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Membranoproliferative glomerulonephritis with masked monotypic immunoglobulin deposits

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The diagnosis of membranoproliferative glomerulonephritis (MPGN) has recently undergone change from an electron microscopy-based classification scheme to one based largely on immunofluorescence findings. This change is due to the recognition that many of these cases are driven by abnormalities of the alternative complement cascade, resulting in the concept of C3 glomerulopathy. Here we reviewed our case files to identify those with an MPGN pattern that show false negative staining for monoclonal immunoglobulins by routine immunofluorescence. Monoclonal immunoglobulin deposits were unmasked by performing immunofluorescence on formalin-fixed paraffin embedded tissue after protease digestion. Clinico-pathological details of 16 such cases with a mean serum creatinine of 2.7 mg/dl and mean 24 h proteinuria of 7.1 g were then determined. Hypocomplementemia was present in two-thirds of patients. Fourteen patients had a paraprotein on serum immunofixation, all of which matched the biopsy immunofluorescence staining pattern. Bone marrow biopsy showed plasma cell dyscrasia or B-cell lymphoproliferative disorder in 13 patients. Ten of these patients had findings on biopsy most consistent with C3 glomerulonephritis prior to performing paraffin immunofluorescence. Thus a high index of suspicion is necessary to avoid misdiagnosis in these cases, as many would have been mistakenly diagnosed as C3 glomerulopathy or unclassified MPGN if paraffin immunofluorescence was not performed.

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Glomerular involvement is not uncommonly present in patients with paraproteinemia. The paraprotein-associated glomerulopathies are classified according to the findings on renal biopsy utilizing light, immunofluorescence (IF), and electron microscopy in combination with the clinical information. Glomerular disorders in this category include immunoglobulin (Ig)-related amyloidosis, immunotactoid GN, type 1 cryoglobulinemic GN, monoclonal Ig deposition disease, proliferative GN with monoclonal Ig deposits, and C3 glomerulopathy with monoclonal gammopathy.[1] These cases commonly fall into the category of monoclonal gammopathy of renal significance when the associated hematological process does not meet diagnostic criteria for overt multiple myeloma or B-cell lymphoma.[2] Even in the lack of a diagnostic hematological process, the monoclonal Ig can have serious renal consequences and treatment of the underlying clonal process is frequently warranted.[3]

It has recently been reported that Ig proteins occasionally show false negative staining by routine IF.[4,5] These deposits can be 'unmasked' by performing IF on the formalin-fixed paraffin-embedded tissue after protease digestion (paraffin IF). Applying this technique to cases that show a membranoproliferative glomerulonephritis (MPGN) pattern has enabled this case series detailing the first clinicopathological description of glomerulopathy with an MPGN pattern by light microscopy and masked monotypic Ig deposits by IF. All these patients had an associated underlying clonal hematological disorder and many of them would have been misdiagnosed as C3 glomerulopathy if paraffin IF was not performed and the masked Igs detected.

RESULTS

The Nephropath renal biopsy database was reviewed from 1 August 2013 to 1 December 2014 for cases with an MPGN pattern by light microscopy that showed 'masked' monotypic Ig deposits on IF (little to no staining for Igs by routine IF and positive Ig staining on paraffin IF with light chain restriction; Figure 1). Nine cases were identified who fulfilled these criteria and were included in this series. During this same time period, there were also six cases of true C3 GN in adults aged >40 years (evidence of MPGN with C3-only staining who did not show Ig unmasking on paraffin IF).

