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ORIGINAL ARTICLE

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C4d Immunohistochemistry in glomerulonephritis with different antibodies

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Abstract
Background. The presence of C4d in the kidney is generally detected particularly for the diagnosis of antibody-mediated rejection in renal transplants. In frozen sections of immunofluorescence (IF) staining with anti-C4d monoclonal antibodies (mAbs), we noted intrinsic C4d deposition even in normal glomeruli though their pathogenic or an intrinsic role is unkown. An anti-C4d polyclonal antibody (C4dpAb), which is suitable for paraffin immunoperoxidase (IP) staining, is less used than mAbs, and it has demonstrated that intrinsic C4d is not evident. To establish a stable and reproducible procedure for C4d detection with the C4dpAb and to determine the staining characteristics of it, the present study aimed to test whether the method was comparable with IF with a mAb.
Methods. We compared the C4dpAb with the mAb in adjacent sections of human diseased kidneys, and then compared IP with IF of C4dpAb. Two ways of antigen retrieval was examined for IP.
Results. On comparing the two antibodies for glomerular staining with IF, we found that the pattern and intensity (C4dpAb showed intrinsic C4d with IF) were similar. In addition, C4dpAb staining with IP and IF demonstrated that the intrinsic staining in the normal glomerulus was mostly undetectable by IP, whereas IF showed distinct staining. Likewise, C4d deposition with IP in some cases was apparently weaker than that on IF, suggesting that this deposition is not intrinsic but indicates pathogenic complement activation.
Conclusions. The advantage of the C4dpAb for immunohistochemistry is of value for reconsidering the role of C4d in glomerular diseases.

Key words C4d · Glomerulonephritis · Paraffin embedded section · Polyclonal antibody

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Introduction

Complement activation is known to be involved in both the onset and progression of immune-mediated renal diseases.[1,2] Immunostaining reveals the deposition of complement components, including C1q, C3, C4, and C5b-9, which indicates the activation of complement pathways.[3]

C4d is a stable complement split product covalently bound to vascular endothelium and is produced via two pathways in the accelerated complement cascade system; the classical and the lectin pathways.[3] The deposition of C4d is found in several types of glomerulonephritis.[4–6]

C4d immunostaining is generally used for the diagnosis of antibody-mediated rejection of renal allografts on frozen sections.[7] There are three types of antibodies available for the detection of tissue C4d. There are two monoclonal antibodies, one manufactured by Quidel (cat no. A213; San Diego, CA, USA) and one manufactured by Biogenesis (cat no. 2222-8004; Kingston, NH, USA); these are used for immunofluorescence (IF) examination on frozen sections. The third type is a polyclonal antibody (C4dpAb; Biomedica, Vienna, Austria, cat no. BI-RC4D); this is usually used for immunoperoxidase (IP) methodology on formalin-fixed, paraffin-embedded tissue sections. For the detection of C4d in peritubular capillary (PTC) staining, these antibodies showed similar staining features.[8] However, it has been noted that glomerular C4d staining patterns for these antibodies are different. In the normal kidney, both monoclonal antibodies show mesangial and arteriolar fluorescence for C4d; however, IP with polyclonal antibodies lacks these signals. We have noted that lupus nephritis and IgA glo-

merulonephritis occasionally show distinct C4d deposition within the mesangium, which is thought to be excessive in relation to intrinsic C4d deposition. Because complement involvement in glomerular disease is based on its presence and on its pattern of deposition, intrinsic C4d staining by monoclonal antibodies may cause problems of interpretation; therefore it is better to determine C4d deposition with a polyclonal antibody. Furthermore, because immunostaining can show many glomeruli with a structural background, we surmise that the use of a polyclonal antibody with IP would be better than using monoclonal antibodies to visualize relevant C4d deposition, if the polyclonal antibody is equivalent to the monoclonal antibodies for the detection of deposition.

