Comparative Study for the Detection of C4d in Paraffin-Embedded Renal Allograft Biopsies by Immunohistochemical Techniques

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Background: *Peritubular capillary complement C4d deposition is one of the criteria for diagnosis of acute humoral rejection in kidney allografts. Little information is provided for the effective technique to stain C4d protein.*
Objective: *To compare C4d staining results by using indirect immunofluorescence detection (IF) with immunoperoxidase methods (IP) in formalin-fixed, paraffin-embedded renal allograft tissue.*
Material and Method: *C4d protein was detected in renal allograft tissues by IF and IP methods. The antigen unmarking procedures were used including (i) heating with 0.05% citraconic anhydride in a water bath plus proteinase K digestion, (ii) heating with 10mM citrate buffer in a microwave and (iii) digesting with proteinase K for comparing the deposition of C4d in paraffin-embedded tissues.*
Results: *The results showed that the unmarking solution containing 0.05% citraconic anhydride pH7.4 and heating in a water bath revealed a signal enhancement in the IP method whereas the solution containing 0.05% citraconic anhydride pH7.4 and heating in a water bath plus proteinase K digestion showed a greatly enhanced signal in the IF method. The prevalence of C4d staining detected in peritubular capillaries was 68% (17/25) and the results observed for both methods were similar.*
Conclusion: *0.05% citraconic anhydride in a water bath with or without proteinase K digestion is useful for unmasking C4d deposition in peritubular capillaries of renal allografts performed by IP and IF methods.*

Keywords*: Antigen unmarking, Citraconic anhydride, Protease K, Allograft*

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The number of patients with end-stage renal disease is continuously increasing worldwide every year. Renal transplantation is the appropriate treatment of choice to improve survival and quality of life for these patients[1]. The problem of this strategy, however, is kidney allograft rejection. Rejection of transplanted organs is a complex process involving both cellular T-cell mediated and humoral antibody-mediated pathways[2]. Acute humoral rejection (AHR) is the most important threat to transplanted kidneys in the early phase after transplantation that has a poorer prognosis than cellular rejection and the treatment is substantially different from conventional treatment for cellular rejection[2,3]. The gold standard for the diagnosis of graft rejection and for guiding patient management is the histological evaluation of a renal allograft biopsy. Peritubular capillary complement C4d deposition is one of the criteria for diagnosis of AHR in kidney allografts[4]. C4d is a depredating product of the complement generated *in vivo* after antibody binding to its antigen. C4d binds covalently to adjacent cell membranes and can be detected with immunohistochemical techniques[2,5-8]. Detection of C4d along peritubular capillaries (PTC) in renal allograft biopsies is a prognostic marker of poor long-term graft survival. It is typically associated with circulating donor-specific antibodies[6, 7]. Several antibody reagents for detection of C4d in allograft tissue are commercially available, but little information is provided for the effective technique to stain C4d protein[8]. To date, indirect immunofluorescence (IF) detection of any antigen on a frozen section is developed as a gold standard technique to evaluate renal biopsy specimens[9]. It is however the fact that IP technique has advantages over IF because

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of better histological detail, convenience and ability to apply the staining on archival as well as prospectively collected tissues in paraffin-embedded tissue blocks[5,10]. In addition, the handling of frozen tissue is somewhat difficult. Therefore, formalin-fixed, paraffin-embedded (FFPE) tissue is used to detect the antigen by both IF and IP with some modifications. In histopathology labs, formalin is a commonly used fixative for tissue preservation. A major adverse effect of this fixative is the masking of tissue antigens by protein cross-linkage through the formaldehyde reaction with the protein amino groups. Tissue antigens are denatured, and effective immunological analysis is precluded. For this reason, various antigen unmasking procedures also referred to as antigen retrieval (AR) methods have been developed. There are widely used methods of retrieving antigens from formaldehyde-fixed and paraffin-embedded tissues, such as proteolytic digestion[10,11], heat-induced treatment[12-18] and a mixed method of both treatments[19]. Different variations of the antigen retrieval technique using different retrieval solutions have been evaluated for their effectiveness in restoring the antigenicity. For the present study, it was aimed to modify the unmarking procedures in order to obtain the maximum immunological signal with well-preserved tissue structure using formaldehyde-fixed and paraffin-embedded renal tissue. Here, two retrieval solutions were used including 0.5% citraconic anhydride, pH7.4 and 10 mM citrate buffer, pH6 with high-temperature heating treatment with or without protease K digestion to unmask the antigens in renal allograft biopsies fixed in the universal fixative, formaldehyde, and compared the C4d staining results by IF and IP detection.

