Immunofluorescence on paraffin-embedded sections in evaluation of immune complex deposits in renal biopsy specimens

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The data focused on the value of immunofluorescence on paraffin-embedded sections are controversial, and it is still difficult to obtain reproducible results. The aim of our study was to evaluate the usefulness of immunofluorescence on paraffin-embedded renal section in detecting immune complex deposits in IgA nephropathy (n = 24), membranous glomerulopathy (n = 22) and lupus nephritis (n = 24). Our study revealed that direct immunofluorescence on paraffin-embedded sections pre-treated with proteinase K for 30 or 60 min is a less sensitive method than immunofluorescence on frozen sections; therefore a number of glomerulopathies may be overlooked. Immunofluorescence on paraffin sections showed dominant or co-dominant fluorescence of Riga only in 41.7% of cases of Riga nephropathy. In the studied glomerulopathies the number of positive immunofluorescences of IgA, IgG, IgM and C3 was significantly lower in immunofluorescence on paraffin sections in comparison with findings obtained from immunofluorescence on frozen sections. Irrespective of glomerular disease the rate of agreement between immunofluorescence on paraffin sections and immunofluorescence on frozen sections with respect to the presence of IgA was 56.5%, IgM – 44.4%, IgG – 73.9%, and C3 – 51.5%. In conclusion, our study revealed that immunofluorescence on paraffin sections cannot replace immunofluorescence on frozen sections in the assessment of human renal biopsies, and must be interpreted with great caution.

Introduction

The fluorescent antibody labelling technique of Coons and Kaplan [1] reported in 1950 permitted for the first time identification of antigens and antibodies in freshly harvested animal and human tissue. Within a few years this technique was applied to renal biopsy specimens. Immunopathological renal lesions are crucial for diagnosis, because some diseases, such as IgA nephropathy, C1q nephropathy, and anti-GBM glomerulonephritis, can only be diagnosed by immunohistology, whereas the diagnosis of other diseases is confirmed or refined by immuno-morphological findings. In the evaluation of glomerular diseases, immunohistology is usually directed at the identification of pathogenic immunoglobulin and complement molecules. An immunofluorescence method is used to determine the distribution, pattern, and composition of glomerular immune deposits [2]. By this method, fluorescein-labelled antibodies direct against human immunoglobulin, components of complement cascade, fibrinogen and albumin are applied to frozen sections of kidney and examined under a fluorescent microscope. Antibodies that are used routinely in the evaluation of glomerular diseases include antibodies specific for IgG, IgA, IgM, κ light chains, λ light chains, C3, C4, C1q, and fibrin/fibrinogen [3, 4]. For half a century immunofluorescence on frozen sections (IF-F) has

been the gold standard for immunohistochemical evaluation of renal biopsy specimens, but traditional immunofluorescence has been replaced by immunoperoxidase methods applied to the paraffin sections of formaldehyde-fixed tissue, or by IF performed on formalin-fixed paraffin-embedded tissue [5-8]. It is well known that immunofluorescence on frozen sections is technically easier than immunofluorescence on paraffin sections. Immunofluorescence methods on paraffin sections have the disadvantage that sections have to be treated with proteolytic enzymes before immunostaining, to unmask antigens and to remove the plasma fixed in blood vessels [9]. The data referred to the value of immunofluorescence performed on paraffin sections in diagnosis of glomerulonephritis is controversial, and it is still difficult to obtain reproducible results [5-8]. In view of the above we decided to assess the usefulness of immunofluorescence on formalin-fixed, paraffin-embedded protease-treated and microwave oven heated renal tissue sections in detecting immune complex deposits in three types of human glomerulopathies.