To determine relevant C4d deposition without the intrinsic deposition, the present study tested whether, in glomerulonephritis, IP with a polyclonal antibody showed C4d staining patterns similar to those seen with IF with a monoclonal antibody. Because a monoclonal antibody is not suitable for use with paraffin sections, differences between two antibodies with different applications cannot be directly compared. Using biopsy samples of glomerulonephritis, we first compared the staining patterns of C4d in glomerular diseases with monoclonal antibodies and polyclonal antibodies with IF to determine whether they had similar characteristics. We then analyzed the C4d deposition pattern with a polyclonal antibody by IF and IP.

Our results suggest that the polyclonal C4d antibody with IP by pressure-cooking shows significant C4d staining without any vague intrinsic background, and this method is useful to access the role of this complement in glomerulonephritis.

Patients and methods

Patients

Eleven renal biopsy samples for frozen-tissue analysis (comparing two antibodies) were obtained from patients attending Tokyo Women's Medical University: there were two cases of IgA glomerulonephritis (IgAGN), two cases of membranoproliferative glomerulonephritis (MPGN), two cases of lupus nephritis with membranous deposition (LNV+), one case of lupus nephritis without membranous deposition (LNV–), two cases of membranous glomerulonephritis (MN), and two cases of minimal change disease (MCD). In addition, kidney biopsy samples from 49 patients with various renal diseases were obtained from Matsuyama Red Cross Hospital for C4dpAb analysis (comparing IP and IF with a single antibody): there were 11 cases of IgAGN, 5 of Henoch-Shönlein purpura nephritis (HSPN), 11 of MPGN, 2 of LNV+, 9 of LNV– and 11 of MN. Pathological diagnosis was made using standard paraffin sections, using data obtained from immunofluorescent staining, and from electron microscopy (EM) coupled with clinical data. Classification of lupus nephritis was performed according to the 2003 ISN/RPS Classification of lupus glomerulonephritis.[9]

Frozen-tissue analysis

The methodology was as previously described. Briefly, frozen sections were cut at 3-μm thickness and air-dried for 30 min. The slides were not fixed in acetone. After a rinse in phosphate-buffered saline (PBS), sections for IF analysis (the sections from patients at Tokyo Women's Medical University) were incubated with the Quidel anti-C4d monoclonal antibody or C4dpAb, both at a dilution of 1:50, for 1 h at room temperature. After PBS washes, a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG (H+L; Invitrogen, Carlsbad, CA, USA) or a fluorescein-conjugated goat antibody to rabbit IgG (ICN/Cappel, Aurora, OH, USA), was applied at a dilution of 1:80 for 30 min at room temperature. After PBS washes, the slides were coverslipped with Shandon PermaFluor (Waltham, MA, USA).

C4dpAb analysis

We used the C4dpAb for IP of paraffin sections and for indirect IF of frozen sections. IP methodology was performed on all samples from the Matsuyama Red Cross Hospital. Formalin-fixed paraffin sections were processed by IP methodology as previously described. Antigen retrieval was done by using two protocols. One was by pressure-cooking (PC) in a microwave oven at a maximum pressure of 0.0392 MPa, and at a maximum temperature of 110°C for at least 15 min in 10 mM citrate-buffer (pH 6.0). For the other protocol, we used a microwave processor (MW) at 98°C for 15 min, with citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with hydrogen peroxide and methanol. After overnight incubation with C4dpAb (1:100; Biomedica, Vienna, Austria), bound IgG was visualized using the Histofine SAB-PO(R) kit (Nichirei, Tokyo, Japan) and Liquid DAB+ Substrate Chromogen System (Dako Denmark, Glostrup, Denmark), according to the manufacturers' protocols. We analyzed C4d staining patterns using light microscopy.

The indirect IF study, using standard procedures, was performed on frozen sections that were available from the samples of the Matsuyama Red Cross Hospital patients: we examined five cases of IgAGN, three of HSPN, four of MPGN, one of LNV+, four of LNV– and five of MN. Dilutions of C4dpAb and secondary antibody (fluorescein-conjugated goat antibody to rabbit IgG; ICN/Cappel) were 1:50 and 1:80, respectively. Specimens were viewed with an Olympus AX 80 (Olympus, Tokyo, Japan).

