal L chains were observed on plasma/serum, as well as on urine protein maps. Interestingly, relatively acidic monoclonal L chains (p*I* 5.0–5.3) were found in three cases, as exemplified for one case in Fig. 16A. In patients with AL and nephrotic syndrome, 2D-PAGE analysis also permits identification of the monoclonal L chain as well as of other proteins that are lost by the damaged kidneys (Fig. 16B).

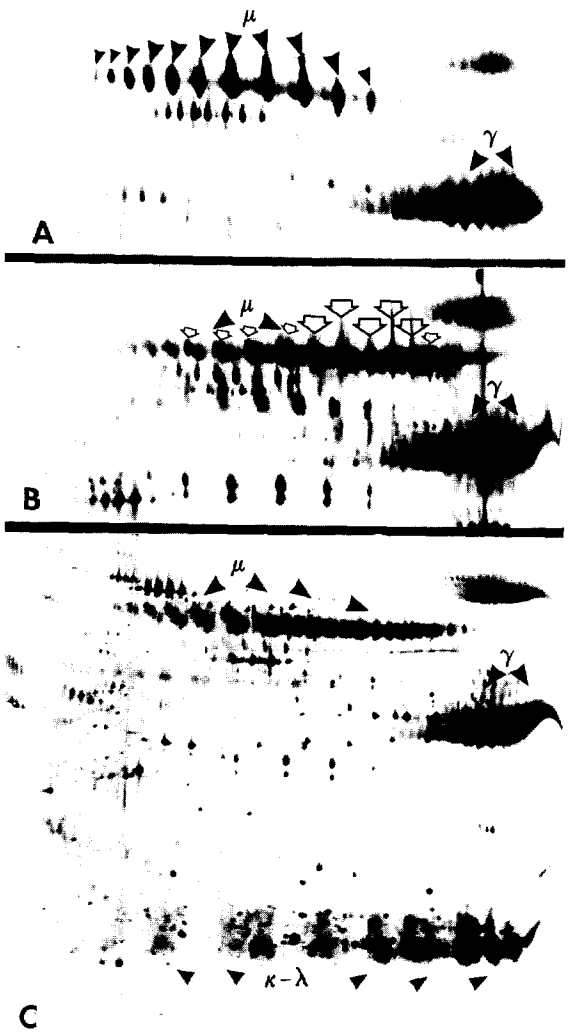

Fig. 15. Two-dimensional electrophoretic patterns of mixed cryoglobulins. Details of the μ chain areas of the protein maps of (A) type II cryoglobulins; monoclonal μ chain pattern; (B) type II–III cryoglobulins; monoclonal μ chain pattern superimposed on polyclonal μ chain pattern; (C) the protein map of type III cryoglobulins; presence of polyclonal μ chains. $\mu = \mu$ Chains; γ = polyclonal γ chains; κ-λ = polyclonal Ig light chains. Open arrows (panel B) highlight prominent clonal μ chain spots. First dimension: IPG.

4. Conclusions and perspectives

What are the implications of 2D-PAGE analysis concerning the understanding of the immune humoral system? With the current 2D-PAGE technologies, individual Ig L chains can be detected when the concentration of the monoclonal Ig is above 25 mg/l [117]. Even if the technique does not permit definitive conclusions regarding clonality on a single case basis because of L chain microheterogeneity, 2D-PAGE analysis is useful to appreciate the apparent clonal diversity of Igs in many different clinical settings. In patients with hypergammaglobulinemia, many small and well-defined L chain spots can be observed, suggesting that a limited number of Ig-secreting B cell clones are preferentially expanded (schematized in Fig. 17C). On the other hand, in some patients, homogeneous representation of L chains spots are observed, suggesting a more polyclonal B cell stimulation (schematized in Fig. 17D). These observations suggest that different mechanisms are implicated in the production of hypergammaglobulinemia in humans.

Little pertinent information is available on the patho-physiological importance of the microheterogeneity of monoclonal L chains observed at 2D-PAGE analysis [94,113,114,123,125]. Biochemical properties of monoclonal L chains may be important in the development of renal insufficiency that is frequently observed in patients with multiple myeloma. Renal failure has been reported in approximately 20–50% of patients with multiple myeloma, resulting primarily from the excretion of free L chains [52,177–184]. Several studies have suggested that the p*I* of the monoclonal L chain is a particularly important determinant of its nephrotoxicity, and that basic L chains are more prone to tubular precipitation than acidic L chains [185–187]. Other investigators have found no such relation [188–192]. Yet, the specific molecular features responsible for the renal deposition of certain Bence Jones proteins (L chains) are unknown [183,184].

Approximately 15 to 20% of monoclonal L chains appear to be amylogenic in that they have the property of precipitating as fibrillar material resembling amyloid [191]. Nevertheless, no specific amino acid sequence common to all amyloidogenic L chains has yet been identified [45–48,49,175,191]. However, a non-random ex-

Table 2
Clinical findings in five patients with immunoglobulin light chain amyloidosis

	Patient (age, sex)				
	52, m	58, m	41, f	61, f	67, m
Cardiac manifestations	+	+	–	+	+
Nephrotic syndrome	+	–	–	+	+
Peripheral neuropathy	+	–	–	–	–
Bone marrow plasmocytosis	<10%	<10%	30%	15%	<10%
Monoclonal light chain (serum/urine)	+ / +	+ / –	+ / –	+ [a] / +	+ [a] / +
Light chain type	λ	λ	κ	λ	λ
p*I* of the light chain spot(s)	5.0	5.1	7.5	5.9 and 6.3 [b]	5.3

[a] Monoclonal L chain only detected at 2D-PAGE analysis.
[b] L Chain appearing as two spots.

pression of the V_λII subgroup of L chains has been recently reported in AL, implying a relationship between V_λ gene usage and certain types of lymphocytic dyscrasias [192,193]. Interestingly, Bellotti et al. [194] showed that determination of the L chain class, M_r and p*I* allowed prediction of the amyloidogenicity in about 81% of the cases. The propensity of L chain nephrotoxicity or amyloidogenicity was shown to be related to the formation of high-molecular-mass aggregates under physiological conditions [195]. It is possible that some particular nephrotoxic or amyloidogenic monoclonal L chains could be identified at 2D-PAGE analysis depending on their apparent M_r, p*I* and microheterogeneous patterns.