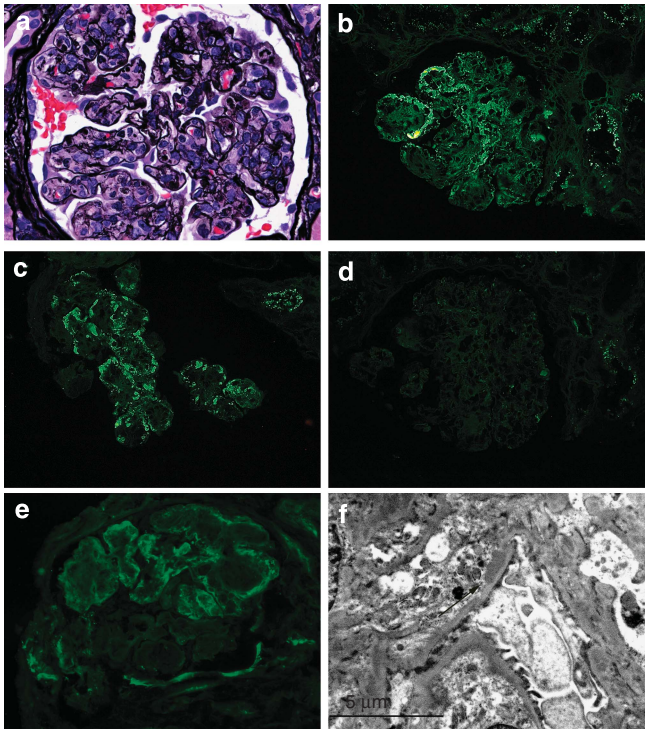

Figure 1 | Light and immunofluorescence microscopic findings in a case of membranoproliferative glomerulonephritis with masked immunoglobulin G (IgG) kappa deposits (patient 2). (**a**) Glomerulus with mesangial expansion, endocapillary hypercellularity, and extensive basement membrane duplication (Jones methenamine silver; original magnification ×400). (**b**) Positive IgG staining within a glomerulus in the paraffin-embedded tissue after protease digestion (direct immunofluorescence). (**c**) Kappa was positive while (**d**) lambda was negative within glomeruli by paraffin immunofluorescence (direct immunofluorescence). (**e**) The C3 was the dominant stain in glomeruli by routine immunofluorescence (direct immunofluorescence). (**f**) Subendothelial electron dense deposits (arrow) were present by electron microscopy (original magnification ×12,000).

The renal biopsy database (from January 2000 to January 2015) of the division of Anatomic Pathology at Mayo Clinic was searched for patients with a known monoclonal gammopathy who had a kidney biopsy showing C3-only or GN with negative glomerular staining for IgG, IgM, IgA, kappa, lambda and C3 by routine IF. Twenty-six cases were identified who fulfilled these criteria, of which 21 had residual paraffin tissue to undergo paraffin IF, including 11 cases of C3 GN, 6 dense deposit disease (DDD), and 4 unclassified MPGN/cryoglobulinemic GN cases. Paraffin IF identified 7 (33%) cases (4 of the 11 C3 GN cases and 3 of the 4 unclassified MPGN/cryoglobulinemic GN cases) with positive staining for Igs (all monoclonal) while the remaining 14 (67%) cases (7 of the C3 GN cases, all 6 DDD cases, 1 of the 4 unclassified MPGN/cryoglobulinemic GN cases) were negative for Igs.

Clinical data around the time of biopsy is presented in Table 1. A total of 16 patients were identified who met inclusion criteria for the study, including 9 cases from Nephropath and 7 cases from Mayo Clinic. The cohort consisted of 9 females and 7 males with a mean age of 61.6 years at the time of biopsy. The vast majority of patients presented with renal insufficiency, proteinuria, and hematuria. Serum creatinine was elevated in 14 of the 16 patients (88%) with a mean value of 2.7 mg/dl. All patients had proteinuria with a mean value of 7.1 g per 24 h and 13 of the 16 patients met criteria for full nephrotic syndrome. Hematuria was present in all 15 patients with this result available. Testing for serum C3 and C4 was performed in 15 patients: 10 (67%) had hypocomplementemia, including 5 with low C3 only, 2 with low C4 only, and 3 with low C3 and C4, while the remaining 5 patients had normal C3 and C4. A single patient had a weakly positive antinuclear antibody with a negative double-stranded DNA. The remaining patients were antinuclear antibody negative. Antineutrophil cytoplasmic antibody was negative in all the 13 patients tested. All patients were negative for hepatitis B and C virus. A cryoglobulin test was positive in 2 (13%) patients.

