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Autoantibodies from mixed cryoglobulinaemia patients bind glomerular antigens

M. P. DOLCHER, B. MARCHINI, A. SABBATINI, G. LONGOMBARDO, C. FERRI, L. RIENTE, S. BOMBARDIERI & P. MIGLIORINI *Clinical Immunology and Rheumatology Units, University of Pisa, Pisa, Italy*

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SUMMARY

Mixed cryoglobulinaemia (MC) is a disorder characterized by the presence of large amounts of cryoprecipitating IgM–IgG complexes. An immune complex glomerulonephritis develops in one third of all patients, but its occurrence does not seem related to the amount of cryoglobulins in the sera, nor to their complement-fixing ability. In this study we investigated the presence of IgG antibodies reactive with kidney antigens in 33 MC patients (11 with glomerulonephritis, 22 without renal involvement). A total glomerular extract was run on a 10% acrylamide gel, blotted to nitrocellulose and probed with the patients' sera. Sera from half of the patients without renal involvement reacted with several glomerular antigens whose molecular weight ranged between 200 and 29 kD. In the group with renal involvement, sera from 7/11 patients reacted with an antigen of 50 kD, which is also expressed in thymus, but not in the heart or liver. In a follow-up study of four patients with renal involvement, the amount of serum antibody specific for the 50-kD antigen fluctuated, either spontaneously or in response to therapy. These results show that antibodies specific for glomerular antigens are detectable in MC sera. The immune response against a 50-kD antigen expressed in the kidney and thymus seems to be restricted to a subset of MC patients with renal involvement. Circulating autoantibodies specific for glomerular antigens might contribute to the induction of glomerulonephritis in MC forming immune complexes *in situ*.

Keywords mixed cryoglobulinaemia autoantibodies glomerulonephritis

INTRODUCTION

Mixed cryoglobulinaemia (MC) is a systemic disorder characterized by the presence of cryoprecipitating complexes formed by a monoclonal or polyclonal rheumatoid factor and polyclonal immunoglobulins. Almost one third of all patients develop renal involvement, histologically characterized by a membranoproliferative glomerulonephritis [1–3]. Deposition of circulating immune complexes has been suggested to play a major role in inducing renal damage [4–6]. However, no correlation has ever been observed between the cryocrit, complement or immune complex levels and the presence and/or severity of renal manifestations [7–9].

Autoantibodies can also cause renal damage by direct binding to kidney-specific antigens or to circulating antigens deposited in the kidney (planted antigens).

Further insights into the mechanisms of renal damage have recently been obtained in systemic lupus. MoAbs from lupus-prone mice and from lupus patients were derived; although originally selected for their DNA binding activity, it was shown that these antibodies can bind several antigens, including phospholipids, cell membrane and cytoskeleton proteins [10]. Heparan sulphate, a constituent of the glomerular basement membrane, is one of the antigens recognized by anti-DNA antibodies [11]. A MoAb derived from NZB × NZW mice reacts with proteins expressed on the surface of glomerular cells [12]. In MRL-*lpr*/*lpr* mice, an anti-DNA MoAb has been found that binds directly to the glomerular antigens and which forms renal immune deposits when injected in a normal animal [13]. The pathogenic role of polyreactive anti-DNA antibodies was further demonstrated by the analysis of immunoglobulins eluted from the nephritic kidneys of MRL-*lpr*/*lpr* mice [14].

In IgA nephropathy and Henoch–Schönlein nephritis, IgG antibodies recognizing glomerular antigens have been described. These autoantibodies bind an antigen(s) of 55 and 48 kD expressed on mesangial cells [15].

Autoimmune responses against renal glomeruli are elicited by sequences of the streptococcal M protein [16]; antibodies to basement membrane collagen and laminin have been detected in sera of patients with post-streptococcal nephritis [17]. The role of these antibodies in the pathogenesis of post-streptococcal nephritis is currently under investigation.

On the other hand, antibodies with a cationic charge may preferentially localize in the glomeruli, which have anionic

Correspondence: Paola Migliorini MD, Clinical Immunology Unit, Istituto di Patologia Medica, Via Roma 67, 56126 Pisa, Italy.

basement membranes [18]. A prevalence of cationic antibodies has in fact been described in renal eluates from lupus mice [19,20], but it is not a constant feature of nephritogenic antibodies [14]. Taken together, these data suggest that in systemic lupus anti-DNA antibodies can bind glomerular antigens and form immune deposits.