Results

In the frozen-tissue analysis, specimens of both the MCD cases showed mesangial C4d staining with the Quidel monoclonal antibody. However, in one of the cases, mesangial C4d staining was weak with C4dpAb (Fig. 1F). Mesangial C4d deposition was also seen in most glomerulonephritis specimens, with the exception of one LNV+ case and one MN case. Other than the mesangial area, in MPGN, MN,

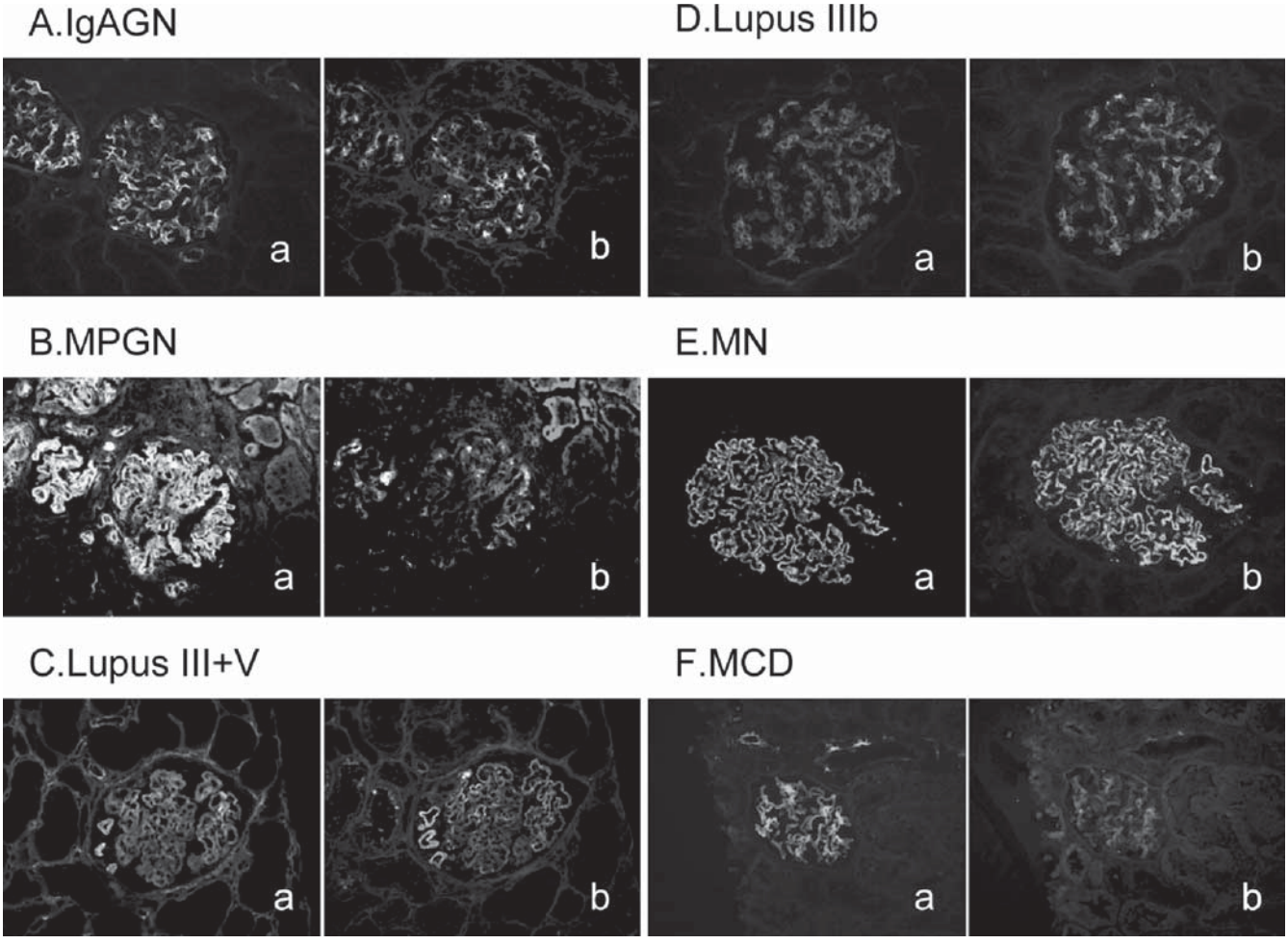

Fig. 1A–F. Representative immunofluorescence (IF) micrographs of C4d staining comparing the Quidel anti-C4d monoclonal antibody (mAb) and the C4d polyclonal antibody (pAb). **A** IgA glomerulonephritis (*IgAGN*): mesangial pattern is obvious in the glomerulus stained with Quidel antibody (*a*). A similar pattern of less brightness is seen in glomeruli stained with C4dpAb (*b*). **B** Membranoproliferative glomerulonephritis (*MPGN*): a peripheral pattern is prominent and tubular cells are also C4d-positive (*a*, Quidel antibody). C4dpAb staining shows a similar pattern, but is weaker (*b*). **C** Lupus nephritis with membranous deposition (LNV+; *lupus nephritis III+V*): C4d-positive area is seen along the glomerular basement membrane (GBM) and TBM in *a* and *b*. An area of focal high brightness is seen in the GBM area (*a*, Quidel antibody; *b*, C4dpAb). **D** Lupus nephritis without membranous deposition (LNV–; *lupus nephritis IIIb*): mesangial pattern and some peripheral patterns are seen (*a*, Quidel antibody; b, C4dpAb). **E** Membranous glomerulonephritis (*MN*): granular membranous pattern (*a*, Quidel antibody; *b*, C4dpAb). **F** Minimal change disease (*MCD*): mesangial and peripheral pattern is seen in the glomerulus stained with Quidel antibody (*a*). A similar pattern, of less intensity, is seen in the glomerulus stained with C4dpAb (*b*)