Fourteen (88%) patients had a paraprotein on serum protein electrophoresis/immunofixation. This paraprotein matched that of biopsy IF staining pattern in 13 cases while in 1 case the immunofixation result was not known (see Table 1). The remaining 2 (12%) patients had negative serum immunofixation but both had evidence of a clonal B-cell population on bone marrow biopsy. Bone marrow biopsy was abnormal in 13 (81%) patients. This includes 9 patients diagnosed with plasma cell dyscrasia, including 4 with multiple myeloma and 5 patients with small clonal plasma cell populations (⩽10%). Four patients were diagnosed with clonal B-cell populations, including two with lymphoplasmacytic lymphoma, one with chronic lymphocytic lymphoma, and one with a small clonal low grade B-cell population. Two patients did not have evidence of clonal hematological processes on bone marrow biopsy but did have a positive paraprotein by serum protein electrophoresis/immunofixation.

Histopathological findings are presented in Table 2. All cases had an MPGN pattern of glomerular injury by light microscopy. There were four cases with crescent formation, all of which were focal. Ten cases had findings most consistent with C3 GN based on the routine IF. In five additional cases, the MPGN was unclassified because of the negative routine IF staining for Igs and complement. Two of these cases (#10 and #12) had light and electron microscopic features suggestive of cryoglobulinemic GN, including intracapillary protein thrombi, phagocytized deposits within glomerular macrophages (Figure 2), focal deposits with substructure, and in one case protein thrombi in vessels. Serum cryoglobulin test was positive in the past in one of these two patients (#10) and was negative in the other patient. The 'masked' deposits were positive for IgG kappa in 12 cases, IgG lambda in 2 cases, IgM lambda in 1 case, and IgM kappa in 1 case. IF staining of the fresh tissue for IgG subtypes 1–4 was completely negative in all nine cases it was performed in.

By electron microscopy, all cases had deposits in the subendothelial space and 12 also had mesangial deposits. Six cases had evidence of subepithelial deposits and 5 cases

Table 1 | Clinical characteristics and follow-up

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Age/gender	64/F	58/M	76/M	70/F	60/F	61/F	55/M	57/F	59/M	52/M	71/F	66/M	53/F	60/F	77/F	47/M
Cr mg/dl	0.8	2.0	1.6	2.2	5.4	1.6	5.2	1.3	2.3	2.2	6	4	0.9	3.2	2.7	1.4
Full nephrotic syn	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes
Prot g/day	1.4	4.6	3.9	5.4	3.5	8.5	7	8	15	4.4	0.6	28	4.5	2.8	3.8	12
Hematuria	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes
C3	Normal	Low	Low	Low	Normal	Normal	Normal	Normal	NA	Low	Low	Normal	Normal	Low	Low	Low
C4	Low	Normal	Normal	Low	Normal	Normal	Normal	Normal	NA	Low	Normal	Normal	Low	Low	Normal	Normal
Serum paraprotein	Neg	IgG K	+M-spike	IgG K	IgG K	IgG K	IgG K	Neg	IgM λ	IgG λ	IgG K	IgG K	IgG K	IgG K	IgM K	IgG K
BM biopsy diagnosis	CLL	MM	5% clonal PCs	Neg	10% clonal PCs	MM	MM	Small clonal B-cell population	LPL	5% clonal PCs	MM	Neg	5% clonal PCs	Neg	LPL	5% clonal PCs
Serum cryoglobulin	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg
Treatment	RTX, bendamustine	Methylpred/carfilzomib	CTX/bortez/DXM	Bortez/RTX/DXM	NA	NA	NA	NA	NA	NA	DXM/bortez	Pred/CTX	MMF/pred then CTX/pred	R-CVP, MMF, bortez, PMP	Bortez/RTX/CTX/pred	CyBorD then pred/CTX
Postbiopsy follow-up (months)	16	2	8	8	NA	NA	NA	NA	NA	NA	16	15	27	17	8	5
Follow-up Cr mg/dl	0.6	1.5	1.5	2.3	NA	NA	NA	NA	NA	NA	0.8	ESRD	1.4	1.4	1.4	2
Follow-up proteinuria	0.9	1.4	0.2	20	NA	NA	NA	NA	NA	NA	NA	NA	0.8	2.9	2.9	12

Abbreviations: BM, bone marrow; Bortez, bortezomib; CLL, chronic lymphocytic lymphoma; Cr, serum creatinine; CTX, cyclophosphamide; CyBorD, cyclophosphamide, bortezomib and dexamethasone; DXM, dexamethasone; ESRD, end-stage renal disease; F, female; IgG, immunoglobulin G; LPL, lymphoplasmacytic lymphoma; M, male; Methylpred, methylprednisolone; MM, multiple myeloma; MMF, mycophenolate mofetil; NA, not applicable; Neg, negative; PC, plasma cell; PMP, plasmapheresis; Pos, positive; Pred, prednisone; R-CVP, rituximab, cyclophosphamide, vincristine, prednisone; RTX, rituximab.