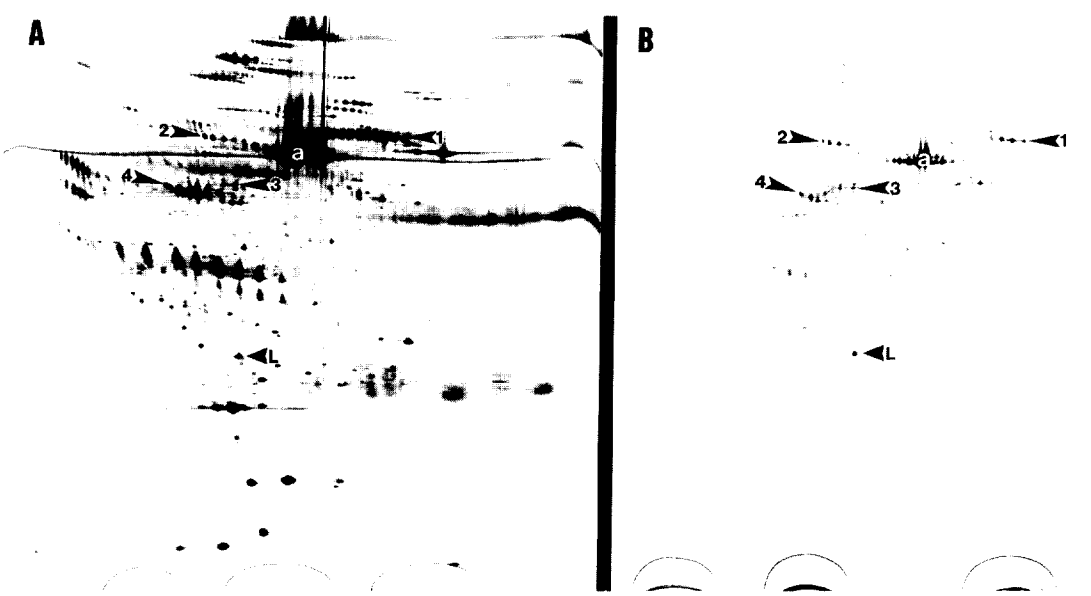

Fig. 16. Serum (A) and urine (B) protein maps from a patient with amyloidosis and nephrotic syndrome. The same amount (0.3 μl of serum and urine were loaded). 1 = Transferrin; 2 = α1-B-glycoprotein; 3 = antithrombin III; 4 = α1-antitrypsin; a = albumin; L = monoclonal light chain. The monoclonal L chain was not detected at serum IFE analysis, but was found in a 40 × concentrated urine sample. First dimension: CAG.

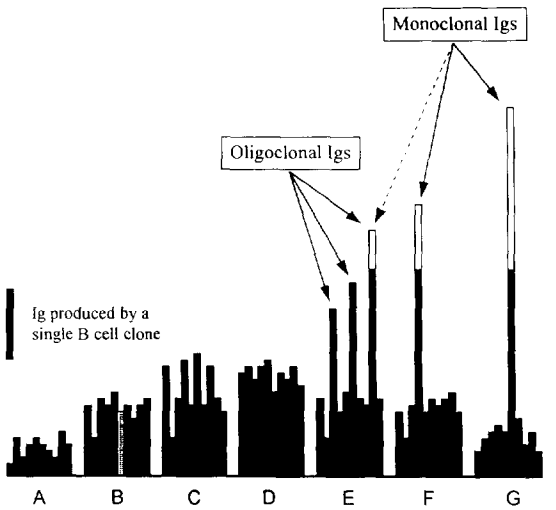

Fig. 17. Schematic representation of the clonal diversity of immunoglobulins. A = Polyclonal hypogammaglobulinemia; B = "normal" polylconal Igs; C = heterogeneous hypergammaglobulinemia; D = homogeneous polyclonal hypergammaglobulinemia; E = oligoclonal gammopathy; F = monoclonal gammopathy without suppression of the polyclonal background; G = monoclonal gammopathy with suppression of the polyclonal background (myeloma pattern). Bars represents the Ig production by a single B cell clone. The open part of the bars (histograms E–G) arbitrarily highlight clonal Igs that are in sufficient amount to be detected using standard methods. The gray bar (histogram B) illustrates why some "low" concentration monoclonal Igs such as cold agglutinins or the monoclonal component of type II cryoglobulins may be not detected in serum samples. Depending on the sensitivity of the methods, pattern E can be either diagnosed as a monoclonal gammopathy or as an oligoclonal gammopathy.

2D-PAGE analysis of CAs and cryoglobulins is an other fascinating area of research. About 5% of monoclonal IgM have been identified as CAs, and between 7 and 10% of monoclonal IgM are cryoglobulins precipitating on cooling and redissolving upon rewarming. In some patients, monoclonal IgMs are directed against determinants shared by red blood cells and γ-globulins, usually carbohydrate determinants present on the surface of erythrocytes and the constant region of Igs [66,67]. Such monoclonal IgMs are both cryoglobulins and CAs. CAs define autoantibodies characterized by their ability to interact with red blood cell antigens at low temperature, but they are not always monoclonal Igs. Although they can be detected in normal individuals and that their titers increase during viral and mycoplasmal infections, most of the clinical symptoms appear in the context of MG secondary to a wide array of B cell dyscrasias, from benign proliferation to malignant, rapidly expanding lymphomas [66,196]. It has been known that most anti-I CAs preferentially use κ chains, and more recent analyses narrowed this restriction to V_κIII families [197–199]. Regardless of whether the autoantibody recognizes the i or the I red cell antigen, restriction at the V_H4–21 gene segment is absolute for pathogenic monoclonal CAs [200]. By contrast, naturally occurring polyclonal anti-i/I CAs can be encoded to a large extend by different V_H3 genes [201]. These results suggest that naturally occurring, polyclonal CAs may derive from B cells that are distinct from the V_H4–21 expressing cells, which can rise to B cell neoplasms. Whatever the mechanisms involved in the production of CAs, it is of clinical relevance to have tools such as 2D-PAGE that allow assessment of their clonality.

The finding of type II–III cryoglobulins at 2D-PAGE analysis in some patients with virus C hepatitis may be important in the understanding of the sequence of events leading to the development of "essential" type II cryoglobulinemia. It is likely that the persistence of hepatitis virus C in the host leads to continuous B cell stimulation and hyperproduction of polyclonal Igs with the appearance of type III cryoglobulinemia in some patients. Possibly directly or indirectly related to infection of blood mononuclear cells by hepatitis C virus [202,203], transformation of polyclonal to oligoclonal and finally to monoclonal IgM may occur, progressively leading to type II–II and finally to type II cryoglobulinemia (Fig. 18). Further studies are need to confirm this hypothesis.

