

Role of two-dimensional electrophoretic analysis in the diagnosis and characterization of IgD monoclonal gammopathy

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Over a period of 5 years, an isolated light chain ($\kappa = 9$, $\lambda = 12$) was detected in 21 sera by immunofixation electrophoresis. Further analysis with anti- δ - and anti- ϵ -specific antisera identified four δ heavy chains, all associated with a λ light chain, and no ϵ heavy chains. For evaluation of the role of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in the diagnosis of IgD paraproteins, as a possible alternative or complement to immunofixation, IgD paraproteins were retrospectively analyzed by 2D-PAGE. δ Heavy chains migrated to gel areas clearly distinguishable from other heavy chains α , γ , or μ , and in a wide range of isoelectric points (pI: 5.4–8). In one serum, the monoclonal δ chain had a pI range comparable to that of albumin and was undetectable. However, all four δ chains were easily identified when analyzed from affinity-purified immunoglobulin fractions. These observations showed the following:

- 1) IgD paraproteins are not rare among apparently isolated monoclonal light chains detected by routine immunofixation, strongly confirming the need for further analysis with anti- δ antisera, before assumption of a light-chain disease.
- 2) 2D-PAGE analysis of affinity-purified immunoglobulin fractions allowed correct identification of IgD monoclonal gammopathies in all cases.
- 3) However, although 2D-PAGE analysis is now easy to perform, well standardized, and highly sensitive, this technique remains time-consuming and expensive, and does not appear suitable for routine practice as a first-line diagnostic procedure.

2D-PAGE should find its place as a complement to immunofixation and in the definitive demonstration, in selected ambiguous cases, of the clonal pattern of a suspected gammopathy at immunofixation.

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IgD is a rare paraprotein that represents less than 1% of all myeloma (5, 15). First described by Rowe & Fahey (20), IgD myeloma is characterized by a high frequency of association with Bence Jones proteinuria and renal insufficiency (7), a high rate of extraosseous spread, and amyloidosis (4, 5, 10, 18, 33). The prognosis of IgD myeloma is generally considered to be poor, with a median survival time of only 11 months as opposed to 29 months for myelomas of other isotypes (17). In most reports, λ light chains largely prevail over κ light chains (6, 14, 17), an observation strongly predictive of a decreased survival rate (22).

IgD paraproteins would be overlooked in routine

immuno-electrophoretic analysis, when only polyclonal antisera against γ , α , and μ heavy chains and κ and light chains are used (9). The large preponderance of λ light chains in IgD myeloma and their frequent association with renal failure could be erroneously interpreted as light-chain disease. Thus, it is now a well-established and long-standing recommendation to investigate further sera with apparently isolated λ light chains for IgD or IgE paraproteins (19, 32). We have tried for the last few years to define precisely the place of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in the diagnosis of monoclonal gammopathies (29). 2D-PAGE has been considerably facilitated by the use

of well-standardized apparatus and ready-to-use preparations, and is now available even for routine diagnostic laboratories (12). In particular, the use of immobilized pH gradients for the first-dimensional separation has considerably improved the resolution and the reproducibility of 2D-PAGE (13, 26), now a powerful, high-resolution tool for analyzing monoclonal gammopathies and determining the clonal diversity of serum immunoglobulins (1, 25, 27). For evaluation of the role of 2D-PAGE in the diagnosis of IgD paraproteins as a possible alternative or complementary analysis to immunofixation, IgD gammopathies were retrospectively analyzed by 2D-PAGE. In parallel, the study of the four IgD paraproteins described in this paper allowed us further to characterize the still poorly described 2D electrophoretic pattern of these rare paraproteins. Analyzing monoclonal IgD by the classical two-step immunofixation, as well as by 2D-PAGE, we demonstrated here the following:

- 1) that the prevalence of IgD monoclonal gammopathies in serum samples with apparently isolated light chains was not low, confirming that full analysis of serum samples with an apparently isolated light chain by immunofixation is mandatory before assumption of a light-chain disease
- 2) that 2D-PAGE allowed correct identification of monoclonal IgD heavy chains in all samples.

2D-PAGE analysis was highly sensitive and reproducible although time-consuming and expensive. Taken together, our findings suggest that 2D-PAGE should be considered to complement classical routine analysis of IgD paraproteins by immunofixation and, in a prepared laboratory, should be reserved for those rare paraproteins in which clonality does not readily appear from immunofixation. Apart from "morphologic" information on IgD paraproteins, 2D-PAGE also provided data on the isoelectric characteristics of the four monoclonal gammopathies. The clinical relevance of this finding still needs to be evaluated.

