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Clinical implications of the types of cryoglobulins determined by two-dimensional polyacrylamide gel electrophoresis

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Abstract

Background and Objective. **Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a new method which can be used to study cryoprecipitates from the sera of cryoglobulinemic patients. It led to the identification of a new type of cryoprecipitate, tentatively named II-III, characterized by polyclonal IgG associated with a mixture of polyclonal and monoclonal IgM. Some discrepancies with the conventional classification of cryoglobulins were revealed. The association of particular clinical features with the classification of cryoglobulins by 2-D PAGE is examined.**

Design and Methods. **Sixty consecutive patients affected by cryoglobulinemic syndrome with mixed cryoglobulins were included in the study. All patients were evaluated for cutaneous, articular, hepatic, renal and nervous involvement. The washed cryoprecipitates were typed using both techniques: immunofixation electrophoresis (IFE) and 2-D PAGE.**

Results. **Sixteen (6 cases of type II and 10 of type III by IFE) of 60 cryoprecipitates (26.6%) appeared as type II-III by 2-D PAGE analysis. Nine cases were classified differently by IFE and 2-D PAGE. Mixed cryoglobulins of type II-III were not associated with a particular clinical pattern. Examining the clinical findings in the *mono* group (those with monoclonal IgM alone) and the *poly* group (those with polyclonal IgM alone or polyclonal and monoclonal IgM) we found clearly significant differences: more severe liver involvement in the *poly* group, and higher cryocrit and creatinine values, lower C4 level and more severe purpura in the *mono* group.**

Interpretation and Conclusions. **Our results confirm the reliability of 2-D PAGE in characterizing cryoprecipitates. This sensitive method can demonstrate a higher number of monoclonal components, undetectable by IFE. Type II-III cryoglobulins are not associated with a particular clinical pattern. The presence or absence of polyclonal IgM in mixed cryoglobulins seems to be correlated with some clinical findings.**

Key words: cryoglobulins, 2-D PAGE, cryoglobulinemic syndrome

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The relationship between the presence of cryoglobulins and a systemic immunologic disorder has been well documented. Tissue deposition of circulating immune complexes may lead to cutaneous and systemic vasculitis, glomerulonephritis or peripheral neuropathy.[1-3] However, it is important to keep in mind that cryoglobulinemia is a descriptive term of an *in vitro* artifact, related to the precipitation of immunoglobulins in the cold.[4] The sequence of events that leads to cryoprecipitation of immunoglobulins remains undefined. Each cryoglobulin has unique properties which indicate that different molecular explanations might be necessary to explain cold insolubility. In 1974, Brouet *et al.*[5] defined 3 types of cryoglobulins: type I contained a single monoclonal Ig, type II a mixture of a monoclonal Ig with polyclonal Igs of a different isotype, and type III a mixture of polyclonal Igs of different isotypes, most frequently IgG and IgM. Type II and type III have also been called mixed cryoglobulins. High prevalence of hepatitis C virus (HCV) markers in patients presenting both secondary and essential cryoglobulinemia has been well documented,[6-9] but the role of HCV in the pathogenesis is not clearly defined.[10] A study of the early clinical and laboratory manifestations of cryoglobulinemia revealed that renal and cutaneous involvement as well as laboratory abnormalities differed significantly between type II and type III.[11] The recent finding, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), of washed cryoprecipitates that contained polyclonal IgG associated with a mixture of polyclonal and monoclonal IgM, tentatively named type II-III cryoglobulins,[12] prompted us to investigate whether these intermediate forms between type II and type III cryoglobulins are associated with particular clinical or laboratory findings.

Materials and Methods

Patients

Sixty consecutive patients referred to us because of cryoglobulinemic syndrome with mixed cryoglobulins were selected for this study; 39 were women and 21 were men. Their mean age was 62.8 years (range 45-79), the mean duration of disease from diagnosis was 9.9 years (range 1-22). The diagnosis of cryoglobu-