The presence of antibodies with such a binding specificity has never been investigated in MC. The aim of the present study was to explore the possibility that circulating antibodies can contribute to renal damage in MC, binding renal antigens and thereby forming immune complexes *in situ*.

MATERIALS AND METHODS

Sera

Sera were obtained from 33 patients with MC who were being followed in the Rheumatology and Clinical Immunology Units of the University of Pisa. The diagnosis was based on the typical clinical findings (purpura, weakness, arthritis/arthralgia) and on the presence of serum IgG–IgM cryoglobulins. Sera were clotted at 37°C, centrifuged and stored at −20°C. Renal involvement was established on the basis of repeated abnormal urinalysis, reduced creatinine clearance (at least 15% less than 80 ml/min) and elevated arterial blood pressure (above 145/95 mmHg).

In those patients in whom renal involvement was diagnosed, a kidney biopsy was performed. Light microscopy showed membranoproliferative glomerulonephritis in all of these cases.

Tissue extracts

Bovine kidney, rat liver, rat heart (Sigma Chemical Co., St Louis, MO) and rabbit thymus (Pel Freeze Biologicals, Rogers, AR) extracts were prepared from lyophilized organ powders. Briefly, the powder was resuspended in 50 mM Tris buffer pH 6·8, 20 mM EDTA, 2 mM phenylmethylsulphonylfluoride (PMSF) and 10 μg/ml of leupeptin, sonicated in an ice bath and centrifuged at 30000 *g* for 90 min at 4°C. The protein concentration of the supernatant (organ extract) was determined by the BCA Pierce method (Pierce Chemical Company, Rockford, IL).

Glomeruli were isolated from the cortex of normal human kidneys by differential sieving, as previously described [21]. Glomerular cells were then lysed in the same Tris buffer described above.

The extract (20 μg of protein per mm of gel) was separated on a 10% acrylamide gel under reducing conditions and transferred to nitrocellulose. The nitrocellulose filters were cut into 0·5-mm strips and saturated for a 1-h incubation period in 0·05 M Tris, 0·15 M NaCl and 5% dry non-fat milk. The same buffer was used for the sera dilutions and washings. Sera diluted 1:250 or purified immunoglobulins and $(Fab)_2$ fragments at 30 μg/ml were incubated on filters for 4 h at room temperature. After repeated washings, an alkaline phosphatase-conjugated antiserum was added and the filters were incubated overnight at 4°C. Serum or purified immunoglobulins were detected by goat anti-human IgG $(Fab)_2$ fraction (Sigma); $(Fab)_2$ binding was detected by goat anti-human κ and λ antiserum (Sigma). The immunoactive bands were visualized using 5-bromo-4-chloro-indoxyl-phosphate and nitroblue tetrazolium as substrate [22].

$(Fab)_2$ preparation

The immunoglobulin fraction was isolated from sera by affinity chromatography on a protein A column. The immunoglobulins eluted by 0·1 M glycine pH 2·8 were digested with pepsin-agarose (Sigma). The reaction was stopped after 3 h at 37°C and the Fc fragments were removed by centrifugation on a membrane with a cut-off of 30 kD (Centricon). As shown by SDS gel electrophoresis, the $(Fab)_2$ fraction was not contaminated by undigested immunoglobulins and Fc fragments.

Fibronectin removal

Sera were repeatedly absorbed on a gelatin-Sepharose column (Sigma) and the fibronectin was eluted by 4 M urea. Absorbed sera were dot blotted on nitrocellulose and probed with an anti-fibronectin MoAb [23] to check the efficiency of the absorption.

Isoelectric focusing

Isoelectric focusing of the sera from MC patients was performed as previously described [14] with minor modifications. Focusing was performed in agarose 1%, 10% sorbitol, 4% ampholine (pH 3·5–9·5; Pharmacia LKB, Uppsala, Sweden). The pH gradient of the gel was determined from the migration of pre-stained IEF standards (Biorad, Brussels, Belgium). Twenty microlitres of serum diluted 1:100 in distilled water were applied to each lane. After focusing, the proteins were transferred to nitrocellulose by passive absorption. The nitrocellulose was then saturated in Tris 5% dry non-fat milk, and incubated with an alkaline phosphatase-conjugated goat anti-human IgG $(Fab)_2$ fraction. The immunoactive bands were visualized as described above.