Material and methods

Sera

All sera referred to the laboratory over the last 5 years for monoclonal gammopathy screening were analyzed by conventional immunofixation as described below and stored at -80°C .

Immunofixation

Sera were analyzed by conventional immunofixation (Beckmann, Paragon, USA, or DAKO A/S, Immunofixation Kit, Glostrup, Denmark). As the first-step analysis, sera are routinely screened with rabbit polyclonal antisera against α , γ , or μ heavy chains and

λ and κ light chains. In the second step, samples with apparently isolated light chains are tested with rabbit antisera against δ or ϵ heavy chains (DAKO A/S, Glostrup, Denmark) for 30 min. at room temperature in a humid chamber. After washing in saline solution, electrophoretic gels are dried and stained with amido black 5 g/l in 5% acetic acid.

2D-PAGE analysis

Serum samples as well as purified immunoglobulin fractions were prepared and analyzed as previously described (11). Proteins, under reducing and denaturing conditions, were separated by isoelectric focusing (carrier ampholytes pH 3.5–10) followed by linear 9 to 16% gradient PAGE and, more recently, with immobilized pH gradients. In all experiments, the final amount of serum loaded on capillary gels was 0.3 μl . An amount of 5 μg of purified immunoglobulin fraction was analyzed. After silver staining, gels or parts of gels were photographed and presented with the standard coordinate system with high-molecular weights at the top, and the acidic side on the left. Proteins were identified as described in detail elsewhere (2, 3, 13, 26).

Immunoglobulin purification

IgD- λ were isolated from serum samples by affinity chromatography on Sepharose beads coated with affinity-purified polyclonal anti- λ -chain immunoglobulins (Calbiochem, San Diego, CA, USA) (24).

IgD levels

Serum IgD levels were measured by nephelometry (Behring, Marburg, Germany).

Western blotting

After 2D-PAGE analysis, affinity-purified immunoglobulin fractions were transferred on PVDF membranes (Millipore, USA), as previously described (23), and developed with horseradish peroxidase-labeled anti-IgD polyclonal antiserum (DAKO AS, Glostrup, Denmark).

Results

Immunofixation

All serum specimens from patients with an apparently isolated monoclonal light chain after conventional immunofixation with polyclonal antisera against α , λ , or μ heavy chains and λ and κ light chains were selected. Among a total of 510 monoclonal gammopathies characterized in our laboratory over the last 5 years, 21 (4.1%) disclosed ap-