linemic syndrome[13] was made when the patients, during a clinical observation of six months or more, had all the following: two out of three classical symptoms (purpura, arthralgia, asthenia), a cryoprecipitable serum with a cryocrit >1% v/v, an elevated rheumatoid factor (RF) activity or a reduced C_4 serum level (<12 mg/dL). All patients with an established diagnosis of malignancy, connective tissue disease or chronic viral (except for HCV infection) or bacterial infection were excluded from this study. All patients, from the diagnosis, were clinically evaluated two or more times per year (mean 2.7) for skin, joint, liver, kidney and nerve involvement. The severity of the cryoglobulinemic syndrome was evaluated by using the following gradation: purpura, 0=absent, 1=limited to the legs, 2=extended to legs and thighs, 3=lower limbs and other sites, 4=presence of necrotic ulcer; arthralgia, 0=absent, 1=occasional, 2=persistent, 3=arthritis; liver involvement, 0=absence of biochemical or ultrasonic signs of liver disease, 1=occasionally abnormal ALT level, 2=persistent elevation of ALT level, 3=clinical or histological diagnosis of chronic evolutive disease, 4=liver cirrhosis; peripheral neuropathy, 0=absent, 1=disesthetic or paresthetic symptoms, 2=sensitive-motor neuropathy with minor EMG abnormalities, 3=sensory-motor neuropathy with major EMG abnormalities. Renal function was evaluated in all patients by measuring the serum level of creatinine. RF was determined by the latex agglutination test (latex reagent RF, Behringwerke AG, Marburg, Germany). Anti-hepatitis C virus (HCV) antibodies were analysed using third generation ELISA from Ortho (Innogenetics, Zwijndrecht, Belgium). HCV-RNA was detected by PCR using the Amplicor HCV-test from Roche (Roche, Diagnostic system, Branchburg, NJ, USA). The serum C_3 and C_4 levels were measured by radial immunodiffusion or by nephelometry (Behringwerke AG, Marburg, Germany).

Cryoprecipitates

Collection of sera, quantification and isolation of cryoglobulins were performed as previously described.[14] Briefly, blood samples were collected and coagulated at 37°C. The serum sample was left for 96h at 4°C. After centrifugation for 15 min at 1700 rpm, the cryocrit was calculated as the percent ratio cryoprecipitate/total serum volume. The precipitate was washed 5 times with phosphate-NaCl buffer at 4°C. Proteins were quantified by the folin assay, and expressed in mg per mL of serum incubated at 4°C.

Immunofixation electrophoresis (IFE)

IFE analysis was performed with undiluted samples, using a commercially available kit, following the indications of the manufacturer (Paragon electrophoresis sytem, Beckman instruments, Brea, CA, USA).

2-D PAGE electrophoresis

Sample preparation

The plasma/serum samples were prepared as described by Hochstrasser *et al*.[15] Briefly, 5 µL of plasma/serum were mixed with 10 µL of a denaturing, reducing solution (*solution D*; SDS 1 g, DTE 0.232 g, H_2O 10 mL), and heated to 95°C for 5 min. After a short cooling time, 485 µL of the *sample solution E* (100 mg of DTT, 400 mg of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 5.4 g urea, 500 mL of pH 3-5-10 ampholytes and 6.5 mL of H_2O) 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. Cryoprecipitates were directly dissolved in *solution D*. Ten µL (25-50 mg of protein) were mixed with 100 ml of *solution E*. Fifty µL of the final diluted sample (12.5-25 µg of protein) were loaded onto the first dimension gels.

Isoelectric focusing (first dimension)

Isoelectric focusing using immobilized pH gradients (IPG) was performed as previously described. The equipment, including Immobiline Dry-Strip pH 3-10 NL, 18 cm, was from Pharmacia-LKB (Uppsala, Sweden). All samples were loaded onto the cathodic end of the rehydrated IPG strips and covered with low viscosity paraffin oil. Isoelectric focusing was performed at 10°C. The voltage was progressively increased from 200 V to 3000 V during the first 3 h, followed by 3500 V for 1 h, and finally increased to 5000 V for a total of 100 kVh. Before the second-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.

SDS-PAGE (second dimension)

The vertical second-dimension was performed on 180×160×1.5 mm 9-16% T gradients gels, also using diacrylylpiperazine as cross-linking agent. 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.