Material and methods

Patients

Kidney tissue biopsies were obtained for diagnostic purposes percutaneously from 24 patients (18 males and 6 females, aged 23-48, mean age = 31) with IgA nephropathy (IgAN), 22 patients (12 males and 10 females, aged 28-53, mean age = 42) with idiopathic membranous glomerulopathy (MGN), and 24 patients (7 males and 17 females, aged 17-37, mean age = 26) with class IV lupus nephritis (LN). Laboratory data including urinalysis, 24 h protein excretion and serum creatinine level were collected from each patient. At the time of biopsy in all patients with IgAN haematuria and albuminuria were noted. All patients with membranous glomerulopathy presented nephrotic syndrome. In 10 patients with lupus nephritis haematuria and proteinuria were noted, whereas 14 patients in this group presented nephrotic syndrome. Renal function impairment was noted in 5 patients with IgAN, in 1 patient with MGN and in 10 patients with LN. In all cases diagnosis of glomerulonephritis was based on characteristic findings by light microscopy (sections stained with Haematoxylin and Eosin, Masson – Trichrome, Jones' silver impregnation and periodic acid-Schiff followed by Alcian Blue), immunofluorescence and electron microscopy using standard protocols. Classification of the histopathological lesions refers to that of the World Health Organization [10] and Renal Pathology Society Working Group on the Classification of Lupus Nephritis [11].

Immunofluorescence

Immunofluorescence was carried out using the following methods: immunofluorescence on frozen tissue (IF-F), immunofluorescence on formaldehyde-fixed tissue and paraffin-embedded sections preceded by microwave treatment (IF-P-MW), immunofluorescence on formaldehyde-fixed tissue and paraffin-embedded sections preceded by microwave treatment plus proteinase digestion (IF-P-MW+P), and immunofluorescence on formaldehyde-fixed tissue and paraffin-embedded sections preceded by proteinase digestion (IF-PP). In all techniques the following polyclonal FITC-labelled rabbit antihuman antibodies (DAKO) were used: IgG (dilution 1 : 20), IgA (dilution 1 : 20), IgM (dilution 1 : 20), and C3 (dilution 1 : 10). Incubation with antibodies was carried out overnight at 4°C in a moist chamber.

Procedure for immunofluorescence on frozen tissue sections (IF-F). A frozen 5-micron cryostat section was attached to Super Frost Plus slides (Menzel-Glaser), air-dried for 15 min and fixed in acetone for 5 min. After three consecutive washes in PBS for 5 min each, sections were incubated with polyclonal FITC-labelled antihuman antibodies.

Procedure for immunofluorescence on paraffin-embedded tissue sections preceded by microwave treatment (IF-P-MW). Paraffin-embedded tissues were cut to a thickness of 3 microns, attached to Super Frost Plus slides (Menzel-Glaser), deparaffinized in xylene and in graded series of ethanol, rehydrated, and afterward were kept in PBS. Microwave treatment with freshly prepared buffer consisting of ethylenediaminetetraacetic acid (EDTA) pH 9.0 (DAKO) for 20 min was carried out. Than slides were cooled at room temperature and were incubated with polyclonal FITC-labelled antihuman antibodies.

Procedure for direct immunofluorescence on formaldehyde-fixed tissue and paraffin-embedded sections preceded by microwave treatment plus proteinase digestion (IF-P-MW+Proteinase). Deparaffinized and rehydrated sections were treated in a microwave oven in buffer EDTA, pH 9.0 (DAKO) for 10 min, and were cooled at room temp. Afterward slides were treated for 10 min or 30 min with proteinase K (DAKO) prepared according to the manufacturer's instruction. Then

slides were incubated with polyclonal FITC-labelled antihuman antibodies.

Procedure for immunofluorescence on formaldehyde-fixed tissue and paraffin-embedded sections preceded by proteinase digestion (IF-PP). Deparaffinized and rehydrated sections were treated for 30 min, 60 min or 120 min with proteinase K (DAKO) prepared according to the manufacturer's instruction. Then slides were incubated with polyclonal FITC-labelled antihuman antibodies.

All sections were mounted in aqueous medium and examined under a dark field ultraviolet fluorescence microscope (Olympus, BX41). Negative controls were carried out with incubation slides with PBS in the absence of the primary antibody and always yielded negative results.