RESULTS

Sera from 33 MC patients were tested for the presence of antibodies binding to human glomerular antigens. In the group of 22 patients who did not show any renal involvement, 11 sera did not react with glomerular antigens, five reacted with one or two antigens, and six reacted with three antigens or more. The molecular weight of the glomerular antigens ranged between 200 and 29 kD (Table 1, group A).

From the group of 11 patients with active renal involvement, four sera did not react with glomerular antigens, and seven (Table 1, group B) reacted with a 50-kD antigen. Three of these seven sera also recognized two additional antigens. These seven sera were tested on a tissue extract prepared with bovine kidney and identical results were obtained (data not shown).

A representative example of the reactivity of MC sera with glomerular extract is given in Fig. 1.

To evaluate the organ specificity of the kidney-reactive antibodies, tissue extracts from rabbit thymus, rat liver and rat heart were used as antigens in an immunoblot. The 11 sera from non-renal patients reactive with glomerular antigens, and the seven sera from renal patients which detected the 50-kD antigen, were tested on these tissue extracts. The results show that some sera reacted with antigens from different organs, but in most cases the molecular weight of these antigens was different from the glomerular antigens. In fact, among the 11 non-renal patients' sera (Table 1, group A), three did not react with heart antigens, five reacted with one or two antigens, and three with three or more; on thymus, eight were negative and three reacted with one antigen; on liver, nine were negative and two reacted with one or two antigens. The only serum in this group that

Table 1. Reactivity with tissue extracts of sera from mixed cryoglobulinaemia patients

Case no.	Kidney	Heart	Thymus	Liver
Group A				
1	72 67 39 38	—	—	—
2	71 67 51 31 29	—	—	—
3	105 53 45 31 28	82 53	—	—
4	55 46	167 104 98 82	—	—
5	200 41	113 72	—	—
6	53 44 39 38 31	75	—	—
7	50	50	50	50
8	37 34	116 104 95 73 53	—	—
9	68 38 31 29	75	—	38 29
10	105 39 38	—	110	—
11	71 29	58 56 31	54	—
Group B				
12	50	—	50	—
13	50	—	50	—
14	50	—	50	—
15	50	—	50	47
16	55 42 50	98	31 50	—
17	57 53 50	—	—	29
18	84 52 50	—	50	—

Sera from mixed cryoglobulinamia patients without renal involvement (group A) or with glomerulonephritis (group B) were tested by immunoblot on kidney, heart, thymus and liver extracts. The number represents the molecular weight in kilodalton of the antigens detected by sera.

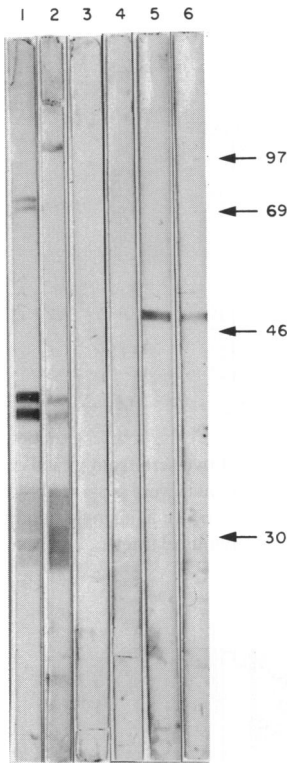

Fig. 1. Reactivity of sera from mixed cryoglobulinaemia patients on glomerular extract. The glomerular extract was separated by SDS gel electrophoresis and transferred to nitrocellulose. Nitrocellulose strips 1, 2 and 3 were incubated with sera from three patients without renal involvement; 4, 5 and 6 with sera from patients with glomerulonephritis. The arrows indicate the molecular weight markers. Several antigens are detected by the first two sera from patients without renal involvement; a single antigen of 50 kD is detected by the last two sera from patients with glomerulonephritis.

reacted with a 50-kD antigen detected a protein of identical molecular weight in the heart, thymus and liver extracts as well (Fig. 2a).

Among the sera from the seven patients with renal involvement (Table 1, group B), six did not react with heart antigens, and one detected a band of 98 kD. On thymus, one was negative and six detected a band of 50 kD; on liver, five were negative and two reacted with antigens of 29 and 47 kD, respectively. An example of a serum reactive with both a glomerular and a thymic antigen of 50 kD is given in Fig. 2b.