Silver staining and spot identification

The ammoniacal silver staining protocols have been described elsewhere.[15] The gels were photographed with higher molecular weights at the top and the acidic side on the left. Polypeptides were identified by comparison with reference protein

Table 1. Laboratory data and clinical scores of the 60 cases of cryoglobulinemic syndrome examined.

	mean ± sd
Cryocrit (% v/v)	8.1±13.5
Rheumatoid factor (UI/mL)	403.7±577.7
C_4 (mg/dL)	11.3±13.1
C_3 (mg/dL)	81.1±36.9
Creatinine (mg/dL)	0.96±0.19
Purpura	0.9±0.9
Arthralgia	0.7±0.8
Liver disease	1.6±1.5
Neuropathy	1.2±0.1

Legend: In our laboratory the normal values were: cryocrit 0%, rheumatoid factor < 20 IU/mL, C_4 12-40 mg/dL, C_3 80-160 mg/dL, creatinine 0.7-1.15 mg/dL. The ranges of the observed scores were: purpura 0-3 (median 1), arthralgia 0-3 (median 1), liver disease 0-4 (median 1) and neuropathy 0-3 (median 1).

maps. Isoelectric points (pI) were determined using the reference values established by Bjellqvists *et al*.[16] Apparent molecular weight (Mr) of polypeptide spots were established by comparison with known proteins used as internal standards.

Statistical studies

Statistical analyses were performed using the Systat (Systat Inc, Evenston, IL, USA) program on an Apple Macintosh Centris 610.

Results

Patients' characteristics

The clinical and laboratory characteristics of the 60 examined patients are shown in Table 1.

HCV-RNA was present in 46 of 57 cases tested (80.7%). Antibodies against HCV antigens were found in 58 of 60 cases. The two negative cases were also negative for HCV-RNA in the whole serum, supernatant and cryoprecipitate.

Two-dimensional electrophoretic pattern

Serum, polyclonal and monoclonal Igs

Figure 1 shows the 2D pattern of a serum sample from a patient presenting a monoclonal IgM. Polypeptides were observed either as isolated spots or as a series of spots, displaying charge and size microheterogeneities. Because proteins were reduced before electrophoresis, Ig heavy chains of different isotypes and light chains were observed at different areas on 2D gels: polyclonal α chains were resolved at an apparent Mr lower than that of albumin, with pI between 4.9 and 6.1, whereas γ chains were found at the basic side of the gel, and in an area corresponding to pI from 6.2 to more than 10. In contrast to polyclonal heavy chains, appearing as *fuzzy* zones without distinct individualizable spots, monoclonal

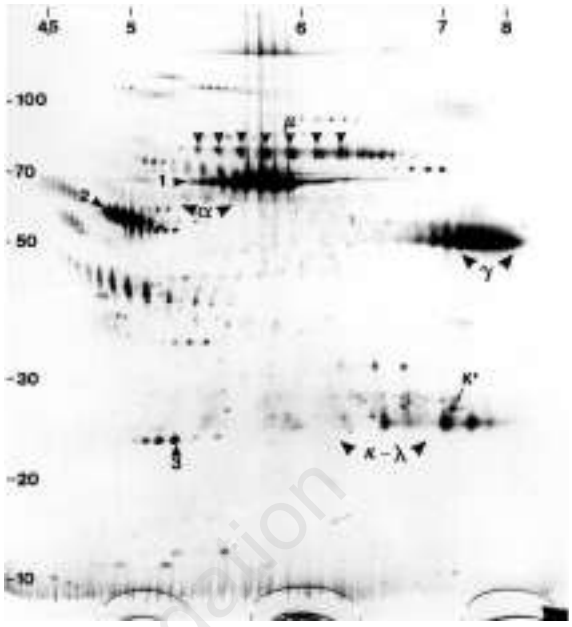

Figure 1. Monoclonal IgM in serum. Polypeptides (0.3 µL of serum) were separated by isoelectric focusing using linear immobilized pH gradient, followed by gradient 9-16% T polyacrylamide gel electrophoresis in the presence of SDS. The ammoniacal silver stained gel was photographed with the higher molecular weight at the top and the acidic side on the left. 1, albumin; 2, α1-antitrypsin; 3, apo A-I; µ, spots of the heavy chain of the monoclonal IgM; α, polyclonal heavy chains of IgA; γ, polyclonal heavy chains of IgG; κ-λ, polyclonal Ig light chains; κ', κ-light chain spot of the monoclonal IgM. pI and Mr (x10³ KDa) scales are respectively presented at the top and on the left of the figure.

heavy chains were detected as sets of well-resolved spots characterized by charge microheterogeneity. An example of a monoclonal µ chain is depicted in Figure 1. Polyclonal as well as monoclonal µ chains were observed in the same region of 2D gels, that is above the albumin spot, and on the acidic side of transferrin, in an area of the gel corresponding to pI of 5.6 to 6.4.