2D-PAGE analysis could also have some role to identify useful features to distinguish patients with MGUS who do not progress from those in whom a malignant transformation will develop. It must be pointed out that neither the level of monoclonal Ig nor the percentage of bone marrow plasma cells had predictive value for malig-

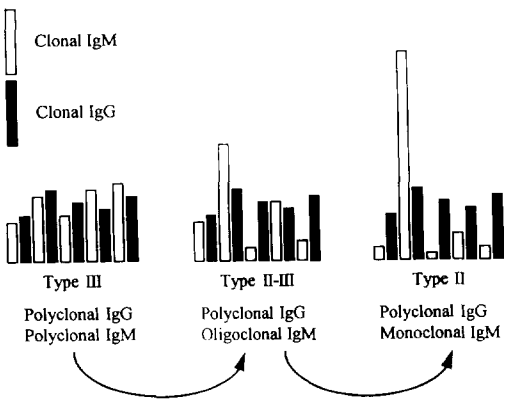

Fig. 18. Hypothetical representation of the evolution of type III to type II–III and finally to type II cryoglobulins. Bars represent the Ig production by a single B cell clone. In type II–III cryoglobulins, the most prominent clonal IgM is detected among polyclonal IgM.

nant transformation [63]. The definition of a MG is generally too imprecise. Radl [36,204] has recently proposed a new classification of MG based on clinical and experimental observations. However, in our opinion, the term MG should be reserved to describe monoclonal Igs that are overproduced by a single B cell clone (Fig. 17F, G), whereas oligoclonal gammopathy (OG) should apply to multiple overproduced Igs (Fig. 17E). Evidently the distinction can be made possible only by using sensitive techniques. In patients with multiple myeloma, monoclonal Igs are easily detected with standard techniques such as HRE or IFE (Fig. 17). On the other hand, in patients with OG, only the most prominent clonal Ig(s) (the tip of the iceberg) is (are) detected by these techniques. Therefore, MG (or more particularly MGUS) may be diagnosed in these patients albeit they present OG [or oligoclonal gammopathy of undetermined significance (OGUS)]. In a series of 36 sera containing a single monoclonal IgG at IFE analysis, Withold and Rick [41] found eight samples that contained additional monoclonal Igs by immunoblotting. Our experience suggests that OG and MG are found in patients presenting readily different clinical conditions. Furthermore, as previously proposed by Radl et al. [98], OG should be considered as forming a separate category of "immunoglobinopathies". MGs are most frequently observed in patients with a single B cell clone dyscrasia, whereas OG may be detected in patients without overt B cell malignancies (Table 3). The sequence of events directly involved in the transformation of a normal B cell into a malignant plasma cell is still unknown. Cytokines, chromosome translocations, genetic factors as well as abnormalities of oncogenes such as *c-myc* and *N-ras* probably play major roles (reviewed in Refs. [13,204]). Separate mechanisms probably contribute to the development of oligoclonal Igs. Oligoclonality may reflect T–B cell imbalance, with restricted B cell help through heterogeneous T cell repertoires (e.g., the Wiskott–Aldrich syndrome [111], acquired immunodeficiency syndrome [205–208], bone marrow transplantation [117,209,210], organ transplantation [211,212], developing infants [124,213] or solid tumors [125]). Oligoclonality may be also related to selective antigenic pressures (e.g. acute or congenital infections [125,214]) or may be the reflect of a limited repertoire of B cell clones (e.g. developing infants, bone marrow transplantation [209]). Finally, oligoclonality may be due to excessive B cell proliferation, directly or indirectly related to outgrowth of Epstein–Barr virus-infected B cell clones [215–218]. It is likely that several mechanisms may operate simultaneously.

A possible link between MG and OG is represented by the "double gammopathies" or biclonal gammopathies [219–221]. Riddell at al. [221] reported an incidence of 2.5% of double gammopathies (at IFE analysis) in a series of 1135 patients. Eleven patients presented myeloma, six lymphoproliferative malignancy, five non-lymphoproliferative malignancy and six a double gammopathy of undetermined significance. Similarly, Kyle et al. [219] found that 37 of 57 (65%) patients with biclonal gammopathy had no features of myeloma or lymphoproliferative disease. It has been postulated that in some patients the monoclonal Igs arise from two separate B cell clones while in others the Igs behave in a concordant manner that is consistent with class switching in a single plasma cell clone. Multiple independent Ig class-switch recombina-

Table 3
Conditions associated with "immunoglobinopathies"

Monoclonal gammopathies	Oligoclonal gammopathies
Multiple myeloma	Normal ontogenesis (developing infants)
Isolated plasmocytoma	Primary immunodeficiency (Wiskott–Aldrich syndrome)
Waldenström's macroglobulinemia	Secondary immunodeficiency with T–B cell imbalance
B Cell lymphoma	· Reconstitution of the immune system (bone marrow transplantation, immunosupression, organ transplantation)
Chronic lymphatic leukemia	· Immunosuppresion
Amyloidosis	· T Cell dyscrasia
Localized plasmocytoma	· Sjögren's syndrome
Biclonal gammopathies[a]	· Acquired immunodeficiency syndrome
	· Aging
	Infections (cytomegalovirus, toxoplasmosis, hepatitis)
	Solid tumors
	Biclonal gammopathies[b]
MGUS/OGUS[c]	
With a single monoclonal component	With multiple clonal components

[a] Biclonal gammopathies derived from a single B cell clone.
[b] Biclonal gammopathies derived from different B cell clones.
[c] MGUS = Monoclonal gammopathy of undetermined significance; OGUS = oligoclonal gammopathy of undetermined significance.

tions occurring within the same clone have been described in 35% of myeloma, suggesting that tumoral expansion may occur either in pre-switch B cells or in more differentiated plasma cells [222]. Recently, Bakkus et al. [223] showed that the clonogenic cell in multiple myeloma originates from a pre-switched but somatically mutated B cell. Different Igs produced by a single B cell clone or by distinct cell populations have been well documented [224–237].

Finally, how should one designate monoclonal CAs or the monoclonal component of type II cryoglobulins that are found in patients without clinical evidence of lymphoproliferative disease and without detectable serum monoclonal Ig at both IFE and 2D-PAGE analysis? In such cases, monoclonal Igs are detected because of their "perverse" biological activities, not because they are overproduced (Fig. 17B). It is possible that such clonal Igs may have similar concentrations that have Igs produced by a single "normal" B cell clone. This notion is important, because the absence of a MG at IFE or at 2D-PAGE analysis does not exclude the presence of low concentration MG with biological activity.

In conclusion, though 2D-PAGE is a powerful technique to study the clonal diversity of Igs as well as many different "immunoglobinopathies", its clinical relevance has yet not been prospectively evaluated. Nevertheless, the technique certainly has a place in laboratories experienced in defining MG, both for diagnostic and for research purposes.

Acknowledgements

The authors thank Mrs. L. Marinkovic and N. Dufour for their excellent technical assistance.