The tissue distribution of this antigen suggests that it is distinct from the 50-kD antigen detected by sera from non-renal patients, which is present in the kidney, thymus, heart and liver.

Immunoglobulins from the sera of patients with renal involvement reactive with the 50-kD antigen were isolated by Protein A chromatography and digested by pepsin. $(Fab)_2$ fragments were incubated on blotted glomerular extract and the bound fragments detected by anti-light chain antibodies labelled with alkaline phosphatase. As shown in Fig. 3, $(Fab)_2$ fragments and serum immunoglobulins recognized the 50-kD antigen, thus showing that the binding is Fab- and not Fc-mediated. Similarly, the sera deprived of fibronectin and fibronectin-containing complexes by absorption on a gelatin-Sepharose column still bound the 50-kD antigen (data not shown).

Sera from 10 MC patients with glomerulonephritis and from 10 patients without renal involvement were run on an agarose isoelectric focusing gel. Focused proteins were transferred to nitrocellulose and IgG were detected by a specific antiserum (see Materials and Methods).

The isoelectric points of the serum IgG were similar in the two groups of patients (data not shown).

Follow-up studies

Four patients with renal involvement were followed for 1–2 years and sequential serum samples were available for testing (Table 2).

In two patients (cases 16 and 18) a slow deterioration of renal function occurred during the follow up. Both patients' sera reacted with three different antigens, one of which was 50 kD; antibodies specific for this antigen became undetectable during follow up, while the other specificities persisted unmodified.

In cases 15 and 12, sera reacted only with the 50-kD antigen. In case 15, as in cases 16 and 18, the anti-kidney antibodies disappeared during the phase of gradual worsening of renal function. However, when the glomerulonephritis entered a phase of rapid deterioration, the antibodies again became detectable in the serum.

The fourth patient (case 12) developed a rapidly progressive glomerulonephritis and was treated with plasma exchange. The antibodies against the 50-kD antigen, initially present, decreased in titre during plasmapheretic treatment. A complete remission of the glomerulonephritis was obtained and renal function has been stable thereafter. Despite the clinical remission, however, antibodies have been repeatedly detectable in the

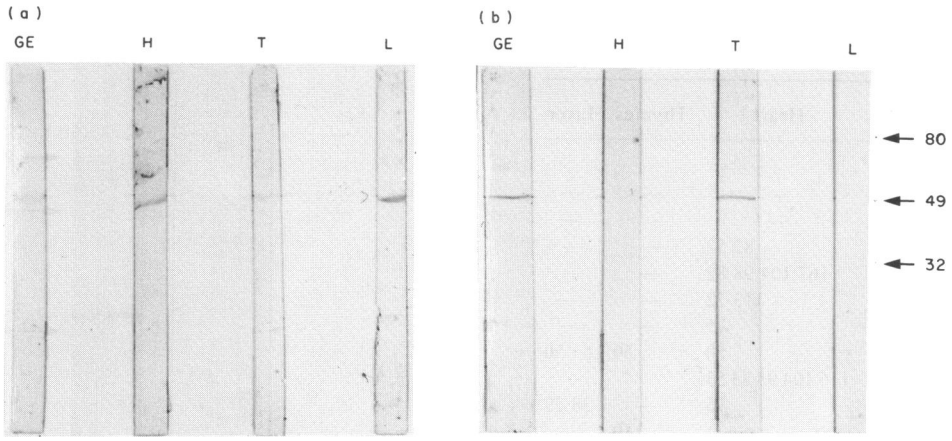

Fig. 2. Reactivity of two sera from mixed cryoglobulinaemia patients with organ extracts. Glomerular extract (GE), heart (H), thymus (T) and liver (L) extracts were separated by SDS gel electrophoresis, transferred to nitrocellulose and incubated with serum from case 7 (a) or from case 12 (b). In a, a single band of 50 kD is detected on all the organs tested; in b the band of 50 kD is present only in the glomerular and thymus extracts.