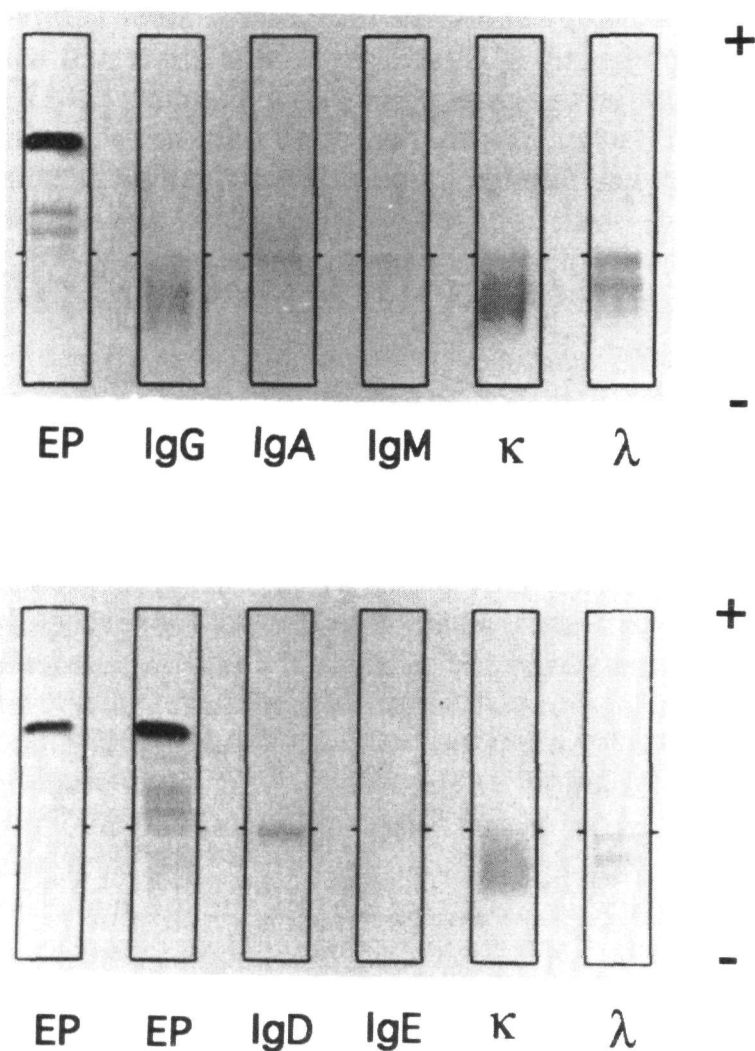


Fig. 1. Conventional strategy: immunofixation electrophoresis analysis of apparently isolated λ light chain (patient OW) (upper panel). Subsequent identification of monoclonal δ chain with anti- δ -chain antiserum (lower panel). EP: protein electrophoresis.

parently isolated λ light chains in 12 cases and κ light chains in nine cases. All 21 sera were subsequently analyzed by immunofixation with anti-IgD and anti-IgE polyclonal antisera as routinely prescribed (Fig. 1). Four of the 21 patients (one female, three males) (0.8% of all monoclonal gammopathies) were positive for a monoclonal δ heavy chain (Fig. 2). A λ light chain was associated with the δ heavy chain in all cases. Interestingly, a free λ light chain was present in three of the four sera. No monoclonal ϵ heavy chains were detected. Serum IgD concen-

trations varied from 1190 to 33 500 U/ml (Table 1). The κ/λ ratio was decreased in three cases and normal in one.

2D-PAGE

All four monoclonal δ -heavy-chain-positive sera were submitted to 2D-PAGE. Fig. 3 shows the 2D-PAGE analysis of patient SO serum (Fig. 3A) and the affinity chromatography-purified immunoglobulin fraction of this patient (Fig. 3B); the λ light chain area was remarkable in that the monoclonal light chain (λ') appeared as an easily detected, well-delimited, large spot. As underlined in previous observations, this pattern was strongly suggestive of monoclonality, in contrast to the fuzzy zone with multiple small and ill-defined spots characteristic of polyclonal light chains (Fig. 3C). Analysis of the immunoglobulin heavy-chain region revealed the presence of a restricted number of small additional spots, located at, and partially covered by, the basic side of albumin and corresponding to the δ heavy chain (Fig. 3A). The electrophoretogram of the purified monoclonal IgD showed charge and size microheterogeneity of the δ chain (Fig. 3B), a pattern confirmed by Western blotting with polyclonal anti-IgD horseradish peroxidase-labeled antiserum (Fig. 3D). In this case, a late-stage IgD myeloma, with medullary infiltration, α and μ chains were not discernible, reflecting polyclonal hypogammaglobulinemia.

In normal serum (Fig. 4A), polyclonal δ chains comigrated with albumin and therefore were not detectable. In contrast, anti- λ purified immunoglobulin fractions migrated in an area easily distinguishable from μ and α heavy chains (Fig. 4A'). 2D-PAGE analysis of serum samples of three patients (MB, OW, VM) and of their purified immunoglobulin fractions are shown in Fig. 4B-D, B'-D'. The IgD migration pattern included a large range of pI from the acidic side of the albumin spot (panel B, pI range 5.4-6.2) to highly cationic areas (panel D, pI range 6-8). Although monoclonal δ chains were easily detected in three serum samples (Figs. 3A and 4C and D), in one case (Fig. 4B) the monoclonal δ heavy chain was completely hidden by the albumin spot. However, after affinity purification with anti- λ chain Sepharose beads, the δ chain was clearly identified (Fig. 4B'). Charge and size microheterogeneities were present in all four analyses (Figs. 3B and 4B'-D').

Discussion

Among all paraproteins, monoclonal gammopathies are infrequently observed. Conventional analysis of monoclonal gammopathies by immunofixation includes initial incubation with anti- γ , α , and μ heavy-

Table 1. Characteristics of monoclonal IgD-positive sera

Patients	Sex/age (years)	Heavy chain	U/ml*	Light chain	κ/λ ratio**
VM	F/79	IgD*	1190	λ	0.44
OW	M/51	IgD	1590	λ	1.39
SO	M/66	IgD	5500	λ	0.44
MB	M/63	IgD	33500	λ	0.09

* Normal range < 100 U/ml.