and LNV+ cases, a loop pattern of C4d deposition was seen in the glomeruli (Fig. 1B, C and E). In MN and LNV+, C4d deposition was seen in a granular pattern. In the LNV+ cases, the C4d-positive area was segmental. Furthermore, there was a relative trend that Quidel monoclonal antibody staining showed stronger intensity than C4dpAb.

For the C4dpAb analysis, representative C4dpAb staining patterns of indirect IF and IP, with the two methods of antigen retrieval, are shown in Fig. 2. The incidence and staining patterns in each of the glomerulonephritides are summarized in Table 1. C4d deposition within the mesangial area was seen in a few cases. One of the 11 cases of IgAGN (Fig.2A-a and-b), one of the 5 HSPN (Fig. 2B-b and -c), and 2 of the 9 LNV– cases showed a mesangial C4d pattern (data not shown). In Fig. 2A, the signal of C4d deposition in the mesangial area of IgAGN was more enhanced by MW than by PC. In contrast, in Fig. 2B-b and -c, the signal of C4d deposition of HSPN was less enhanced by MW than by PC. Thus, we did not find any obvious tendency showing the efficacy of antigen retrieval in glomerular lesions. Among the C3-positive MN cases (10/11), only 1 was C4d-negative; it was stage I MN, with IgG deposition seen on IF and electron-dense deposits aligned sparsely along the glomerular basement membrane (GBM) seen on an electron micrograph. C3 was positive in the glomeruli in all cases of MPGN and lupus nephritis. However C4d, was positive in the glomeruli in about half of the cases of MPGN and in 4 cases of LNV–.