Material and Method

Patients and biopsies

Twenty-five cases of renal allograft biopsies that were diagnostic as acute humoral rejection and were not stained for C4d on frozen sections, were obtained from the Department of pathology, Faculty of Medicine, Khon Kaen University, Thailand. The research protocols were approved by the Human Research Ethics Committee, Khon Kaen University and informed consent was obtained from each subject before surgery. All specimens were fixed in 10% buffered formalin and embedded in paraffin for routine diagnosis. Tissues for immunostaining were cut to a thickness of 3 microns and attached to saline-coated slides. The sections were deparaffinized and rehydrated in a graded series of ethanol and soaked in 3% hydrogen-peroxide in methanol for 5 min and washes for 5 min with PBS. Immunostaining was performed using two methods (IF and IP method) and various pretreatments for antigen retrieval were required before staining.

Indirect immunofluorescence method

For the indirect IF method, the sections were divided into 3 groups and antigen retrieval was performed under the following conditions: i) water bath treatment with 0.05% citraconic anhydride solution pH7.4 at 95°C for 45 min plus protease K digestion 30 min, ii) 10 mM citrate buffer, pH6 in a microwave at power level 10, 3 min and level 3, 10 min plus protease K digestion 30min, and iii) digestion with protease K for 30 min.

Immunoperoxidase method

Antigen retrieval method for immuno-peroxidase was performed under the following conditions: i) water bath treatment with 0.05% citraconic anhydride solution, pH7.4 at 95°C for 45 min, ii) microwave treatment with 10mM citrate buffer, pH6 at level 10, 3 min and level 3, 10 min, and iii) digestion with protease K for 30 min.

After pretreatment and washing with phosphate buffered saline (PBS), rabbit anti-human C4d (AbD serotec, Martinsried, Munich) was applied and samples were incubated 1 h at room temperature. After washing with PBS, the sections were incubated with swine anti-rabbit immunoglobulin antibody conjugated with FITC (DAKO, Glostrup, Denmark) for 1 h. After washing with PBS, the sections were mounted with mounting media (DAKO) for the IF method. For the IP method, after pretreatment and incubation with C4d, the sections were incubated with peroxidase-conjugated Envision™ antibody (DAKO) for 30 min and the color was developed with 0.1% diaminobenzidine tetrahydrochloride (DAB) solution. The sections were then counterstained lightly with Mayer's Haematoxylin, rinsed in water for 3-4 min, dehydrated, cleared and mounted in Permount®.

Interpretation

The perittubular capillary (PTC) staining was defined as diffuse if more than 50% of the capillaries were C4d positive and focal if less than 50% of the PTCs were positive and the intensity of staining was graded from 0 to 3+ (1+, mild staining; 2+, moderate staining; 3+, strong staining). Glomerular staining served as internal quality control and it was not used

for scoring[20, 21]. The most appropriate AR method was estimated by Cohen's Kappa and categorized as poor (Kappa < 0.20), fair (0.21 < Kappa < 0.40), modurate (0.41 < Kappa < 0.60), substantial (0.61 < Kappa < 0.80), or almost perfect (Kappa 0.80)[22].

Results

Peritubular capillary C4d staining by IP

Of 25 cases kidney transplant biopsies in IP tested, it was found that 17 (68%) revealed peritubular capillary positive staining with 0.05% citraconic anhydride, 14 (56%) with 10 mM citrate buffer and 15 (60%) with proteinase K digestion (Table 1). Three cases had some dissimilarities in staining results between three methods, including 3 cases that were positive for focal staining patterns and intensity 1+ with 0.05% citraconic anhydride solution, 3 and 2 cases showed negative staining when 10 mM citrate buffer and proteinase K digestion was used. When compared, the best method was 0.05% citraconic anhydride pH7.4 in the water bath, proteinase K digestion and 10 mM citrate buffer pH6, for the staining between the three different antigen retrieval methods. The degrees of intensity staining by using 0.05% citraconic anhydride pH7.4 in water bath were insignificantly higher than those using proteinase K digestion. When 0.05% citraconic anhydride buffer was used as antigen retrieval solution, the background staining was higher than 10 mM citrate buffer and enzyme digestion. Renal tubular epithelia and serum proteins within peritubular capillaries were found as light brown staining whereas in two other methods, both were clear (Fig. 1). The deposit of C4d in glomerular capaillary wall served as an internal positive control (Fig. 1).