** Normal range 0.7-3.0.

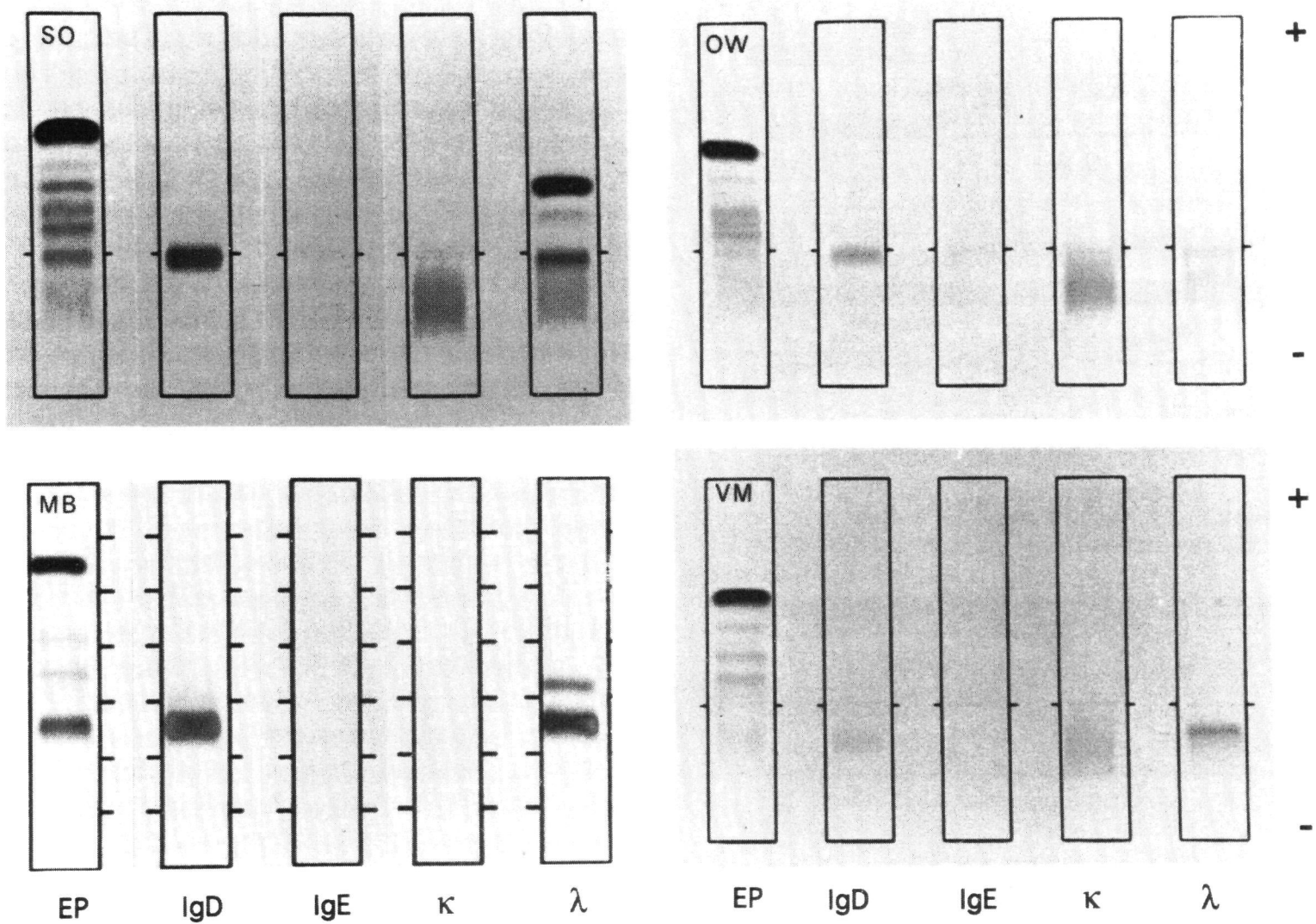


Fig. 2. Immunofixation electrophoresis analysis of four sera positive for apparently isolated light chain. Incubation with anti- δ -chain antiserum in second-step analysis demonstrated presence of associated monoclonal δ chain. EP: protein electrophoresis.