References

[1] C. Brack, M. Hirma, R. Lenhard-Schuller and S. Tonegawa, *Cell*, 15 (1978) 1.
[2] S. Tonegawa, *Nature*, 308 (1983) 575.
[3] A. Rolink and F. Melchers, *Cell*, 66 (1991) 1081.
[4] G. Winter and C. Milstein, *Nature*, 349 (1991) 293.
[5] F.W. Alt, E.M. Oltz, F. Young, J. Gorman, G. Taccioli and J. Chen, *Immunol. Today*, 13 (1992) 306.
[6] J. Banchereau and F. Rousset, *Adv. Immunol.*, 52 (1992) 125.
[7] R. Lerner, A. Kang, J. Bain, D. Burton and C. Barbas, *Science*, 258 (1992) 1313.
[8] H.D. Ochs, S. Nonoyama, M.L. Farrington, S.H. Fischer and A. Aruffo, *Semin. Hematol.*, 30 (1993) 72.
[9] V. Pascual and J.D. Capra, *Adv. Immunol.*, 49 (1991) 1.
[10] E.A. Hendrickson, *Am. J. Pathol.*, 143 (1993) 1511.
[11] G. Betz, C. Milstein, A. González-Fernández, R. Pannell, T. Larson and M.S. Neuberger, *Cell*, 77 (1994) 239.
[12] N. Motoyama, T. Miwa, Y. Suzuki, H. Okada and T. Azuma, *J. Exp. Med.*, 179 (1994) 395.
[13] P.D. Burrows, J.F. Kearney, H.W. Schroeder, Jr. and M.D. Cooper, *Baillière's Clin. Haematol.*, 6 (1993) 785.
[14] S. Desiderio, *Nature*, 361 (1993) 202.
[15] S. Jung, K. Rajewsky and A. Radbruch, *Science*, 259 (1993) 984.
[16] F. Brière, C. Servet-Delprat, J.M. Bridon, J.M. Saint-Remy and J. Banchereau, *J. Exp. Med.*, 179 (1994) 757.
[17] F. Melchers, H. Karasuyama, D. Haasner, S. Bauer, A. Kudo, N. Sakaguchi, B. Jameson and A. Rolink, *Immunol. Today*, 14 (1992) 60.
[18] R.C. Allen, R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, C.M. Disteche, D.K. Simoneaux, W.C. Fanslow, J. Belmont and M.K. Spriggs, *Science*, 259 (1993) 990.
[19] J.P. DiSanto, J.Y. Bonnefoy, J.F. Gauchat, A. Fischer and D. de Saint Basile, *Nature*, 361 (1993) 541.
[20] U. Korthäuer, D. Graf, H.W. Mages, F. Brière, M. Padayachee, S. Malcolm, A.G. Ugazio, L.D. Notarangelo, R.J. Levinsky, R.A. Kroczek, *Nature*, 361 (1993) 539.
[21] D. Vetrie, I. Vorechovský, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarström, C. Kinnon, R. Levinsky, M. Borbroe, C.I.E. Smith and D.R. Bentley, *Nature*, 361 (1993) 226.
[22] E.A. Clark and J.A. Ledbetter, *Nature*, 376 (1994) 425.
[23] S. Desiderio, *Curr. Opin. Immunol.*, 6 (1994) 248.
[24] J. Hagman and R. Grosschedl, *Curr. Opin. Immunol.*, 6 (1994) 222.
[25] L. Law and E.A. Clark, *Curr. Opin. Immunol.*, 6 (1994) 238.
[26] F. Melchers, D. Haasner, U. Grawunder, C. Kalberer, H. Karasuyama, T. Winkler and A.G. Rolink, *Annu. Rev. Immunol.*, 12 (1994) 209.
[27] R.S. Schwartz and B.D. Stollar, *Immunol. Today*, 15 (1994) 27.
[28] A. Turner, Jr., D.H. Mack and M.M. Davis, *Cell*, 77 (1994) 297.
[29] P.W. Kincade, G. Lee, C.E. Pietrangeli, S.I. Hayashi and J.M. Gimble, *Ann. Rev. Immunol.*, 7 (1989) 111.
[30] N. Rosenberg and P.W. Kincade, *Curr. Opin. Immunol.*, 6 (1994) 203.
[31] R.A. Kyle, *Clin. Haematol.*, 11 (1982) 123.
[32] R.A. Kyle and J.P. Garton, *Mayo Clin. Proc.*, 62 (1982) 719.
[33] R.A. Kyle, *J.A.M.A.*, 251 (1984) 1849.
[34] R.A. Kyle, *Baillière's Clin. Haematol.*, 1 (1987) 533.
[35] R.A. Kyle and J.A. Lust, *Semin. Hematol.*, 26 (1989) 176.
[36] J. Radl, *Immunol. Today*, 11 (1990) 234.
[37] R. Creyssel, A. Gibaud, J.F. Cordier and J.P. Boissel, *Biomedicine*, 22 (1975) 41.
[38] J.M. Fine and P. Lambin, *Biomedicine*, 23 (1975) 323.
[39] S. Makni, R. Zouari, M.R. Barbouch, K. Ayed, M. Moalla and L. Zakraoui, *Rev. Fr. Transfus. Hémobiol.*, 33 (1990) 31.
[40] R.A. Kyle, *Hematol. Oncol. Clin. North Am.*, 6 (1992) 347.
[41] W. Withold and W. Rick, *Eur. J. Clin. Chem. Clin. Biochem.*, 31 (1993) 17.
[42] E.C. Franklin, J. Lowenstein, B. Bigelow and M. Meltzer, *Am. J. Med.*, 37 (1964) 332.
[43] M. Seligmann, F. Danon, D. Hurez, E. Mihaesco and J.L. Preud'homme, *Science*, 162 (1968) 1396.
[44] R.A. Kyle, R.A. Robinson and J.A. Katzmann, *Am. J. Med.*, 71 (1981) 999.
[45] S.M. Rosen, J.N. Buxbaum and B. Frangione, *Semin. Oncol.*, 13 (1986) 260.
[46] A. Solomon, *Semin. Oncol.*, 13 (1986) 341.
[47] J.N. Buxbaum, J.V. Chuba, G.C. Hellman, A. Solomon and G.R. Gallo, *Ann. Intern. Med.*, 112 (1990) 455.
[48] J. Buxbaum, *Hematol. Oncol. Clin. North Am.*, 6 (1992) 323.
[49] M. Cogné, C. Silvain, A.A. Khamlichi and J.L. Preud'homme, *Blood*, 79 (1992) 2181.
[50] D.L. Wahner-Roedler and R.A. Kyle, *Am. J. Hematol.*, 40 (1992) 56.
[51] P. Aucouturier, A.A. Khamlichi, G. Touchard, E. Justrabo, M. Cogné, B. Chauffert, F. Martin and J.L. Preud'home, *N. Engl. J. Med.*, 329 (1993) 1389.
[52] A. Solomon and D.T. Weiss, *N. Engl. J. Med.*, 329 (1993) 1423.
[53] T. Somer, *Baillière's Clin. Haematol.*, 1 (1987) 695.
[54] U. Axelsson, R. Bachmann and J. Hällen, *Acta Med. Scand.*, 179 (1966) 235.