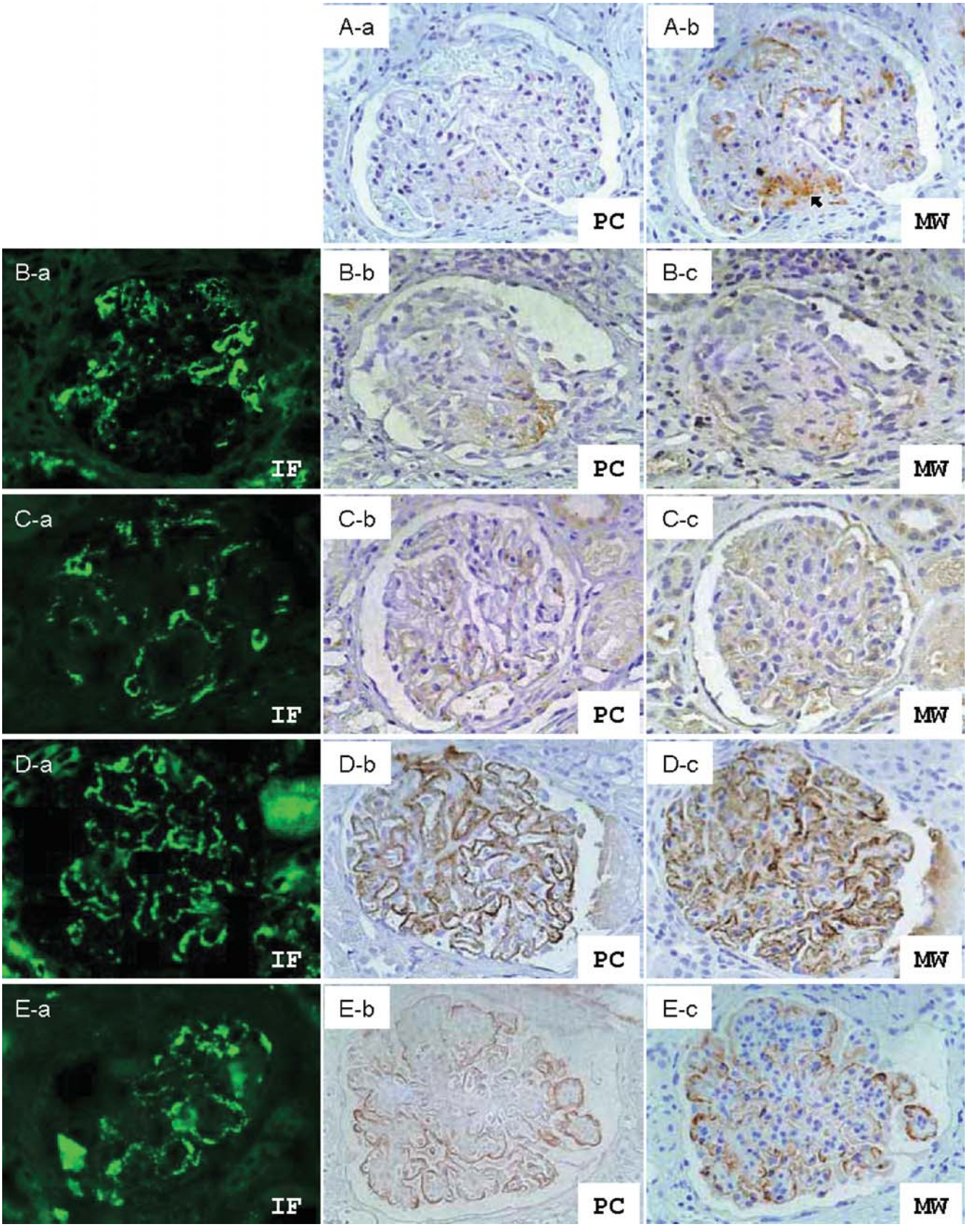

Fig. 2A–E. Representative immunoperoxidase (IP) and IF micrographs of C4dpAb staining. **A** IgAGN: microwave (*MW*)-retrieved glomerulus (**A**-*b*) shows mesangial deposition, which is only faintly detectable in the pressure-cooker (*PC*)-retrieved one (**A**-*a*). IF was not undertaken. **B** Henoch-Shönlein purpura nephritis (HSPN): C4d staining in the mesangial area adjacent to sclerosis is seen in PC-retrieved glomerulus (**B**-*b*), but is only mildly detectable in the MW-retrieved one (**B**-*c*). **C** LNV–: on IF (**C**-*a*), mesangial and subendothelial C4d deposition is definitely detected; however, C4d deposition is not clearly defined with IP (**C**-*b* and -*c*). **D** MN stage III: a similar pattern and intensity of C4d deposition is seen along the GBM on IF and IP (**D**-*a*, -*b,* and -*c*). **E** MPGN: a fringe-like pattern with a similar intensity of C4d deposition is seen on IF and IP (**E**-*a*, -*b,* and -*c*)