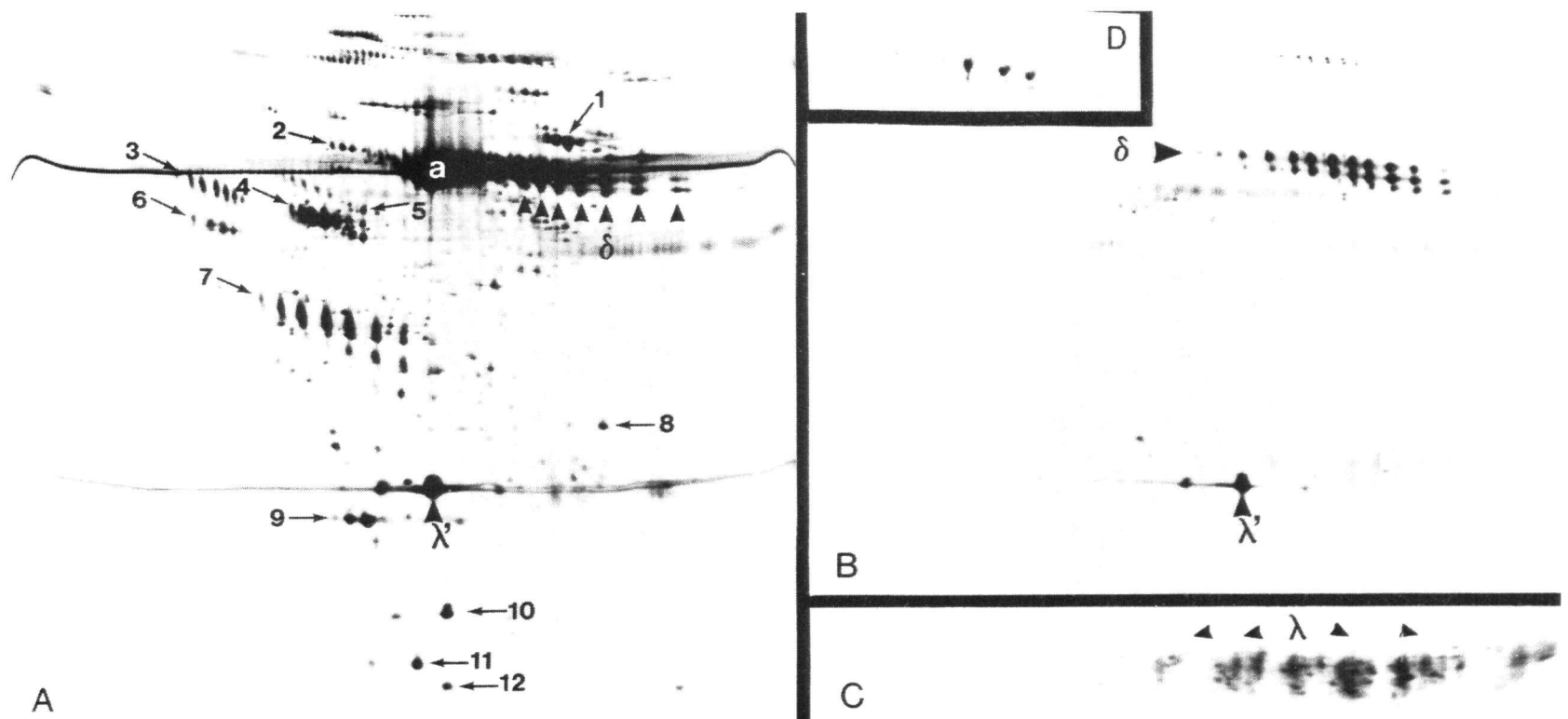


Fig. 3. 2D-PAGE of monoclonal IgD. (A) Serum and (B) purified IgD (anti- λ Sepharose column). Purified polyclonal λ chains (anti- λ Sepharose column) are shown in panel C. Purified immunoglobulin fraction was transferred on PVDF membrane and revealed with horseradish peroxidase-labeled anti- δ -chain rabbit antiserum (panel D). 1) transferrin; 2) β -1 glycoprotein; 3) α -1 antichymotrypsin; 4) α -1 antitrypsin; 5) antithrombin III; 6) α -2 HS-glycoprotein; 7) haptoglobin (β chain); 8) complement C4 (γ chain); 9) apolipoprotein A-I; 10) haptoglobin (α -2 chain); 11) transthyretin (prealbumin); 12) haptoglobin (α -1 chain); a) albumin; δ) monoclonal immunoglobulin δ chain; γ) polyclonal immunoglobulin λ chains; λ') monoclonal λ chain.