[55] R.A. Kyle, S. Finkelstein, L.R. Leveback and L.T. Kurland, *Blood*, 40 (1972) 719.
[56] J.M. Fine, P. Lambin and P. Lerous, *Vox. Sang.*, 23 (1976) 336.
[57] J.M. Fine, P. Lambin, C. Derycke, J.Y. Muller and M. Marneux, *Transfusion*, 19 (1979) 332.
[58] N.M. Papadopoulos, R.J. Elin and D.M. Wislon, *Clin. Chem.*, 28 (1982) 707.
[59] J.P. Saleun, M. Vicariot, P. Deroff and J.F. Morin, *J. Clin. Pathol.*, 35 (1982) 63.
[60] D. Sinclair, T. Sheehan, D.M.V. Parrott and D.I. Stott, *Br. J. Haematol.*, 64 (1986) 745.
[61] J. Crawford, M.K. Eye and H.J. Cohen, *Am. J. Med.*, 82 (1987) 39.
[62] J. Singh, A.W. Dudley, Jr. and K.A. Kulig, *J. Lab. Clin. Med.*, 116 (1990) 785.
[63] J. Blade, A. Lopez-Guillermo, C. Rozman, F. Cervantes, C. Salgado, J.L. Aguilar, J.L. Vives-Corrons and E. Montserrat, *Br. J. Heamtol.*, 81 (1992) 391.
[64] M. Bowden, J. Crawford, H.J. Cohen and O. Noyama, *J. Am. Geriar. Soc.*, 41 (1993) 11.
[65] R.A. Kyle, *Mayo Clin. Proc.*, 68 (1993) 26.
[66] D.B. Duggan and A. Schattner, *Am. J. Med.*, 81 (1986) 864.
[67] M. Farhangi and G. Merlini, *Semin. Oncol.*, 13 (1986) 366.
[68] G. Merlini, M. Farhangi and E.F. Osserman, *Semin. Oncol.*, 13 (1986) 350.
[69] D.F. Keren, *Hematol. Oncol. Clin. North Am.*, 7 (1993) 1153.
[70] G.D. Miralles, J.R. O'Fallon and N.J. Talley, *N. Engl. J. Med.*, 327 (1992) 1919.
[71] R.A. Kyle, *Clin. Haematol.*, 11 (1982) 151.
[72] D.F. Keren, *High-Resolution Electrophoresis and Immunofixation: Techniques and Interpretation*, Butterworths, Boston, 1987.
[73] P. Grabar and C.A. William, *Biochim. Biophys. Acta*, 10 (1953) 193.
[74] S.L. Shore, D.J. Phillips and C.B. Reimer, *Immunochemistry*, 8 (1971) 562.
[75] E. Afonso, *Clin. Chim. Acta.*, 10 (1964) 114.
[76] C.A. Alper and A.M. Johnson, *Vox. Sang.*, 17 (1969) 445.
[77] L.P. Cawley, B.J. Minard, W.W. Tourtelotte, B.I. Ma and C. Chelle, *Clin. Chem.*, 22 (1976) 1262.
[78] R.F. Ritchie and R. Smith, *Clin. Chem.*, 22 (1976) 497.
[79] R.F. Ritchie and R. Smith, *Clin. Chem.*, 22 (1976) 1982.
[80] N.S. Pedersen and H.N. Axelsen, *J. Immunol. Methods*, 30 (1979) 257.
[81] T. Sun, Y.Y. Lien and T. Degnan, *Am. J. Clin. Pathol.*, 72 (1979) 5.
[82] M.O. Marshall, *Clin. Chim. Acta*, 104 (1980) 1.
[83] A.M. Smith, R.A. Thompson and M. Haeney, *J. Clin. Pathol.*, 33 (1980) 500.
[84] G. Merlini, P. Piro, F. Pavesi, R. Epsi and F. Aguzzi, *Clin. Chem.*, 27 (1981) 1862.
[85] C.M. Reichert, D.F. Everett, Jr., P.I. Nadler and N.M. Papadopoulos, *Clin. Chem.*, 28 (1982) 2312.
[86] J.T. Whicher, *Clin. Chem.*, 29 (1983) 402.
[87] R.T. Roberts, *Clin. Lab. Med.*, 6 (1986) 601.
[88] S.K. Gerard, K.H. Chen and H. Khayam-Bashi, *Am. J. Clin. Pathol*, 88 (1987) 198.
[89] J. Duc, B. Morel, R. Peitrequin and P.C. Frei, *J. Clin. Lab. Immunol.*, 26 (1988) 141.
[90] S.N. Kahn and M. Bina, *Clin. Chem.*, 34 (1988) 1633.
[91] D.F. Keren, J.S. Warren and J.B. Lowe, *Clin. Chem.*, 34 (1988) 2196.
[92] J.E. Guinan, D.F. Kenny and P.A. Gatenby, *Pathology*, 21 (1989) 35.
[93] D.M. Monos, M. Bina and S.N. Kahn, *Clin. Biochem.*, 22 (1989) 369.
[94] H.H. Harrison, *Clin. Chem.*, 36 (1990) 1526.
[95] H.H. Harrison, K.L. Miller, A. Abu-Alfa and S.J. Podlasek, *Am. J. Clin. Pathol.*, 100 (1993) 550.
[96] D. Sinclair, D.S. Kumararatne, J.B. Forrester, A. Lamont and D.I. Stott, *J. Immunol. Methods*, 64 (1983) 147.
[97] D. Sinclair, D.S. Kumararatne and D.I. Stott, *J. Clin. Pathol.*, 37 (1984) 255.
[98] J. Radl, J. Wels and C.M. Hoogeveen, *Clin. Chem.*, 34 (1988) 1839.
[99] F. J Fasullo Jr, H.A. Fritsche, F.J. Liu and R.G. Hamilton, *Clin. Chem.*, 35 (1989) 364.
[100] F.J.M. Nooij, A.J. van der Sluijs-Gelling, C.M. Jolvan der Zijde, M.J.D. van Tol, H. Haas and J. Radl, *J. Immunol. Methods*, 134 (1990) 273.
[101] P.H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
[102] A.K. Thorsrud, H.F. Haugen and E. Jellum, in B.J. Radola (Editor), *Electrophoresis '79*, Walter de Gruyter, Berlin, 1980, p. 425.
[103] A.L. Latner, T. Marshall and M. Gambie, *Clin. Chim. Acta*, 103 (1980) 51.
[104] A.L. Latner, T. Marshall and M. Gambie, *Electrophoresis*, 1 (1980) 82.
[105] N.L. Anderson, *Immunol. Letters*, 2 (1981) 195.
[106] E. Jellum and A.K. Thorsrud, *Clin. Chem.*, 28 (1982) 876.
[107] R.P. Tracy, R.M. Currie, R.A. Kyle and D.S. Young, *Clin. Chem.*, 28 (1982) 900.
[108] P. Blangarin, P. Deviller, K. Kindbeiter and J.J. Madjar, *Clin. Chem.*, 30 (1984) 2021.
[109] R.P. Tracy, R.A. Kyle and D.S. Young, *Hum. Pathol.*, 15 (1984) 122.
[110] J.D. Tissot and P. Schneider, *Rev. Fr. Transfus. Hémobiol.*, 32 (1989) 345.
[111] J.D. Tissot, P. Schneider, B. Pelet, P.C. Frei and D.F. Hochstrasser, *Br. J. Haematol.*, 75 (1990) 436.
[112] J.D. Tissot, P. Schneider, R.W. James, R. Daigneault and D.F. Hochstrasser, *Appl. Theor. Electroph.*, 75 (1991) 7.