Table 2 | Histopathology of membranoproliferative glomerulonephritis with masked monotypic immunoglobulin deposits

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
LM pattern	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN	MPGN
% crescents	0	0	0	0	11	0	0	8	0	0	0	0	4	40	0	0
Fresh IgA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fresh IgG	0	1	0	0	0	tr	0	0	0	0	0	0	0	0	0	0
Fresh IgM	0	0	0	0	0	0	0	0	0	0	0	tr	1	0	0	0
Fresh C3	3	3	2	2	0	tr	0	2	1	0	0	0	3	2	3	3
Fresh K	0	1	0	0	0	tr	0	0	0	0	0	0	0	0	0	0
Fresh λ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Par IgA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Par IgG	2	3	2	2	3	3	3	3	0	3	3	3	2	3	tr	2
Par IgM	0	0	0	0	0	0	0	0	2	tr	0	tr	1	tr	2	0
Par C3	NP	2	NP	NP	1	1	0	1	NP	1	0	NP	NP	NP	NP	0
Par C1q	NP	NP	NP	0	0	0	NP	NP	0	tr	0	NP	NP	NP	NP	0
Par K	2	3	0	2	2	3	3	3	0	0	3	3	2	3	3	1
Par λ	0	0	2	0	0	0	0	0	2	3	tr	0	0	0	0	0
Deposit type	Fib, G	G	G	G	G	G	Crys, G	G	G	MT	G	Fib, crys	MT	MT	G	G
Deposit location	SEN, SEP, mes	SEN, mes	SEN, SEP, mes	SEN, mes	SEN, mes	SEN, mes	Lum, SEN, SEP	SEN	SEN, SEP, mes	Lum, SEN, mes, SEP	SEN, mes	Lum, SEN	Lum, SEN	Lum, SEN, mes	SEN, SEP, mes	SEN, mes
Concurrent lesions	None	None	None	None	None	None	None	None	None	None	LCCN	NDG	None	None	LHCDD	None
Glomerular Dx before Par IF	C3 GN	C3 GN	C3 GN	C3 GN	MPGN, unclass	MPGN, unclass	TMA	C3 GN	C3 GN	MPGN, unclass/Cryo GN	MPGN, unclass	MPGN, unclass/Cryo GN	C3 GN	C3 GN	C3 GN	C3 GN
Glomerular Dx after Par IF	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg	MPGN with mIg

Abbreviations: C3 GN, C3 glomerulonephritis; Cryo GN, cryoglobulinemic glomerulonephritis; Crys, crystalloid; Fib, vague fibrillar; G, granular without substructure; IF, immunofluorescence; LCCN, light chain cast nephropathy; LHCDD, light and heavy chain deposition disease; LM, light microscopy; Lum, luminal; Mes, mesangial; mIg, monoclonal immunoglobulin; MPGN, membranoproliferative glomerulonephritis; MT, microtubular; NDG, nodular diabetic glomerulosclerosis; NP, not performed; Par, paraffin; SEN, subendothelial; SEP, subepithelial; tr, trace; TMA, thrombotic microangiopathy; unclass, unclassified.