The intensity of immunofluorescence in renal tissue was scored semiquantitatively on a scale of 0 (lack of deposits), trace (+0.5), weak (+1), mode-rate (+2) and strong (+3).

Statistical methods

The differences between groups were tested using non-parametric χ^2 test. Results were considered statistically significant if $p < 0.05$.

Results

Immunofluorescence on paraffin-embedded sections preceded by microwave (IF-P-MW) treatment for 20 min, and immunofluorescence on paraffin-embedded sections preceded by microwave treatment for 10 min plus proteinase digestion (IF-P-MW+Proteinase) for 10 min as well as 30 min did not unmask the antigens, and revealed only trace amounts of fluorescence in the renal tissues. Similarly, immunofluorescence on paraffin-embedded sections preceded by proteinase digestion (IF-PP) for 120 min probably destroyed antigens and revealed trace amounts (+0.5) of deposits in the renal biopsy specimens. In view of the above the results of immunofluorescence on paraffin-embedded sections preceded by microwave (IF-P-MW) treatment, immunofluorescence on paraffin-embedded sections preceded by microwave treatment plus proteinase digestion (IF-P-MW+ Proteinase) for 10 min and 30 min, as well as immunofluorescence on paraffin-embedded sections preceded by proteinase digestion (IF-PP) for 120 min, are not mentioned in this study. Immunofluorescence on paraffin-embedded sections preceded by proteinase digestion (IF-PP) for 30 and 60 min yielded similar findings; therefore the present data include results of both methods. Results of immunofluorescence on frozen as well as paraffin-embedded section in patients with IgAN, MGN and LN are shown in Tables I-IV.

IgA nephropathy

Immunofluorescence on frozen sections. The IgAN group revealed strong (+3) granular

Table I. The results of IgA, C3 and IgM immunofluorescence on frozen and paraffin-embedded sections in 24 cases of IgA nephropathy (IgAN).

	Number of cases with fluorescence of		
	IgA	C3	IgM
IF-F	24	22	16
IF-PP	18	10	6
positivity agreement (%)	75%	45.5%	37.5%
p value	< 0.009	< 0.001	< 0.004

IF-F – immunofluorescence on frozen sections
IF-PP – immunofluorescence on paraffin-embedded sections pre-treated with proteinase K for 30 or 60 min

Table II. The results of IgG, C3 and IgM immunofluorescence on frozen and paraffin-embedded sections in 22 cases of membranous glomerulopathy (MGN)

	Number of cases with fluorescence of		
	IgG	C3	IgM
IF-F	22	22	2
IF-PP	16	6	2
Positivity agreement (%)	72.7%	27.3%	100%*
p value	< 0.009	< 0.001	= 1.0 (NS) *

IF-F – immunofluorescence on frozen sections
IF-PP – immunofluorescence on paraffin-embedded sections pre-treated with proteinase K for 30 or 60 min
** Only two cases with positive IF on frozen, as well as on paraffin sections.*

Table III. The results of IgG, C3 and IgA immunofluorescence on frozen and paraffin-embedded sections in 24 cases of lupus nephritis (LN)

	Number of cases with fluorescence of		
	IgG	C3	IgA
IF-F	24	22	22
IF-PP	18	6	8
Positivity agreement (%)	72.7%	27.3%	36.4%
p value	< 0.009	< 0.001	< 0.001

IF-F – immunofluorescence on frozen sections
IF-PP – immunofluorescence on paraffin-embedded sections pre-treated with proteinase K for 30 or 60 min

Table IV. The results of the intensity of immunoglobulin and C3 immunofluorescence on frozen and paraffin-embedded sections in IgA nephropathy, membranous glomerulopathy and lupus nephritis