[113] M. Goldfarb, *Electrophoresis*, 12 (1992) 440.
[114] H.H. Harrison, *Clin. Biochem.*, 25 (1992) 235.
[115] D.F. Hochstrasser, O. Huber, A. Kaelin, A. Toussi, A. Pavlovic, M. Funk, R.D. Appel, C. Pasquali, F. Ravier, J.D. Tissot, A. Goodman and A.F. Muller, in M.J. Dunn (Editor), *2-D PAGE '91*, Zebra Printing, Perivale, 1992, p. 224.
[116] J.D. Tissot, F. Clément, J.A. Schifferli, P.C. Frei, D.F. Hochstrasser and P. Schneider, *Am. J. Hematol.*, 40 (1992) 171.
[117] J.D. Tissot, C. Helg, B. Chapuis, R.H. Zubler, M. Jeannet, D.F. Hochstrasser, P. Hohlfeld and P. Schneider, *Bone Marrow Transplant.*, 10 (1992) 347.
[118] J.D. Tissot, P. Schneider, P. Hohlfeld, J.F. Tolsa, A. Calame and D.F. Hochstrasser, *Appl. Theor. Electroph.*, 3 (1992) 67.
[119] M. Golfarb, *Electrophoresis*, 14 (1993) 1379.
[120] D.F. Hochstrasser and J.D. Tissot, *Adv. Electrophoresis*, 6 (1993) 268.
[121] J. Stulík, M. Tichy and H. Kovárová, *Clin. Chim. Acta.*, 218 (1993) 149.
[122] J.D. Tissot and D.F. Hochstrasser, *Clin. Immunol. Newsletter*, 13 (1993) 97.
[123] J.D. Tissot, D.F. Hochstrasser, F. Spertini, J.A. Schifferli and P. Schneider, *Electrophoresis*, 14 (1993) 227.
[124] J.D. Tissot, P. Hohlfeld, D.F. Hochstrasser, J.F. Tolsa, A. Calame and P. Schneider, *Electrophoresis*, 14 (1993) 245.
[125] J.D. Tissot, P. Schneider, P. Hohlfeld, F. Spertini, D.F. Hochstrasser and M.A. Duchosal, *Electrophoresis*, 14 (1993) 1366.
[126] A. Zahnd, J.D. Tissot and D.F. Hochstrasser, *Appl. Theor. Electroph.*, 3 (1993) 321.
[127] J.A. Schifferli, L.A. French and J.D. Tissot, *Adv. Nephrol.*, in press.
[128] J.D. Tissot, J.A. Schifferli, D.F. Hochstrasser, C. Pasquali, F. Spertini, F. Clément, S. Frutiger, N. Paquet, G.J. Hughes and P. Schneider, *J. Immunol. Methods*, 173 (1994) 63.
[129] D.F. Hochstrasser, M.G. Harrington, A.C. Hochstrasser, M.J. Miller and C.R. Merril, *Anal. Biochem.*, 173 (1988) 424.
[130] D.F. Hochstrasser and C.R. Merril, *Appl. Theor. Electroph.*, 1 (1988) 35.
[131] D.F. Hochstrasser, A. Patchornik and C.R. Merril, *Anal. Biochem.*, 173 (1988) 412.
[132] A. Görg, W. Postel and S. Günther, *Electrophoresis*, 9 (1988) 531.
[133] B. Bjellqvist, C. Pasquali, F. Ravier, J.C. Sanchez and D.F. Hochstrasser, *Electrophoresis*, 14 (1993) 1357.
[134] B.R. Oakley, D.R. Kirsch and N.R. Morris, *Anal. Biochem.*, 105 (1980) 361.
[135] T. Rabilloud, *Electrophoresis*, 13 (1992) 429.
[136] G.J. Hughes, S. Frutiger, N. Paquet, F. Ravier, C. Pasquali, J.C. Sanchez, R. James, J.D. Tissot, B. Bjellqvist and D.F. Hochstrasser, *Electrophoresis*, 13 (1992) 707.
[137] O. Golaz, G.J. Hughes, S. Frutiger, N. Paquet, A. Bairoch, C. Pasquali, J.C. Sanchez, J.D. Tissot, R.D. Appel, C. Walzer, L. Balant and D.F. Hochstrasser, *Electrophoresis*, 14 (1993) 1223.
[138] N.L. Anderson and N.G. Anderson, *Proc. Natl. Acad. Sci. USA*, 74 (1977) 5421.
[139] P.M. Clark, L.J. Kricka, A.R. Gomo, T.P. Whitehead, D.G. Bullock and H. Sadi, *Clin. Chim. Acta*, 103 (1980) 219
[140] D.L. Emerson, C.C. Chapuis and P. Arnaud, *Electrophoresis*, 1 (1980) 159.
[141] K. Felgenhauer and D. Hagerdorn, *Clin. Chim. Acta.*, 100 (1980) 121.
[142] D. Goldmann, C.R. Merril and M.H. Ebert, *Clin. Chem.*, 26 (1980) 1317.
[143] N.L. Anderson, S.L. Nance, T.W. Pearson and N.G. Anderson, *Electrophoresis*, 3 (1982) 135.
[144] G.B. Germer, L.M. Silverman and J.F. Chapman, *Clin. Chem.*, 28 (1982) 759.
[145] R.P. Tracy, R.M. Currie and D.S. Young, *Clin. Chem.*, 28 (1982) 890.
[146] M.I. Jones and S.P. Spragg, *Electrophoresis*, 4 (1983) 291.
[147] B.B. Rosenblum, J.V. Neel and S.M. Hanash, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 5002.
[148] N.L. Anderson and N.G. Anderson, *Clin. Chem.*, 30 (1984) 1898.
[149] N.L. Anderson, R.P. Tracy and N.G. Anderson, in F.W. Putnam (Editor), *The Plasma Proteins —Structure, Function, and Genetic Control*, Vol. IV, Academic Press, Orlando, FL, 1984, p. 221.
[150] J.A. Daufeldt and H.H. Harrison, *Clin. Chem.*, 30 (1984) 1972.
[151] R.P. Tracy and D.S. Young, in J.E. Celis and R. Bravo (Editors), *Two-Dimensional Gel Electrophoresis of Proteins —Methods and Applications*, Academic Press, Orlando, FL, 1984, p. 193.
[152] D. Goldman, L.R. Godlin, P. Rathnagiri, S.J. O' Brien, J.A. Egeland and C.R. Merril, *Am. J. Hum. Genet.*, 37 (1985) 898.
[153] D. Goldman and C.R. Merril, *J. Psychiatr. Res.*, 21 (1987) 597.
[154] N.L. Anderson and N.G. Anderson, *Electrophoresis*, 12 (1991) 883.
[155] H.H. Harrison, K. Miller, C. Ober, S. Refetoff, M. Dick and S. Elias, *Am. J. Hum. Genet.*, 48 (1991) 362.
[156] H.H. Harrison, K.L. Miller, C. Dickinson and J.A. Daufeldt, *Am. J. Clin. Pathol*, 97 (1992) 97.
[157] H.H. Harrsion, K.L. Miller, M.D. Harbison and A.E. Slonim, *Clin. Chem.*, 38 (1992) 1390.
[158] J.D. Tissot, P. Hohlfeld, F. Forestier, J.F. Tolsa, A. Calame, E. Plouvier, H. Bossart and P. Schneider, *Appl. Theor. Electroph.*, 3 (1993) 183.
[159] J.D. Tissot, D.F. Hochstrasser, B. Schneider, J.J. Morgenthaler and P. Schneider, *Br. J. Haematol.*, 86 (1994) 143.
[160] Z.L. Awdeh, A.R. Williamson and B.A. Askonsas, *Nature*, 70 (1968) 219.