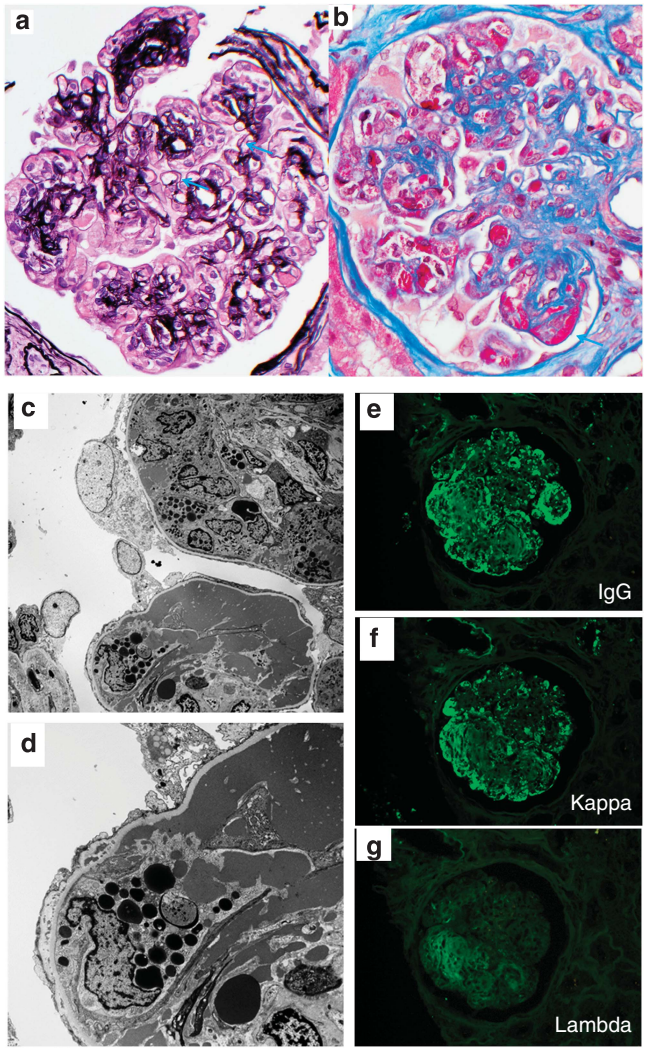

Figure 2 | Renal biopsy findings in patient #12 who had histological features on light microscopy and electron microscopy (EM) compatible with cryoglobulinemic glomerulonephritis. (**a**) A glomerulus on silver stain shows lobular accentuation due to mesangial hypercellularity and sclerosis. There is segmental duplication of the glomerular basement membrane (arrows) with associated cellular interposition (original magnification ×400). (**b**) A glomerulus on trichrome stain shows numerous intracapillary macrophages. A large subendothelial immune deposit is also seen (arrow) (original magnification ×600). (**c**) On EM, large subendothelial and mesangial electron dense deposits are seen. Glomerular peripheral capillaries are occluded by infiltrating monocytes/macrophages (original magnification ×3100). (**d**) A higher magnification image highlighting the large electron dense deposits in the subendothelial space that severely narrow the glomerular capillary lumen. A macrophage with intracellular phagocytized deposits and large phagolysosomes is seen (original magnification ×7830). Panels (**e**–**g**) show bright glomerular capillary wall and mesangial positivity for immunoglobulin G (IgG) (**e**) and kappa (**f**) with negative staining for lambda (**g**) by immunofluorescence on paraffin tissue after pronase digestion, corresponding to a monoclonal IgG kappa on serum immunofixation. The glomerular deposits were negative for IgG, IgA, C3, C1q, kappa, and lambda by routine immunofluorescence on frozen tissue (not shown) (original magnification ×400 for panels (**e**–**g**)).

had intraluminal deposits (4 of them had corresponding 'pseudothrombi' by light microsocopy). No subepithelial 'humps' were identified in any case. Ten cases had deposits of the immune complex type while 3 cases had deposits with a microtubular substructure. One case (#7) showed endocapillary aggregates of dense needle-shaped crystalline deposits that could be mistaken for fibrin tactoids or hyaline thrombi. Another case (#1) had a mixture of granular and vaguely fibrillar deposits. With regards to the 2 cases of cryoglobulinemic GN, the deposits on electron microscopy in one of them (#10) were composed of long microtubules with a mean diameter of 20 nm and parallel arrangement. In the other one (#12), most of the deposits were granular but few show ill-defined fibrillar substructure. Some of the intraglomerular infiltrating macrophages in this case contained large needle-shaped crystalline inclusions.