Differences between the 2D patterns of polyclonal and monoclonal light chains were also noticed. Monoclonal light chains appeared as well resolved and distinguishable spots, contrasting with polyclonal light chains which appeared as clustered non discrete spots and formed cloudy zones with unevenly distributed densities. The apparent Mr of light chains was between 21 and 27 kDa, and their pI were between 5 and 11.

Cryoglobulins

Two-dimensional analysis of mixed cryoglobulins (containing IgG or IgM) revealed 3 different electrophoretic patterns that we have previously classified as type II, II-III, and III. All types of cryoglobulins were characterized by the presence of polyclonal IgG with

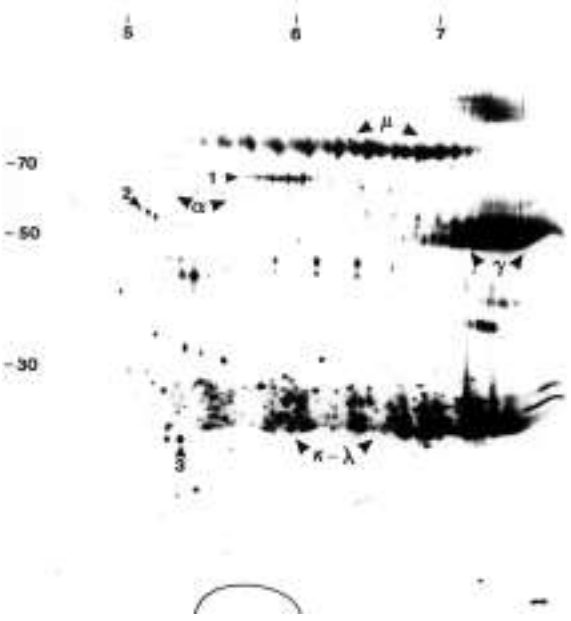

Figure 2. Two-dimensional electrophoretic patterns of mixed type III cryoglobulins. 1, albumin; 2, α1-antitrypsin; 3, apo A-I; μ, polyclonal heavy chains of IgM; α, polyclonal heavy chains of IgA; γ, polyclonal heavy chains of IgG; κ-λ, polyclonal Ig light chains. pI and Mr (x10³ KDa) scales are respectively presented at the top and on the left of the figure.

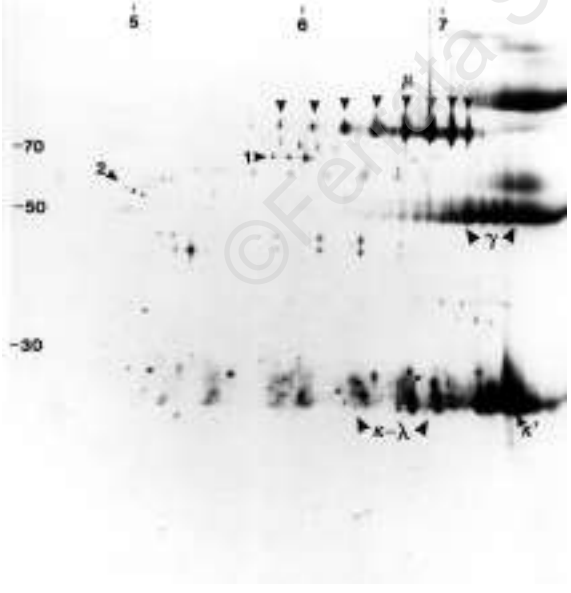

Figure 3. Two-dimensional electrophoretic patterns of mixed type II cryoglobulins. 1, albumin; 2, α1-antitrypsin; μ, spots of the heavy chain of the monoclonal IgM; γ, polyclonal heavy chain of IgG; κ-λ, polyclonal Ig light chains; κ', κ-light chain spot of the monoclonal IgM (hidden by polyclonal chains). pI and Mr (x10³ KDa) scales are respectively presented at the top and on the left of the figure.