[161] Z.L. Awdeh, A.R. Williamson and B.A. Askonsas, *Biochem. J.*, 70 (1970) 241.
[162] M.T. Cwynarski, J. Watkins and P.M. Johnson, in J.P. Arbuthnott and J.A. Beeley (Editors), *Isoelectric Focusing*, Butterworth, 1975, p. 319.
[163] W. Pruzanski and K.H. Shumack, *N. Engl. J. Med.*, 297 (1977) 538.
[164] W. Pruzanski and K.H. Shumack, *N. Engl. J. Med.*, 297 (1977) 583.
[165] D. Crisp and W. Pruzanski, *Am. J. Med.*, 72 (1982) 915.
[166] J.C. Brouet, J.P. Clauvel, F. Danon, M. Klein and M. Seligmann, *Am. J. Med.*, 57 (1974) 775.
[167] M. Pasqual, L. Perrin, E. Giostra and J.A. Schifferli, *J. Infect. Dis.*, 162 (1990) 569.
[168] V. Agnello, R.T. Chung and L.M. Kaplan, *N. Engl. J. Med.*, 327 (1992) 1490.
[169] A. Pechère-Bertschi, L. Perrin, P. De Saussure, J.J. Widmann, E. Giostra and J.A. Schifferli, *Clin. Exp. Immunol.*, 89 (1992) 419.
[170] G. Abel, Q. -X. Zhang and V. Agnello, *Arthritis Rheum.*, 36 (1993) 1341.
[171] R. Misiani, P. Bellavita, D. Fenili, D. Marchesi, M. Massazza, G. Vendramin, B. Comotti, E. Tanzi, G. Scudeller and A. Zanetti, *Ann. Intern. Med.*, 117 (1993) 573.
[172] J.M. Levey, B. Bjornsson, B. Banner, M. Khuns, R. Malhotra, N. Whitman, P.L. Romain, T.G. Cropley and H.L. Bonkovsky, *Medicine*, 73 (1994) 53.
[173] L. Musset, M.C. Diemert, F. Taibi, L. Thi Huong Du, P. Cacoub, J.M. Leger, G. Boissy, O. Gaillard and J. Galli, *Clin. Chem.*, 38 (1992) 798.
[174] Cacoub, F. Lunel Fabiani, L. Musset, M. Perrin, L. Frangeul, J.M. Leger, J.-M. Huraux, J.-C. Piette and P. Godeau, *Am. J. Med.*, 96 (1994) 124.
[175] Lunel, L. Musset, P. Cacoub, L. Frangeul, P. Cresta, M. Perrin, P. Grippon, C. Hoang, J.C. Piette, J.-M. Huraux and P. Opolon, *Gastroenterology*, 106 (1994) 1291.
[176] M. Gallo, M. Picken, J. Buxbaum and B. Frangione, *Semin. Hematol.*, 26 (1989) 234.
[177] M.A. Gertz, P.R. Greipp and R.A. Kyle, *J. Lab. Clin. Med.*, 118 (1991) 33.
[178] R.A. DeFronzo, C.R. Cook, J.R. Wright and R.L. Humphrey, *Medicine*, 57 (1978) 151.
[179] MRC Working Party on Leukaemia in Adults, *Br. Med. J.*, 288 (1984) 1411.
[180] L.S.T. Fang, *Kidney Int.*, 27 (1985) 582.
[181] M. Cavo, M. Baccarani, P. Galieni, M. Gobbi and S. Tura, *Nouv. Rev. Fr. Hematol.*, 28 (1986) 147.
[182] S. Rota, B. Mougenot, B. Baudouin, M. de Meyer-Brasseur, V. Lemaitre, C. Michel, F. Mignon, E. Rondeau, P. Vanhille, P. Verroust and P. Ronco, *Medicine*, 66 (1987) 126.
[183] A. Solomon, D.T. Weiss and A.A. Kattine, *N. Engl. J. Med.*, 324 (1991) 1845.
[184] A. Kyle, *Annu. Rev. Med.*, 45 (1994) 71.
[185] D.H. Clyne, K.S. Kant, A.J. Pesce and Pollak, *Curr. Probl. Clin. Biochem.*, 1 (1979) 299.
[186] D.H. Clyne, A.J. Pesce and R.E. Thompson, *Kidney Int.*, 16 (1979) 345.
[187] R.A. Coward, I.W. Delamore, N.P. Mallick and E.L. Robinson, *Clin. Sci.*, 66 (1984) 229.
[188] C. Melcion, B. Mougenot, B. Baudoin, P. Ronco, L. Moulonguet-Doleris, P.H. Vanhille, M. Beaufils, L. Morel-Maroger, P. Verroust and G. Richet, *Clin. Nephrol.*, 22 (1984) 138.
[189] E.A. Johns, R. Turner, E.H. Cooper and I.C.M. MacLennan, *J. Clin. Pathol.*, 39 (1986) 833.
[190] A.G.W. Norden, F.V. Flynn, L.M. Filcher and J.D.M. Richards, *J. Clin. Pathol.*, 42 (1989) 52.
[191] M.J. Stone, *Blood*, 75 (1990) 531.
[192] M. Abe, S. Ozaki, D. Wolfenberger, M. DeBram-Hart, D.T. Weiss and A. Solomon, *J. Clin. Lab. Anal.*, 8 (1994) 4.
[193] Ozaki, M. Abe, D. Wolfenbarger, D.T. Weiss and A. Solomon, *Clin. Immunol. Immunopathol.*, 71 (1994) 183.
[194] V. Bellotti, G. Merlini, E. Bucciarelli, V. Perfetti, S. Quaglini and E. Ascari, *Br. J. Haematol.*, 74 (1990) 65.
[195] A. Myatt, F.A. Westholm, D.T. Weiss, A. Solomon, M. Schiffer and F.J. Stevens, *Proc. Natl. Acad. Sci. U.S.A.*, 91 (1994) 3034.
[196] D. Roelcke, *Transfusion. Med. Rev.*, 2 (1989) 140.
[197] G.J. Silverman and D.A. Carson, *Eur. J. Immunol.*, 20 (1990) 351.
[198] L.E. Silberstein, E.C. Jefferies, J. Goldman, D. Friedman, J.S. Moore, P.C. Nowell, D, Roelcke, W. Pruzanski, J. Roudier and G.J. Silverman, *Blood*, 78 (1991) 2372.
[199] L.C. Jefferies, G.J. Silverman, C.M. Carchidi and L.E. Silberstein, *Clin. Immunol. Immunopathol.*, 65 (1992) 119.
[200] V. Pascual, K. Victor, M. Spellerberger, T.J. Hamblin, F.K. Stevenson and J.D. Capra *J. Immunol.*, 149 (1992) 2337.
[201] L.C. Jefferies, C.M. Carchidi and L.E. Silberstein, *J. Clin. Invest.*, 92 (1993) 2821.
[202] C. Ferri, M. Monti, L. La Civita, G. Longombardo, F. Greco, G. Pasero, P. Gentilini, S. Bombardieri and A.L. Zignego, *Blood*, 82 (1993) 3701.
[203] J. Moldvay, P. Deny, S. Pol, C. Brechot and E. Lamas, *Blood*, 83 (1994) 269.
[204] J. Radl, *Neth. J. Med.*, 28 (1985) 134.
[205] J. Epstein, *Hematol. Oncol. Clin. North Am.*, 6 (1992) 249.
[206] N.M. Papadopoulos, H.C. Lane and R. Costello, *Clin. Immunol. Immunopathol.*, 35 (1985) 43.
[207] D. Sinclair, E. Galloway, S. McKenzie, E.A.C. Follet and L. Wallace, *Clin. Chem.*, 35 (1989) 1669.
[208] J.J. Lefrère, M. Debbia and P. Lambin, *Br. J. Heamatol.*, 84 (1993) 20.