Table 1. C4d deposition pattern of IP staining in glomerulonephritides

	IgAGN	HSPN	MN	MPGN	Lupus nephritis
(1) Negative	10	4	1	5	5
(2) Focal segmental	1	1	2	1	2
(3) Diffuse global					
a. GBM	0	0	8	5	4
b. Mesangium	0	0	0	0	0
a + b	0	0	0	0	0
Sum	11	5	11	11	11

Discussion

We tested whether the C4dpAb IP procedure was comparable with IF using the Quidel anti-C4d monoclonal antibody for the detection of C4d in glomeruli.

The first point of our study was the comparison between IF with Quidel anti-C4d mAb and the C4dpAb to determine whether detection of C4d by the latter was consistent with the former. In the cases of MCD where there was no complement activation, the staining patterns of these two antibodies were similar, but the intensity was somewhat stronger with the monoclonal antibody. In the cases of glomerulonephritis, regardless of the underlying disease, the staining patterns were identical in each case, but intensities were variable. Thus, the detection of C4d with C4dpAb was equivalent to that with the monoclonal antibody on IF.

We next tested whether IF and IP with the C4dpAb were comparable in glomerulonephritis. If there had been no differences in terms of staining pattern, intensity, and intrinsic mesangial expression, we would have inferred that the C4dpAb with IP was as powerful to detect tissue C4d as the Quidel anti-C4d monoclonal antibody with IF. For this comparison, adequate methods of antigen retrieval for IP examinations of paraffin sections are needed. Thus, we tested two methods of antigen retrieval, pressure-cooking (PC) and microwave (MW), and found that the staining patterns with both methods were similar, but MW was a little more sensitive for C4d detection. In addition, our preliminary experiment revealed that only MW, but not PC, was applicable for detecting C4d in the PTC of renal grafts (data not shown). Thus, we recommend MW for antigen retrieval in the IP methodology with C4dpAb. When we compared IF and IP with MW with this antibody, the intrinsic staining pattern of mesangium that was found on IF was not seen wth IP. However, the nonintrinsic C4d expression in MN, MPGN, and LN with IP was comparable with that shown on IF.

The implications of the presence of C4d in glomerulonephritis have not been well clarified. Of note, in the present study, C3 was exclusively found in all cases of MPGN and lupus nephritis, whereas C4d was present in only half of these. This difference was also found in the case of stage I MN. The absence of C4d deposition may occur because C4d is present at undetectable levels. The lack of C4d in cases of MPGN and lupus nephritis may be explained as follows. Activation of the C4 component initiated the classical pathway on a small scale at the beginning, and the main pathway was switched to an alternative pathway later, and thus the quantity of C4d deposition was too small to be detected.

Of note, we identified an apparent positive C4d signal within the mesangial area on IP staining in some cases. From this phenomenon, we inferred that there must be significant C4 activation in this area, other than intrinsic C4, which we could identify by the IP methodology with the C4d polyclonal antibody (pAb). Additionally, we observed that such deposition was seen in association with segmental sclerosis. This finding suggests that the kinetics of C4d deposition in the mesangial area is slow, and C4d deposition remains for a long time.

In conclusion, we demonstrated the usefulness and reproducibility, along with some limitations, of a commercially available polyclonal antibody for C4d that is particularly applicable for use with paraffin sections. Because the IP staining with this polyclonal anti-C4d antibody shows several patterns of deposition, without intrinsic background, it will help to characterize the role of C4 activation in glomerulonephritis.

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