IgM. An example of type III cryoglobulins is shown in Figure 2. In this case, as well as in those depicted in Figure 4 a-e, detailed analysis of the IgM μ chain areas revealed fuzzy cloudy zones of unresolved spots, corresponding to polyclonal IgM μ chains. By contrast, sets of spots corresponding to monoclonal IgM μ chains were observed after 2D-PAGE of type II cryoglobulins (Figures 3, 4 k-o). Finally, the μ chains area of type II-III cryoglobulins showed sets of μ chains spots (corresponding to monoclonal μ chains) mixed with polyclonal μ chains (Figure 4 f-j).

Classification of cryoglobulins by 2-D PAGE or by IFE

At IFE examination, cryoglobulins were typed according to the classification of Brouet *et al.* and results were compared with those obtained by 2-D PAGE analysis (Table 2). Thirty-five of the 60 cases were identically classified by both 2-D PAGE and IFE techniques. Sixteen of 60 cryoprecipitates appeared as type II-III by 2-D PAGE analysis; 6 of these were type II and 10 type III by IFE examination.

Discrepancies in the interpretation of the types of cryoglobulins depending on the technique employed were observed in 9 cases. Six apparently type III samples by IFE analysis were classified as type II by 2-D PAGE, and inversely, 3 samples appearing as type II by IFE were diagnosed as type III by 2-D PAGE. All these data result in a higher number of monoclonal components (47/60, 78.3% by 2D-PAGE vs 36/60, 56.6% by IFE) in mixed cryoprecipitates.

Laboratory and clinical correlates

The laboratory and clinical data observed at time of cryprecipitate analysis with 2D-PAGE did not differ between type II or III classified according the IFE, except for the C_3 serum level that was significantly higher in type II cryoglobulinemias than in type III.

Table 3 presents the laboratory and clinical variables of patients grouped according to the classification by 2-D PAGE. No significant differences between the three types (II, II-III and III) were observed with the exception of C_4 concentration and of purpura severity (at not parametric analysis: p=0.043 and 0.009, respectively). Among the three groups the age and the duration of disease from the diagnosis were not different. Statistical analysis of clinical and laboratory parameters showed no differences between type III and type II-III patients. This fact suggests that the presence of polyclonal IgM is strictly related to similar clinical patterns.

The results obtained examining the data of type II, i.e. those with monoclonal IgM alone, the *mono*, versus the *poly*, i.e. type III and II-III together, both with polyclonal IgM, are presented in Table 4.

The differences between *mono* and *poly* are clearly significant. Only arthralgia and peripheral neuropathy are not different. The RF activity in *mono* is higher than in *poly*, but not statistically significant. The