To determine whether the ultrastructural findings can predict whether paraffin IF will show monoclonal deposits or not in patients with clinical evidence of monoclonal gammopathy and renal biopsy findings that would have been consistent with C3 GN based on frozen IF only, we compared the ultrastructural findings of seven cases of true C3 GN with monoclonal gammopathy (without masked monoclonal deposits) versus four cases of MPGN with masked monoclonal deposits and staining for C3 only on frozen tissue from the Mayo cohort. As evident from the data in Supplementary Table S1 online, subepithelial 'humps', intramembranous deposits, or deposits with only mild electron density, seen in 43, 57, and 29% cases of true C3 GN with monoclonal gammopathy, respectively, were not present in any case of MPGN with masked monoclonal deposits, while organized deposits were only seen in MPGN with masked monoclonal deposits.

Follow-up data was available in 10 patients with a mean follow-up of 12.2 months (2–27 months). The medications used in the treatment of each patient are listed in Table 1. Most of these patients were treated with a therapy directed against the underlying hematological neoplasia, if present. At follow-up, 2 (20%) had partial remissions, 6 (60%) had persistent renal dysfunction, and 1 patient progressed to end-stage renal disease. The remaining patient had normalization of serum creatinine but follow-up 24-h urinary protein collection was not available.

DISCUSSION

MPGN is a pattern of glomerular injury recognized on kidney biopsy when glomeruli show mesangial expansion and basement membrane duplication by light microscopy. Traditionally, MPGN has been divided into three categories based on the appearance and location of the immune deposits by electron microscopy (types 1, 2, and 3). More recently, a shift has been made toward a pathogenesis-based classification system based on the pattern of staining by IF.[6,7] Specifically, biopsies that show the light and electron microscopic findings of MPGN are now categorized as C3 glomerulopathy when there is C3-only or dominant staining

as identified by IF. This is an important distinction, as this designation implies a unique pathogenesis in which the glomerular disease is driven by abnormalities of the alternative complement cascade. 'C3 GN with monoclonal gammopathy' is a subtype of C3 glomerulopathy characterized by C3 deposition in the glomeruli with little to no Ig in the setting of a monoclonal gammopathy.[1] The paraprotein in these cases is thought to cause GN indirectly through interacting with complement regulatory proteins leading to overactivation of the alternative complement pathway.[8,9] However, based on the findings in this study, it is likely that at least some of these cases are caused by paraprotein deposition in the glomeruli resulting in injury. The underlying pathogenic driver of this disease is the presence of a clonal plasma cell or B cell population in most cases, and it is therefore best considered a hematological disorder-associated GN as far as treatment is concerned.

We strongly recommend performing paraffin IF in all patients with clinical evidence of monoclonal gammopathy in whom kidney biopsy shows C3 GN or MPGN with negative IF findings (including cases with light and electron microscopic features of cryoglobulinemic GN), as this technique unmasks glomerular monoclonal deposits in about a third of these patients (see Results). This would provide the treating nephrologists and/or hematologists compelling evidence that the monoclonal protein is the cause of GN and would justify treating with chemotherapy, which can be toxic. This is an important point as the hematological disorder in these patients is frequently a low-grade lymphoproliferative disorder (present in 9 of the 16 patients in our series) in whom chemotherapy would not be given unless MPGN could be proven to be a complication of the underlying hematological disorder.[1] In these patients, paraffin IF establishes the diagnosis of monoclonal gammopathy of renal significance. Monoclonal gammopathy of renal significance is a term that was recently introduced by the International Kidney and Monoclonal Gammopathy Research Group in order to distinguish monoclonal gammopathies associated with severe renal complications due to deposition of monoclonal proteins in the kidney from benign 'monoclonal gammopathy of undetermined significance', which cannot lead to end-organ damage.[1,2]

We found that the ultrastructural findings can help distinguishing MPGN with masked monoclonal deposits from true C3 GN with monoclonal gammopathy. The presence of subepithelial 'humps', intramembranous deposits, or deposits with only mild electron density favor true C3 GN with monoclonal gammopathy. Conversely, the absence of these findings or the presence of organized deposits (the latter being exceedingly rare in true C3 GN) favor MPGN with masked monoclonal deposits. However, as we only studied a small number of these cases and until larger studies become available, we still recommend performing paraffin IF in every case of 'C3 GN with monoclonal gammopathy' regardless of the ultrastructural appearance and location of deposits. Of note, in this study we only evaluated C3 GN associated with monoclonal gammopathy; we do not know if these distinguishing ultrastructural findings can predict whether paraffin IF will show Ig deposits or not in patients with C3 GN without monoclonal gammopathy.