	Strong IF (+3)		Moderate IF (+2)		Weak IF (+1)		Trace IF (+0.5)	
IgA Nephropathy (n = 24)	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**
IgA	18	0	6	6	0	6	0	6
C3	4	2	12	2	6	0	0	6
IgM	0	0	0	2	14	0	2	4
Membranous glomerulopathy (n = 22)	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**
IgG	12	4	8	2	2	8	0	2
C3	8	0	8	2	6	2	0	2
IgM	0	0	0	0	2	2	0	0
Lupus nephritis (n = 24)	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**	**IF-F**	**IF-PP**
IgG	14	2	8	8	2	6	0	2
C3	8	0	10	2	4	0	0	4
IgA	4	0	12	0	6	2	0	6

IF-F – immunofluorescence on frozen sections
IF-PP – immunofluorescence on paraffin-embedded sections pre-treated with proteinase K for 30 or 60 min

fluorescence of IgA in mesangium and in paramesangial areas in 18 cases. In 6 renal tissues in this group the intensity of IgA fluorescence was moderate (+2). In all cases IgA was the dominant or co-dominant immunoglobulin in deposits. Mild mesangial, granular (+1) fluorescence of IgM was noted in 14 cases of IgAN, and only a trace of IgM was seen in 2 other cases of this glomerulopathy. The intensity of C3 fluorescence was strong in 4 renal samples, moderate in 12, and mild in 6 cases of IgAN. The fluorescence of C3 was granular and located in mesangial areas. No IgG deposits were seen in the IgAN group in the IF-F technique. In immunofluorescence on paraffin-embedded sections preceded by proteinase digestion (IF-PP) for 30 and 60 min in this group granular deposits of IgA in mesangium and paramesangial areas (Fig. 1) were seen in 18 of 24 cases (75%), granular deposits of C3 in 10 cases (45.5%) and granular deposits of IgM in 6 renal biopsies (37.5%). We did not notice strong fluorescence of IgA in this group. The intensity of IgA fluorescence was moderate and mild. A trace of IgA was noted in 6 renal biopsy specimens in patients with IgAN. The intensity of IgM fluorescence was moderate in 2 cases and very low (+0.5) in 4 cases of IgAN. In 2 renal biopsies deposits consisted of a large amount of C3 (strong fluorescence), whereas the intensity of C3 fluorescence was moderate or very low in 8 cases of IgAN. Only in 41.7% of IgAN cases did the IF-PP method show dominant or co-dominant fluorescence of IgA. Statistical analysis revealed that in the renal biopsies from patients with IgAN the number of positive fluorescences of IgA, C3 and IgM was significantly lower in the IF-PP method than in the IF-F method ($p < 0.009$, $p < 0.001$ and $p < 0.004$, respectively).

Membranous glomerulopathy

In immunofluorescence applied on frozen sections in the MGN group granular deposits along glomerular capillary loops were seen in all cases. The intensity of fluorescence was strong (+3) in 12 renal biopsy specimens, moderate (+2) in 8, and weak (+1) in 2 cases. Deposits of IgM were noted in 2 renal tissues in this group. In renal tissues in all patients with MGN granular deposits of C3 along glomerular capillaries were seen. The intensity of C3 fluorescence was strong in 8 cases, moderate in 8, and weak in 6 biopsy specimens. No IgA deposits were seen in the MGN group. Using immunofluorescence on paraffin-embedded sections in the MGN group granular deposits of IgG along glomerular capillary loops were seen in 16 of 22 cases (72.7%), granular deposits of C3 in 6 cases (27.3%) and granular deposits of IgM in 2 renal biopsies. In 4 renal biopsies deposits of a large amount of IgG (strong immunofluorescence) (Fig. 2) were noted in the IF-PP method. The intensity of IgG fluorescence was moderate in 2 cases, mild in 8 renal tissues and very low (+0.5) in 2 cases of MGN. Statistical analysis showed that in the renal tissue in the MGN group the number of positive fluorescences of IgA and C3 was significantly lower in the IF-PP method than in

the IF-F technique ($p < 0.009$, and $p < 0.001$, respectively).