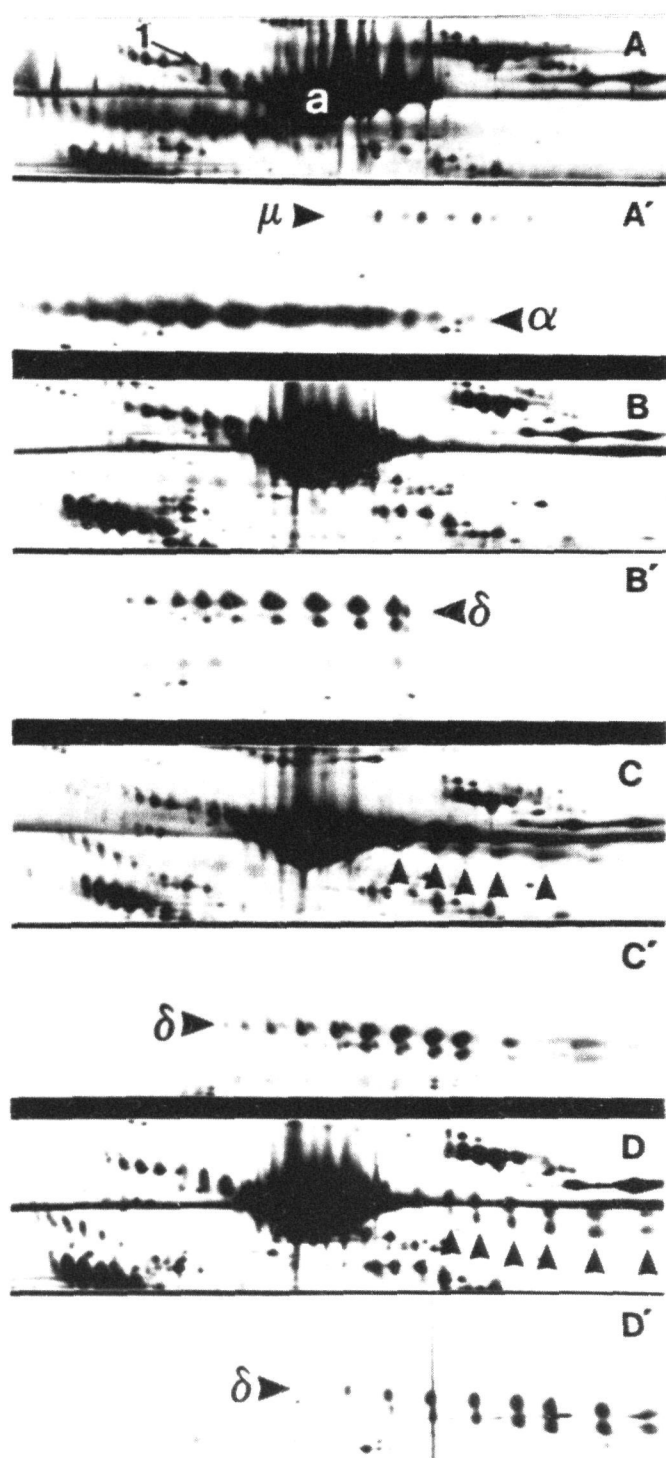


Fig. 4. Detailed regions of two-dimensional gels of A) normal serum; B–D) sera from three patients with monoclonal IgD. A'–D' show two-dimensional patterns of corresponding purified immunoglobulin fractions (anti- λ Sepharose column). a) albumin; μ) polyclonal μ chains of IgM; α) polyclonal κ chains of IgA; δ) monoclonal δ chains. Arrowheads in panels C and D indicate spots corresponding to monoclonal δ chains (not hidden by albumin in serum samples).