Interestingly, in contrast to C3 GN, none of the six cases of DDD associated with monoclonal gammopathy that we tested by paraffin IF showed unmasked monoclonal deposits. These preliminary data suggest that paraffin IF may not be indicated in cases of DDD and support a different pathomechanism of glomerular injury in DDD associated with monoclonal gammopathy than in C3 GN with monoclonal gammopathy.[10]

We currently do not know why some Igs show false negative staining by routine IF. This phenomenon was first identified in the setting of light chain proximal tubulopathy with crystal formation in which the crystals require paraffin IF to uncover the positive light chain restriction.[11,12] A previous report described a case of MPGN in which masked IgG kappa deposits were uncovered by laser microdissection–mass spectrometry.[13] Considering the fact that up to 41% of MGPN cases have an abnormal serum protein electrophoresis in one study,[14] it is possible that many of these cases represent previously unrecognized paraprotein-associated GN. Finally, membranous-like glomerulopathy with masked IgG kappa deposits is a glomerulopathy most commonly seen in young females that also shows false negative staining by routine IF.[4] It is interesting that all cases with masked Igs described thus far involve a monotypic Ig molecule. Apparently there are qualities of the monoclonal Igs that contribute to the negative staining by routine IF. Perhaps these proteins have a tertiary or quaternary structure that does not allow binding to the target epitope by the antibodies employed and a retrieval technique (such as protease digestion) is necessary to uncover the antigenic epitopes. Alternatively, it is possible that the proteins of interest are washed off from the slide during the routine IF staining procedure due to a charge–charge interaction with the slide while the proteins are held in place on paraffin IF due to formalin-induced cross linking. It is also possible that the higher antibody concentration used in paraffin IF might have a role in detection by this modality. Regardless of what underlies this phenomenon, there is compelling evidence that it exists and that it may be a potential pitfall for misdiagnosis if a high index of suspicion is not maintained.

This finding of 'masked' deposits is a rare phenomenon and paraffin IF is certainly not warranted in the vast majority of cases. We recently published our experience with this technique over a 9-month period at Nephropath.[5] During this period, 2% of the 4969 native kidney biopsies were evaluated by paraffin IF for masked deposits. Out of a total of 97 cases evaluated, paraffin IF was useful or had a significant contribution in identifying masked deposits in 22 cases (23%). We find that paraffin IF is often useful when the findings by routine IF do not match either the clinical scenario or electron microscopic findings. In this series, a masked deposit was uncovered in 36% of Mayo cases that initially showed C3 GN with monoclonal gammopathy. The

Nephropath cases show that, in adults with MPGN and C3-only staining on routine IF, there were equal numbers of cases of C3 GN and MPGN with masked Ig deposits. Because of the high frequency of masked deposits in C3 GN in this biopsy series and others,[4] it is our opinion that C3 GN in adults should be considered a diagnosis of exclusion only after masked deposits have been excluded by paraffin IF.

Although paraffin IF is a useful technique in the renal pathology laboratory, there are inherent pitfalls to be aware of with this procedure. One example is the positive staining of intraluminal serum that could lead to a false positive diagnosis if care is not taken to determine the exact location of the deposits. Therefore, it should not be interpreted as positive unless staining can be identified along the capillary walls and within the mesangium (or in the distribution of 'pseudothrombi') with corresponding deposits by electron microscopy. Luminal staining within the capillary loops in cases lacking intracapillary immune deposits by light microscopy or electron microcopy should be regarded as negative regardless of the staining pattern. One should also be aware of the low sensitivity of this technique for the detection of linear IgG staining in antiglomerular basement membrane disease and the staining intensity is generally weaker than routine IF (occasionally falsely negative) in cases of idiopathic membranous nephropathy and IgA nephropathy.[12] Finally, the technique we are describing is based on formalin fixation; performance of this assay has not been tested with non-formalin-based fixatives.