Lupus nephritis

Immunofluorescence on frozen sections revealed in all cases of LN deposits of IgG. Granular deposits of IgG were localized to mesangium and distributed along glomerular capillary loops. In 14 renal biopsies the intensity of IgG fluorescence was strong, moderate in 8 cases, and mild in 2 renal tissues in this group. In the IF-F method IgA and C3 deposits were noted in 22 cases of LN. The intensity of IgA fluorescence was strong in 4 biopsies, moderate in 12, and weak in 6 cases of LN, whereas the intensity of C3 fluorescence was strong in 8 biopsies, moderate in 10, and weak in 4 renal tissues. Immunofluorescence on paraffin sections pre-treated with proteinase at 30 or 60 min revealed in patients with LN granular deposits of IgG in mesangium and along glomerular capillaries in 18 of 24 cases (72.7%), granular deposits of C3 in 6 cases (27.3%), and granular deposits of IgA in 8 renal biopsies (36.4%). The intensity of IgG fluorescence was strong (Fig. 3) in 2 cases, moderate in 8 cases, mild in 6 renal tissues and very low (+0.5) in 2 cases of LN. Weak deposits of IgA were seen in 2 renal tissues in patients with LN, and in 6 cases immunofluorescence performed on paraffin sections revealed only trace amounts of IgA. The intensity of C3 fluorescence was moderate in 2 cases, and very low (+0.5) in 4 cases of LN. Statistical analysis showed that in the LN group

Table V. Results of IgA, IgG, IgM and C3 immunofluorescence on frozen and paraffin-embedded sections in all studied cases irrespective of the type of glomerulopathy

	IgA	IgG	IgM	C3
The number of positive immunofluorescences in IF-F	46	46	18	66
The number of positive immunofluorescences in IF-PP	26	34	8	34
Positivity agreement (%)	56.5	73.9	44.4	51.5

IF-F – immunofluorescence on frozen sections
IF-PP – immunofluorescence on paraffin-embedded sections pre-treated with proteinase K for 30 or 60 min

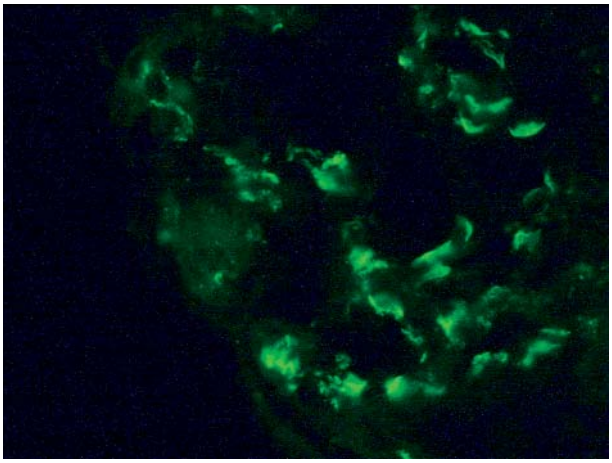

Fig. 1. Renal biopsy specimen from patient with IgA nephropathy. Moderate granular immunofluorescence of immunoglobulin A in mesangium. Immunofluorescence on paraffin-embedded tissue with anti-human FITC-labelled polyclonal IgA antibody and proteinase K predigestion for 30 min. Magnification 400 ×