[209] A.J. Mitus, R. Stein, J.M. Rappeport, J.H. Antin, H.J. Weinstein, C.A. Alper and R.B. Smith, *Blood*, 74 (1989) 2764.
[210] E.J.A. Gerritsen, M.J.D. van Tol, A.C. Lankester, C.P.M. van der Weiden-Ragas, C.M. Jol-van der Zijde, N.J. Oudeman-Gruber, J. Radl and J.M. Vossen, *Blood*, 82 (1993) 3493.
[211] J. Radl, R.M. Valentijn, J.J. Haaijman and L.C. Paul, *Clin. Exp. Immunol.*, 37 (1985) 98.
[212] D. Peest, B. Schaper, B. Nashan, K. Wonigeit, E. Raude, R. Pichlmayr, A. Haverich and H. Deicher, *Transplantation*, 46 (1988) 389.
[213] E. Gerristen, J. Vossen, M. van Tol, C. Jol-van der Zijde, R. van der Weijden-Ragas and J. Radl, *J. Clin. Immunol.*, 9 (1989) 296.
[214] H. Hass, S. Anders, G.W. Bornkamm, E. Mannweiler, H. Schmitz, J. Radl and M. Schlaak, *Clin. Exp. Immunol.*, 81 (1990) 435.
[215] A. Saxon, Macy, E., K. Denis, M. Tary-Lehman, O. Witte and J. Braun, *J. Clin. Invest.*, 87 (1991) 658.
[216] D.W. Hanto, G. Frizzera, K.J. Gajl-Peczalska and R.L. Simmons, *Transplantation*, 39 (1985) 461.
[217] O. W Kamel, M. van De Rijn, L.M. Weiss, G.J. Del Zoppo, P.K. Hench, B.A. Robbins, P.G. Montgomery, R.A. Warnke and R.F. Dorfman, *New Engl. J. Med.*, 328 (1993) 1317.
[218] D.J.L. Joske and H. Knecht, *Blood Rev.*, 7 (1993) 215.
[219] R.A. Kyle, R.A. Robinson and J.A. Katzmann, *Am. J. Med.*, 71 (1981) 999.
[220] J.M. Fine and M. Marneux, *Rev. Fr. Transfus. Immuno.*, 28 (1985) 591.
[221] S. Riddell, Z. Traczyk, F. Paraskevas and L.G. Israel, *Medicine*, 65 (1986) 135.
[222] A. Palumbo, S. Battaglio, M. Astolfi, R. Frieri, M. Boccadoro and A. Pileri, *Br. J. Heamtol.*, 82 (1992) 676.
[223] M.H.C. Bakkus, I. van Riet, B. van Camp and K. Thielemans, *Br. J. Haematol.*, 87 (1994) 68.
[224] B.J. Rosen, T.W., Smith and K.J. Block, *N. Engl. J. Med.*, 277 (1967) 902.
[225] A.C. Wang, S.K. Wilson, J.E. Hopper, H.H. Fudenberg and A. Nisonoff, *Proc. Natl. Acad, Sci. U.S.A.*, 66 (1970) 337.
[226] A.S. Levin, H.H. Fudenberg, J.E. Hopper, S.K. Wilson and A. Nisonoff, *Proc. Natl. Acad. Sci. U.S.A.*, 68 (1971) 169.
[227] R.A. Rudders, V. Yakulis and P. Heller, *Am. J. Med.*, 55 (1973) 215.
[228] J.P. Bouvet, D. Buffe, R. Oriol and P. Liacopoulos, *Immunology*, 27 (1974) 1095.
[229] R. Oriol, J. Huerta, J.P. Bouvet and P. Liacopoulos, *Immunology*, 27 (1974) 1081.
[230] J.P. Bouvet, J. Feingold, R. Oriol and P. Liacopoulos, *Biomedicine*, 22 (1975) 517.
[231] J.E. Hopper, *Blood*, 50 (1977) 203.
[232] S. Sarasombath, J. Mestecky and P. Skvaril, *Clin. Exp. Immunol.*, 29 (1977) 67.
[233] T. Ockhuizen, G. Steen, H.G. Muilerman, J.W. Smit, P. Wietzes, E. Mandema and J. Marrink, *Immunology*, 37 (1979) 863.
[234] J.P. Bouvet, P. Liacopoulos, J. Pillot, R. Banda, E. Tung and A.C. Wang, *J. Immunol.*, 129 (1982) 1519.
[235] N. Miyagawa, K. Morita and T. Azuma, *Microbiol. Immunol.*, 34 (1990) 787.
[236] M. Houdayer, J.P. Bouvet, A. Wolff, C. Magnac, J.C. Guillemot, L. Borche and G. Dighiero, *J. Immunol.*, 150 (1993) 311.
[237] T. Murata, H. Fujita, H. Harano, M. Hukawa, H. Kanamori, M. Matsuzaki, H. Mohri, J. Kudoh, N. Shimizu and T. Okubo, *Am. J. Hematol.*, 42 (1993) 21.