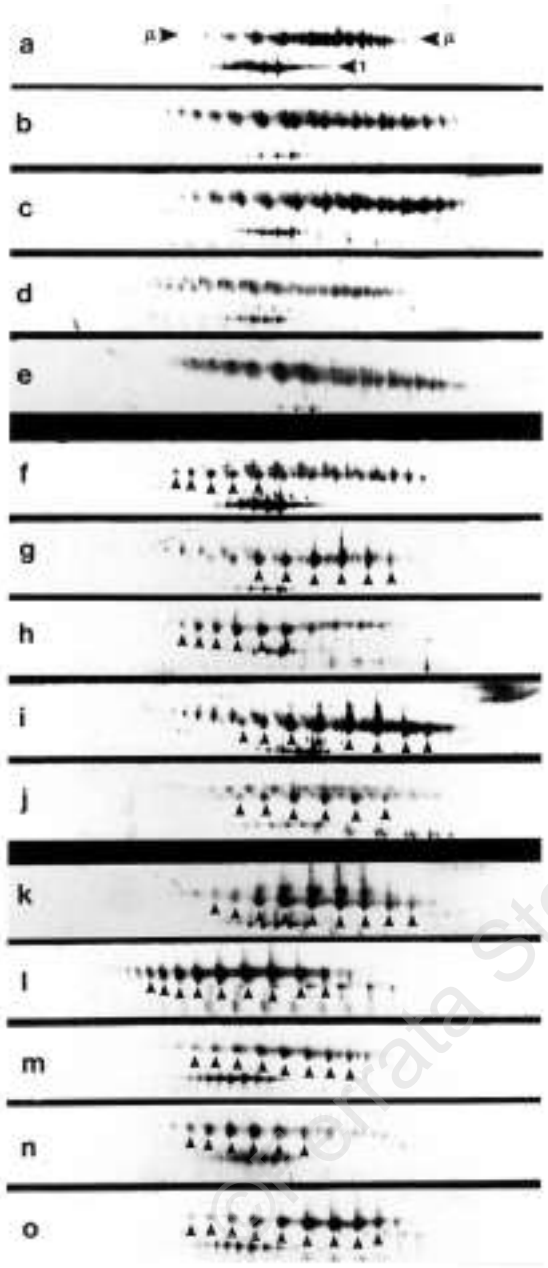

Figure 4. Details of the μ chain areas of the protein maps of (a-e) type III cryoglobulins; polyclonal μ-chain patterns; (f-j) type II-III cryoglobulins; monoclonal μ-chain patterns superimposed on polyclonal μ chain patterns; (k-o), type II cryoglobulins; presence of monoclonal μ-chains. μ, μ-chains; 1, albumin. Arrows highlight spots corresponding to monoclonal μ chains.

difference in liver involvement is also confirmed considering the clinical course: 7 out of 11 patients who never had clinical or biochemical signs of liver inflammation during the follow-up belong to the *mono* group, moreover 15 out of 19 patients with evolutive liver disease are in the *poly* group (the difference is

Table 2. Correspondence of 60 cryoprecipitate classifications by 2D-PAGE with immunotyping by IFE.

	Immunotyping according to Brouet		
2D-PAGE	Type II	Type III	Total
Type II	25	6	31
Type II-III	6	10	16
Type III	3	10	13
Total	34	26	

Table 3. Principal serological data and clinical score° (mean±SD) of MC patients classified according to 2D-PAGE typing.

	Type II #31	*Type II-III #16*	*Type III #13*
Cryocrit % v/v	10.1±14.4	5.5±7.8	6.5±16.9
Rheumatoid factor, IU	554.8±742.1	245.7±205.1	238.5±298.5
C_3 mg/dL	90.1±38.4	64.9±28.4	79.8±37.9
C_4 mg/dL*	7.9±5.9	16.8±22.3	13.0±7.9
Creatinine mg/dL	1.0±0.2	0.9±0.2	0.9±0.2
Purpura*	1.2±0.8	0.6±0.7	0.5±1.0
Arthralgia	0.7±0.7	0.7±0.8	0.5±0.8
Liver involvement	1.1±1.2	2.2±1.7	1.8±1.8
Peripheral neuropathy	1.3±1.0	0.9±1.1	1.5±1.1
Years of illness	9.5±5.6	11.3±3.2	9.2±3.7
Age	62.7±8.5	63.7±8.3	62.0±9.9

*°For the score definition see text in the Materials and Methods section; *differences between groups are significant for C_4 (p= 0.043) and for purpura (p= 0.009) by Kruskall-Wallis rank analysis of variance.*

Table 4. Principal serological data (mean±SD) of 60 MC patients grouped according to the clonality of the IgM component in the cryoprecipitate revealed by the 2-D PAGE typing.

	Type II "mono"° (#31)	*Type II-III & III "poly"° (#29)*
Cryocrit % v/v*	10.0±14.6	5.9±12.5
Rheumatoid factor, IU	554.9±742.1	242.1±246.7
C_3 mg/dL*	90.1±38.4	71.6±33.2
C_4 mg/dL*	7.9±5.9	15.1±17.3
Creatinine mg/dL*	1.01±0.19	0.91±0.19
Purpura*	1.2±0.8	0.6±0.8
Arthralgia	0.7±0.7	0.6±0.8
Liver involvement*	1.1±1.2	2.0±1.7
Peripheral neuropathy	1.3±1.0	1.2±1.1

*°For the score definition see text in material and methods section; *differences are significant for cryocrit (p=0.024), for C_3 (p=0.043), for C_4 (p= 0.017), for creatinine (p=0.037), for purpura (p= 0.002) and for liver involvement (p=0.050) by Kruskall-Wallis rank analysis of variance.*

significant at χ^2, p=0.015). Liver biopsy, indicated for clinical purposes, was performed in 36 patients (22 of the *poly* group), liver cirrhosis was diagnosed in 11 cases, 8 of which belonged to the *poly* group.