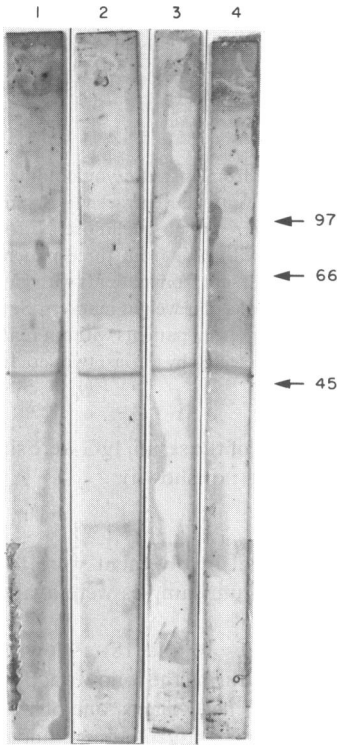

Fig. 3. Reactivity of $(Fab)_2$ fragments with glomerular extract. $(Fab)_2$ fragments were obtained from pepsin digestion of serum immunoglobulins of two renal patients and incubated with blotted glomerular extract. $(Fab)_2$ fragments (lanes 1 and 3) reacted with the 50-kD antigen as the corresponding intact immunoglobulins (lanes 2 and 4).

serum, in amounts comparable to those observed in the active phase of the nephritis (Fig. 4).

DISCUSSION

This study shows that IgG antibodies specific for glomerular antigens are detectable in subjects affected by MC. In particular, the immune response to a 50-kD antigen expressed in kidney and thymus seems to be restricted to a subset of MC patients with renal involvement.

The presence of autoantibodies reactive with glomerular antigens is common in patients with glomerulonephritis, as well as in patients without renal involvement: however, there are clear differences between the two groups. Sera from patients without renal disease either do not react with glomerular antigens or else detect several bands in a wide range of molecular weights. Proteins of 39, 38 and 31 kD are frequently a target of the antibodies in this group; only one serum reacts with an antigen of 50 kD in the kidney, thymus, liver and heart. It is worth noting the reactivity of these sera with a variety of cardiac antigens, whose molecular weight is in most instances different from that of the renal antigens. Such broad reactivity with different tissue extracts may reflect an enhanced immune response against several tissue constituents.

Patients with renal involvement, on the other hand, react in 64% of cases with a band of 50 kD which is also expressed in thymus, but not in heart or liver. In 4/7 patients, the 50-kD antigen is the only one detected on glomerular extract. The binding is not Fc-mediated, since $(Fab)_2$ fragments still bind the antigen. When tested on the other tissue extracts, most sera are negative and only a few react with a low number of antigens. The molecular weight of these antigens is again different from the glomerular ones. Thus, in contrast with the non-renal patients, those with renal involvement show a very restricted immune response. No clinical feature distinguishes the subset of renal patients whose sera react with the 50-kD antigen from the others that do not react with kidney antigens. Disease duration, the severity of organ involvement and disease activity are not different in the two groups. Similarly, the presence of anti-hepatitis C virus (HCV) antibodies and viral RNA [24,25] in serum are identical in the two groups and are not different from non-renal patients.

It is clear from our results that not every anti-glomerular extract antibody detected by immunoblot is potentially nephritogenic. In the 11 patients of Table 1, these antibodies are in fact detected in the absence of any renal involvement. Among the factors that may affect the nephritogenicity of the antibodies that react with glomerular extract by immunoblot, the antibody

Table 2. Follow up of four mixed cryoglobulinaemia patients with renal involvement

	Case 16				Case 18			
	Apr 91	Sept 91	Nov 92	Dec 92	Sept 90	Nov 90	May 91	Jul 92
Reactivity with 50 kD	+	+	−	−	+	+	−	−
Creatinine (mg/dl)	0·9	1	1	1	1·3	1·2	1·5	1·7
Proteinuria (g/24 h)	1	1·4	2·1	2	0·3	0·5	0·3	1

	Case 15				Case 12					
	Jan 90	May 90	Aug 90	Oct 91	Nov 82	Dec 82	Jan 83	Apr 93	Dec 90	Mar 91
Reactivity with 50 kD	+	−	−	+	+	±	+	+	+	+
Creatinine (mg/dl)	3·4	3·5	3·3	6	0·9	0·9	0·9	1	1	1·1
Proteinuria (g/24 h)	0·7	1·6	3	3	2·5	0·3	0·4	0·3	<0·1	<0·1

Serial serum samples from four patients with renal involvement were tested by immunoblot on glomerular extract. At each time point the serum reactivity with the 50-kD antigen, the creatinine level and the 24 h proteinuria were evaluated.