chain and λ and κ light-chain antisera. Detection of an isolated light chain requires serum incubation in a second step with anti- δ and anti- ϵ antisera. Non-observance of this classical strategy is bound to fail to identify IgD or IgE paraproteins, and IgD myeloma would be misinterpreted as an isolated light-chain disease, a clinical condition with different presentation and prognosis (4, 6, 14, 17, 18, 22, 33). Systematic analysis of sera associated with an apparently isolated monoclonal light chain revealed that four of 21 sera were positive for the δ heavy

chain (about 20%). This high proportion should not result from a bias in the recruitment of serum samples in a reference laboratory, since the proportion of 0.8% IgD monoclonal gammopathies among all gammopathies detected is within the range published in other studies (15, 16). The δ heavy chain was associated in all cases with a monoclonal light chain. In three cases, the free λ chain was also identified, as noted by other studies (4, 6, 14, 16, 21). This finding indicates that IgD monoclonal gammopathies are not rare among apparently isolated light-chain disease and further reinforces the general guideline that demonstration of a light-chain reaction in the serum after immunofixation requires further characterization with anti- δ and anti- ϵ polyclonal antisera.

We have recently tried to determine the place of 2D-PAGE in routine diagnosis of monoclonal gammopathies. Technically, 2D-PAGE has considerably improved over the past few years, evolving from a cumbersome exercise based on homemade preparations to a highly sensitive and reproducible analytic tool. The availability of immobilized pH gradients and highly sensitive silver staining has recently proved invaluable in identifying even minute amounts of monoclonal paraproteins. In many instances (29), 2D-PAGE contributed to definitive identification of monoclonal or oligoclonal patterns, which might not readily appear from immunofixation, a considerably less sensitive technique. In particular, 2D-PAGE has demonstrated its superiority to immunofixation in the analysis of cryoglobulins and has helped to define more subtly transitional stages between type II and type III cryoglobulins (28).

Furthermore, 2D-PAGE allowed us to detect as yet unrecognized proteins associated with IgM. Although IgG, IgA, and IgM paraproteins can be readily identified from serum by 2D-PAGE, there is only scant information on IgD paraprotein analysis. In serum, δ chains were found midway between μ and α chains and clearly distinct from them, partially hidden by the albumin spot in three cases. However, one monoclonal δ chain (Fig. 4B) could be easily missed, since it was localized on the plasma/serum protein map within the albumin spot. But in other cases the monoclonal IgD heavy chain migrated in a wide range of pI and was readily identified by its microheterogeneous pattern even in unpurified whole serum. After affinity purification, which allowed avoidance of interference with the albumin spot, the migration area of the four δ chains was clearly distinguishable from the migration areas of the μ and α chains. Thus, 2D-PAGE analysis of IgD paraproteins allowed correct identification of the monoclonal gammopathy in all cases, provided serum proteins were first purified by affinity chromatography. The electrophoretic patterns of monoclonal λ light

chains were not different from those observed in other monoclonal gammopathies of α , γ , or μ isotypes. Monoclonal light chains have been resolved as single spots, probably in relation to the absence of glycosylation of those chains (30, 31).

In this study, we have shown that when analysis was performed on immunoglobulin fractions, 2D-PAGE allowed correct identification of all four IgD paraproteins, but of only three out of four in whole serum. Therefore, the use of 2D-PAGE cannot be recommended as an alternative to immunofixation for routine analysis. However, as shown by the present study on IgD paraproteins, as well as by our previous observations on IgG, IgA, or IgM paraproteins, 2D-PAGE is a powerful technique which complements immunofixation in essentially two aspects. First, its high sensitivity allows the identification of paraproteins that might not be detected by immunofixation. Minimal paraproteins may be masked in a polyclonal background when analyzed by immunofixation, and appear distinctly as monoclonal by 2D-PAGE (27). Conversely, due to its low sensitivity, immunofixation may lead to the diagnosis of monoclonal gammopathy when actually 2D-PAGE demonstrates an oligoclonal immunoglobulin pattern. Second, 2D-PAGE provides information on immunoglobulin migratory patterns. In this paper, we have shown that IgD monoclonal gammopathies were migrating along a very large pI gradient. So far, 2D-PAGE has not been used widely enough in routine analysis of monoclonal gammopathies to provide potential correlation between migratory pattern and clinical expression of paraproteins. However, in experimental models, the pathogenicity of specific autoantibodies, such as anti-DNA antibodies in lupus nephritis, has been related to the cationic charge of the monoclonal immunoglobulin (8). This suggests that 2D-PAGE still has great potential, and, apart from morphologic information, may provide important data on the clinical expression of a given plasma cell dyscrasia, an aspect that justifies further development.

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