Discussion

HCV is the virus most frequently observed in patients suffering from what was, until recently, called essential mixed cryoglobulinemia.[6-9] This observation is confirmed by the present paper. Out of the 60 patients affected by cryoglobulinemic syndrome included in this study, i.e. patients with cryoglobulins in the absence of malignancy, connective tissue disease or chronic infection other than HCV, 58 were infected by HCV, as demonstrated by anti-HCV antibodies or HCV-RNA detected by PCR. HCV infection is frequently detected in patients with cryoglobulins, while cryoglobulins are less frequently found in patients with HCV infection, and have been reported in 20 to 25% of patients with HCV infection or liver disease.[17-20] However, most of these patients were asymptomatic. Their serum complement levels were not significantly different from that of controls and RFs were present only in 27-60% of sera. By contrast, the patients evaluated in this study were selected only if they presented a cryoglobulinemic syndrome. The syndrome was characterized either by purpura, arthralgia, neurological or renal manifestations, and by biochemical modifications that were: cryocrit > 1% v/v, and either elevated RF activity or reduced C_4 levels.

Cryoglobulins are usually classified according to Brouet *et al.*[5] on the basis of the analysis of washed cryoprecipitates by immunoelectrophoretic methods such as immunoelectrophoresis or immunofixation (IFE). Recently, methods such as immunoblotting[21] or 2-D PAGE[12] demonstrated new features by detecting minor monoclonal components that were not detected by more standard tecniques, and more particularly by IFE.

2-D PAGE offered the possibility of identifying monoclonal, polyclonal, as well as mixtures of polyclonal and monoclonal components by analyzing the μ chain zone of the gels. Using this technique, monoclonal components were detected in 16 of 26 cryoprecipitates characterized as type III cryoglobulins by IFE, reflecting the sensitivity of 2-D PAGE in the detection of minor monoclonal components. Contrariwise, in 3 cryoprecipitates defined as type II by IFE, no monoclonal μ chains were observed at 2-D PAGE analysis. These samples were therefore classified as type III by this technique. Discrepancies between IFE and 2-D PAGE, as previously suggested,[12] were observed because only the most prominent IgM clones were detected by IFE or, alternatively, because the minor IgM monoclonal components were hidden by polyclonal IgM. Besides the above described discrepancies in cryoglobulin typing between the two different techniques used in this study, our results provide further evidence that an intermediate form between type II and type III cryoglobulins does exist. These cryoglobulins, named type II-III, were identified in 16 of 60 cases (26.6%).

The higher number of monoclonal components found in this study confirms previous results[22] and suggests that clonal B cell expansion occurs in the cryoglobulinemic syndrome more commonly than previously thought. These clonally expanded B cells populations may not represent the cryoglobulins producing clones. It is possible that other antibody reactivities are present in cryoglobulins directed against unusual epitopes. In sera with type II mixed cryoglobulins epitopes displaying a strong homology with a signaling molecule on the surface of T lymphocytes were recognized by a non rheumatoid monoclonal IgM.[23]

Clinical and laboratory differences have been previously noted among patients with different types of mixed cryoglobulins.[24] In a multi-centre study it was previously shown that type II and type III cryoglobulinemic syndromes (classified on the basis of IFE) differed significantly in terms of prevalence of renal involvement, of cryocrit >3% v/v, C_4 <15 mg/dL and purpura.[11] Furthermore the clinical course between patients presenting either type II or type III cryoglobulins also appeared to be different,[25] membranoproliferative glomerulonephritis with a poor prognosis was almost exclusively present in type II[26] and only in these patients could a lymphoproliferative disease develop.[25,27,28] In the present study, these differences in laboratory and clinical data could not be statistically confirmed when cryoprecipitates were studied by IFE. By the 2-D PAGE analysis, the differences in C_4 level and severity of purpura reached statistical significance. On the basis of these results, type II and III cryoglobulins could be considered the extremes of a spectrum, and type II-III cryoglobulins as the intermediate forms characterized by features of both type II and type III cryoglobulins (Figure 5). Therefore, according to the parameters used in this study, the new group of cryoglobulins classified as type II-III is