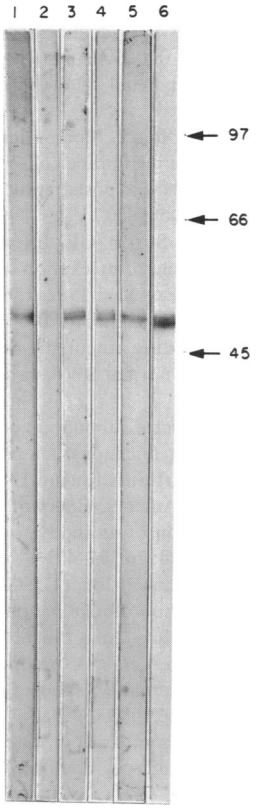

Fig. 4. Follow-up study of case 12. Serial serum samples from patient 12 were tested by immunoblot on glomerular extract. The arrows indicate the molecular weight markers. The reactivity with the 50-kD antigen detectable in the first sample is strongly reduced in the second, and is again present in the last four samples.

class is not relevant, since we measured only IgG antibodies. Similarly, the antibody charge does not seem to be an important factor. Although we did not analyse the isoelectric point of the antibodies specific for kidney antigens, we did not find a prevalence of cationic immunoglobulins in sera from patients with renal involvement. Probably a more important factor is the ability of antibodies reactive in immunoblot to bind the native antigen. Proteins separated by SDS electrophoresis are in fact denatured and unfolded: some conformational epitope is destroyed and new linear epitopes, masked by the folding of the molecule, are exposed [26]. Only some of the antibodies reactive in immunoblot can bind the native antigen, and only some of the antibodies binding the native antigen are detectable by immunoblot.

It is possible that many of the antibodies detected in the sera of patients without renal involvement do not react with native organ proteins.

Reactivity with the 50-kD antigen seemed to be the only factor clearly related to kidney involvement and was therefore more closely analysed. In the four patients for whom we had serial samples, the antibody was present at the beginning of the study and its level fluctuated during the follow up. These fluctuations were either spontaneous (cases 16, 18 and 15) or induced by therapy (case 12). No data are available to establish whether a decreased serum antibody level is related to decreased production or to increased tissue deposition. Moreover, clinical signs of renal disease are late events in immunologically mediated damage, obscuring the correlation between antibody levels and indicators of renal disease. Taking into account these considerations, the follow-up studies suggest that the autoantibodies against the 50-kD antigen might, at least in some patients and in some stage of the disease, play a role in the induction of renal damage. Rheumatoid factors might then contribute to immune complex formation, reacting with the immunoglobulins already bound to renal antigens.

Peripheral vasculitis and glomerulonephritis can be induced in normal mice by the injection of a cryoprecipitating monoclonal rheumatoid factor of the IgG3 class, specific for IgG2a [27]. A hybrid molecule can be engineered, formed by the $\gamma 3$ chain of the original monoclonal and by a different light chain. The injection of this hybrid antibody, which retains the original cryoprecipitating ability but lacks the rheumatoid factor activity, induces glomerulonephritis, but not peripheral vasculitis [28]. In an experimental model of cryoglobulinaemia, distinct pathogenic mechanisms were found to be responsible for two

manifestations of the disease; the deposition of circulating immune complexes seemed to be involved in the induction of skin lesions but not of glomerulonephritis. However, direct evidence for a pathogenic role of circulating autoantibodies in spontaneous or experimental disease can be obtained only from the analysis of tissue-bound immunoglobulins.

The 50-kD antigen detected by MC sera is present in bovine kidney extract and in human glomerular extract, thereby suggesting a glomerular origin. No data are yet available concerning its expression in cells or in the intercellular matrix of the various kidney regions. According to preliminary experiments, the molecular weight of this protein is 50 kD in unreduced form, and does not change after reduction. We can therefore exclude the possibility that we are detecting the heavy chain of an immunoglobulin molecule, as in this case a molecular weight of 150 kD would be expected in the unreduced form. The distribution of the antigen, present in the kidney and thymus but absent in the liver and heart, is not consistent with a serum contaminant such as an immunoglobulin, which should be equally represented in all the organ extracts.

Experiments are in progress to isolate and characterize the 50-kD antigen. The purified antigen could be used to isolate autoantibodies from patients' sera. Immunization-induced antisera could be produced and used to localize the 50-kD protein in kidney. Finally, by immunizing animals with the purified antigen, the possibility of inducing kidney-reactive antibodies can be explored.

All of these experiments should help to clarify the role of the immune response to the 50-kD antigen in the renal disorder of mixed cryoglobulinaemia.

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