We provide the first clinicopathological description of cases with an MPGN pattern of GN on biopsy with masked monotypic Ig deposits. This combination of findings is frequently associated with the presence of an underlying hematological disorder. A high index of suspicion is necessary to avoid misdiagnosis in these cases, as many would have been mistakenly diagnosed as C3 glomerulopathy or unclassified MPGN in the past. Paraffin IF is necessary to identify the Ig component and uncover the true nature of these deposits, avoiding misdiagnosis. It is important to identify the 'masked' Ig as this finding frequently informs our understanding of the true pathogenesis of the patient's disease. As these cases are best considered a paraprotein-associated glomerulopathy, treatment directed against the underlying clonal hematological process is likely warranted to treat the GN.

MATERIALS AND METHODS

All cases were processed by light, IF, and electron microscopy using routine techniques described below. All data were collected according to protocols approved by each institution's internal review board.

Light microscopy

Kidney biopsies were fixed in buffered formalin, dehydrated in graded alcohols, and embedded in paraffin using standard techniques. Serial 3-μm-thick sections were cut and treated with hematoxylin and eosin, Jones methenamine silver, Masson trichrome, and periodic acid–Schiff reagent.

Immunofluorescence

For routine IF, samples were transported in Michel's media, washed in buffer, and frozen in a cryostat. Sections, cut at 3–5 μm, were rinsed in buffer, incubated with fluorescein-tagged polyclonal rabbit anti-human antibodies to IgG, IgA, IgM, C3, C4, C1q, fibrinogen, and κ-, and λ-light chains (all from Dako, Carpenteria, CA) for 1 h, and rinsed, and a coverslip was applied using aqueous mounting media.

Paraffin IF was performed at the Mayo Clinic according to the protocol previously described by Nasr *et al.*[12] and at Nephropath according to the protocol described by Messias *et al.*[5] Three-micron sections were cut from the paraffin-embedded blocks onto organosilane-coated slides. The sections were deparaffinized in xylene for 10 min followed by an alcohol gradient. They were then washed in distilled water and rinsed with buffer. Proteinase K (Dako) or pronase (Sigma-Aldrich, St Louis, MO) was applied for 20 min. The sections were then rinsed in buffer, reacted with fluorescein-tagged polyclonal rabbit anti-human antibodies to IgG, IgA, IgM, κ-, and λ-light chains (all from Dako), and rinsed and a coverslip was applied using aqueous mounting media. The results for both traditional IF and paraffin IF were graded on a scale of 0–3 by trained renal pathologists. Supplementary Tables S2 and S3 online detail the protocol for paraffin IF used currently at the Mayo Clinic and Nephropath.

Electron microscopy

Renal biopsy tissue was embedded in epon/araldite resin. Sections 1-μm thick were cut using an ultramicrotome, stained with toluidine blue (Electron Micrscopy Sciences, Hartfield, PA) and examined with a light microscope. Thin sections were examined in an electron microscope.

Clinical definitions

The following definitions were applied: hematuria, >5 red blood cells per high-power field; nephrotic syndrome, 24-h urine protein >3.5 g/day, peripheral edema, and hypoalbuminemia (<3.5 g/dl); and renal insufficiency, serum creatinine >1.2 mg/dl. Proteinuria is reported in g/day, when available, or urine protein/creatinine ratio. The following definitions were applied for the purpose of outcome analysis and are similar to those we used previously[15]: complete remission, remission of proteinuria to <500 mg/day with normal renal function; partial remission, reduction in proteinuria by at least 50% and to <2 g/day with stable renal function (no more than a 20% increase in serum Cr); persistent kidney dysfunction, failure to meet criteria for complete or partial remission but not reaching end-stage kidney disease (incudes patients with unremitting proteinuria or progressive chronic kidney disease); and end-stage kidney disease, requiring renal replacement therapy.

DISCLOSURE

All the authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Table S1. Ultrastructural findings in C3 GN with monoclonal gammopathy (without masked monoclonal deposits) vs. MPGN with masked monoclonal deposits and staining for C3 only on frozen tissue.

Table S2. Paraffin immunofluorescence staining procedure from Mayo Clinic.

Table S3. Paraffin immunofluorescence staining procedure from Nephropath.

Figure S1. Renal biopsy findings in patient #10 who had histologic features on LM and EM compatible with cryoglobulinemic glomerulonephritis.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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