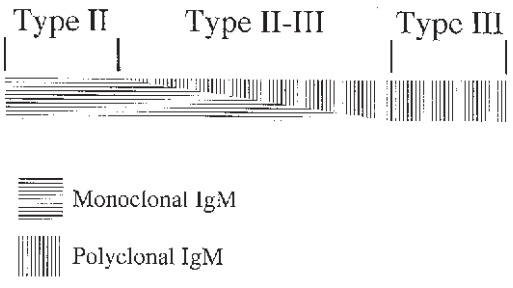

Figure 5. The spectrum of mixed cryoglobulins, typed according to the clonality of their IgM.

not associated with a distinct clinical pattern. On the other hand when we compared two groups of patients (Table 4), those without polyclonal IgM components (type II) and those with polyclonal IgM components (type II-III and type III), the differences were clearly significant.

Unexpectedly, patients with polyclonal components (type II-III and III) had more severe liver involvement. Recently, Agnello[29] proposed a protective effect of monoclonal RF (mRF) based on blocking endocytosis of HCV-VLDL complexes by hepatocytes, via the low density lipoprotein receptor, when mRF reacts with these complexes. At the moment we have no other hypothesis for this result.

The severity of peripheral neuropathy was the only clinical finding that was independent of the type of cryoprecipitate, as previously observed.[11] The pathogenesis of the neuropathy is probably due to inflammatory infiltrates of mononuclear cells around epineural cells as demonstrated by sural nerve biopsies from cryoglobulinemic patients with peripheral neuropathy.[3]

The RFs characterizing the type of cryoglobulins are mono or polyclonal, their presence is not mutually exclusive. The results of this study suggest that monoclonality is associated with more vasculitic pattern: more intense purpura, more severe renal involvement and lower C_4. On the other hand, the presence of polyclonal RF is associated with more severe liver involvement and C_3 consumption.

These facts accord with the concept that cryoglobulinemic syndrome is a melting pot in which ingredients such as HCV, monoclonal RF, polyclonal RF, C_4 consumption, C_3 consumption, high or low cryocrit, are variously mixed in each different patient. Recently, it was suggested that type III cryoglobulinemia represents the earliest step of a progressive process of continuous B cells stimulation by HCV directed towards a monoclonal B cell proliferation.[12] Persistence of HCV infection might be the cause of Ig gene rearrangements with progression to monoclonal B cells with IgM mRF production and cryoglobulin precipitation. However, we found no evidence of this sequence: in our case series poly or monoclonality were not associated with different patients' ages or times from diagnosis.

In conclusion, 2-D PAGE analysis of the sera from 60 patients with cryoglobulinemic syndrome and mixed cryoglobulinemia showed lower prevalences of type II and, more significant, of type III cryoprecipitate than those determined by Brouet's classification; at the same time the number of monoclonal components was higher being an expression of monoclonal B cells expansion.

A complete cryoglobulinemic syndrome can also be present in type III mixed cryoglobulins. Although a distinct syndrome can not be identified as being associated with the various types identified by 2D-PAGE, some differences appear relevant. Patients with type II cryoprecipitate have more severe purpura and a more relevant decrease in C_4 levels than the patients with mono and/or polyclonal IgM in precipitates; the clinical features of purpura and renal involvement appear almost in part related to, and associated with cryocrit levels. In contrast, patients with type II-III and type III cryoprecipitates suffer from more severe liver disease and their serum level of C_3 is lower. No differences are evident in neuropathy. The slight increase in creatinine level in type II patients is difficult to interpret; it may be related to the previous observed more severe membranoproliferative glomerulonephritis in this group of patients.

Although 2-D PAGE analysis of cryoprecipitates is a technique that can not be used routinely in most clinical laboratories, it can be of value in defining the initial events in this syndrome at the border between autoimmunity and lymphoproliferation.

Contribution and Acknowledgments

JDT did 2D-PAGE of all cryoprecipitates and contributed to the writing of the paper. MP was responsible for clinical assessement of the patients, data handling, statistical analysis and interpretation. LT carried out all other studies of cryoprecipitates and was involved in clinical evaluation of the patients. FI formulated the design of the study and was responsible for the writing of the paper. The order of the authors is based on the importance of their contributions. FI is the senior author.

Disclosures

Conflict of interest: none.

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Manuscript processing

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