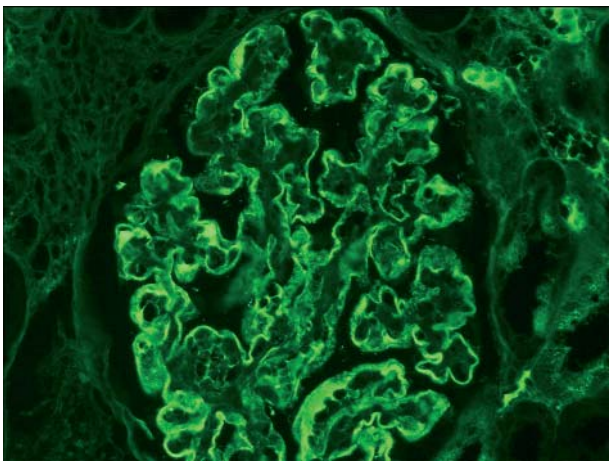

Fig. 2. Renal biopsy specimen from patient with membranous glomerulopathy. Strong granular immunofluorescence of immunoglobulin G along glomerular capillary walls. Immunofluorescence on paraffin-embedded tissue with anti-human FITC-labelled polyclonal IgG antibody and proteinase K predigestion for 30 min. Magnification 400 ×

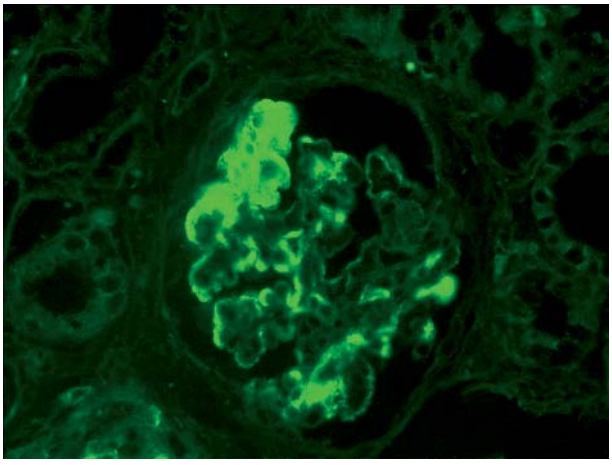

Fig. 3. Renal biopsy specimen from patient with class IV lupus nephritis. Strong granular immunofluorescence of immunoglobulin G in mesangium and along glomerular capillary walls. Immunofluorescence on paraffin-embedded tissue with anti-human FITC-labelled polyclonal IgG antibody and proteinase K predigestion for 60 min. Magnification 400 ×

the number of positive immunofluorescences of IgG, C3 and IgA was significantly lower in the IF-PP method in comparison with the IF-F method ($p < 0.009$, $p < 0.001$, and $p < 0.001$, respectively). The results of IgA, IgM, IgG and C3 immunofluorescence in all studied cases irrespective of type of glomerulonephritis are shown in Table V. Immunofluorescence on paraffin-embedded sections preceded by proteinase digestion (IF-PP) for 30 or 60 min revealed IgA deposits in 26 (56.5%) of 46 renal biopsy specimens with positive staining in IF-F, IgM deposits in 8 (44.4%) of 18 cases positive in IF-F, IgG deposits in 34 (73.9%) from 46 cases, and C3 deposits in 34 (51.5%) of 66 renal biopsy specimens positive in IF-F.

Discussion

Immunofluorescence staining of renal biopsies for the deposition of immunoglobulin and complement components is often the primary approach for a differential diagnosis of glomerular disease [12]. It is postulated that immunomorphological examination must be a part of every diagnostic renal biopsy [4]. Direct immunofluorescence on frozen tissue remains the most widely used technique of immunohistochemistry [13]. This method requires native tissue without fixation, and moreover when the diagnostic material is scanty, the part of renal tissue assigned for immunofluorescence may not contain glomeruli. Recent data concerning immunomorphological methods used in nephropathology suggest that snap-frozen tissues provide the best sensitivity for antigen detection; however, satisfactory results can be obtained with fixed tissue if antigen-retrieval strategies are employed [6, 7, 12, 13]. In our study we used three methods of retrieving antigens from formaldehyde-fixed and paraffin-embedded tissue: proteolytic digestion with proteinase K for 30, 60 and 120 min, microwave treatment for 20 min, and a mixed method of both treatments: microwave treatment for 10 min plus proteinase K for 10 or 30 min. Direct immunofluorescence on paraffin-embedded sections preceded by proteinase digestion (IF-PP) for 30 and 60 min was a method of choice to detect immunoglobulin and complement component in formalin-fixed renal tissue. Unfortunately, using microwave treatment, as well as mixed technique (microwave for 20 min plus proteinase K for 10 or 30 min), and proteinase K for 120 min we obtained false negative results, probably due to damage of the tissue. In the study of Chowdhury *et al.* [7] microwave treatment without protease digestion did not unmask the antigens and protease digestion without microwave treatment was effective when tissue was digested for more than 30 min. In contrast to our results, the above-mentioned authors using microwave treatment plus protease digestion for 30 or 60 min and trypsin digestion for 120 min revealed in more than 80% of cases IgA deposits in IgA nephritis, and IgG deposits in lupus nephritis, as well as in membranous nephropathy. It must be stressed that our results showed good preservation of tissue morphology in the IF-PP method, while the intensity of immunofluorescence was much lower than in IF-F. Statistical analysis showed that in all studied glomerulopathies the number of positive immunofluorescences of IgA and C3 was significantly higher in the IF-F method than in the IF-PP method. The highest percentage agreement of positive cases in IF-F and IF-PP for IgG was noted in membranous glomerulopathy and in lupus nephritis. A high percentage of positivity for IgA fluorescence was obtained in nephropathy IgA; however, in only 41.7% of IgAN cases IF-PP technique showed dominant or co-dominant fluorescence of IgA, which creates a possibility to establish a diagnosis of IgA nephropathy. These results are in contrast to other studies. Using immunofluorescence on paraffin section Nasr *et al.* [13] obtained diagnostic findings in 100% of cases of lupus nephritis, 88% of cases of immunoglobulin A nephropathy and 50% of cases of idiopathic membranous glomerulopathy, but IF-P was less sensitive than IF-F for the detection of C3 in all disease categories and for detection of IgG in cases of membranous glomerulopathy. Irrespective of the type of glomerular disease, our study revealed that positivity agreement between IF-F and IF-PP methods ranged from 44.4% to 73.9%. The positivity agreement for IgA was 56.5%, for IgM – 44.4%, for IgG – 73.9% and for C3 – 51.5%. Fogazzi *et al.* [14] found a high percentage agreement of positive and negative cases and IF intensity for the main antigens: IgG in membranous glomerulopathy, IgA in IgA nephropathy and IgG and C1q in lupus nephritis. Qualman *et al.* [15] reported 80-90% rate of agreement between IF-P after trypsin digestion and IF-F with respect to the presence or absence of IgG, IgM, IgA, and fibrinogen deposition. It is possible that discrepancies between our results and others depend to some extent on the type of enzyme applied on paraffin-embedded tissues for proteolytic digestion. Among proteolytic enzymes used for the pre-treatment of paraffin sections pepsin, trypsin or pronase (*Streptomyces griseus*) is employed in the majority of laboratories

[13-16]. Van der Ven *et al.* [17] revealed that immunofluorescence on bacterial proteinase XXIV-digested paraffin sections is generally more sensitive than IF-P on pronase-digested sections, and less background was observed. These authors demonstrated that in renal biopsies from patients with lupus nephritis staining for IgG and C1q was more sensitive in sections digested with proteinase XXIV than in those digested with pronase.

In conclusion, our results demonstrate that the investigation of immune complexes in paraffin sections harbours the risk of being less sensitive than studies performed on frozen material; therefore the findings obtained from immunofluorescence on formalin-fixed paraffinembedded renal tissue must be interpreted with great caution. Applying proteinase K on paraffin sections for 30 or 60 min is sufficient to unmask antigens and to remove the plasma fixed in blood vessels. Immunofluorescence on formalin-fixed, paraffin-embedded sections cannot replace immuno-fluorescence performed on frozen tissue in the assessment of human renal biopsies, but may be a salvage technique in the diagnosis of glomerulonephritis when frozen tissue is not available.

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