Peritubular capillary C4d staining by IF

The water bath and microwave treatments without proteinase K digestion did not retrieve the antigen (data not shown). The deposition of C4d in peritubular capillary was effectively unmasked by heat-induced treatment plus proteinase K digestion and/or proteinase K digestion only. Of 25 cases of renal allograft biopsies in IF tested, 17 (68%) revealed peritubular capillary positive staining with 0.05% citraconic anhydride plus digestion, 14 (56%) with 10 mM citrate buffer plus digestion and 13 (52%) with proteinase K digestion only (Table 2). In 4 cases that were weakly positive by 0.05% citraconic anhydride plus digestion, three and four cases were negative by using either 10 mM citrate buffer plus digestion or proteinase K digestion only. The IF staining after antigen unmarking by using heat-induced treatment plus digestion showed higher intensity than the digestion only. The condition of 0.05% citraconic anhydride in the water bath plus digestion revealed a significant enhancement over digestion only. Diffuse glomerular staining with C4d along capillary walls was used as an internal positive control (Fig. 2).

Comparison of peritubular capillary C4d staining by IP and indirect IF

The comparisons of C4d staining in formalin-fixed, paraffin-embedded tissues using IP and IF with the most appropriate AR method has shown that the prevalence of peritubular capillaries C4d staining in all biopsies was 68% (17/25) in both IP and IF methods. In positive cases, 53% (9/17) revealed diffused and 47% (8/17) focal positive staining by IP, whereas in the IF method, 35% (6/17) revealed diffuse and 65% (11/17) focal staining patterns. Agreement between the most appropriate AR method for IF and IP was substantial (Kappa 0.61).

Discussion and Conclusion

Antigen retrieval is a necessary step used for immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded tissue. The application of AR methods includes heat-induced or enzyme digestion and the

Table 1. Comparison of the positive cases and staining patterns of PTC C4d staining by using the IP method with different antigen retrieval conditions (n = 25)

Conditions of antigen retrieval	Positive cases	PTC staining pattern	
		Diffuse	Focal
0.05% citraconic anhydride in water bath	17 (68%)	9 (36%)	8 (32%)
10mM citrate buffer in microwave oven	14 (56%)	9 (36%)	5 (20%)
Proteinase K digestion	15 (60%)	8 (32%)	7 (28%)

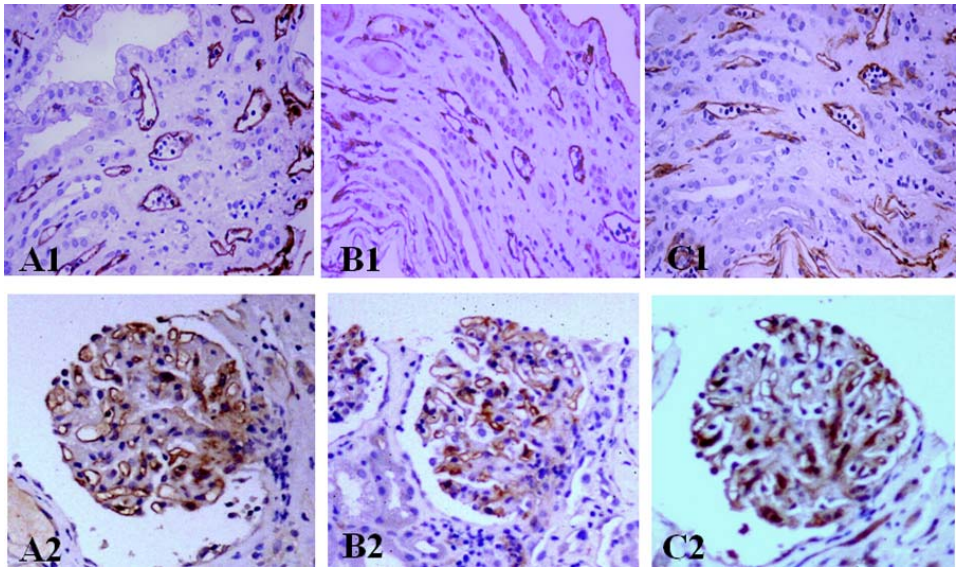

Fig. 1 Representative micrographs showing a comparison between IP staining of formalin-fixed, paraffin-embedded kidney allograft tissue. Peritubular capillary (A1) positive staining (3+) using 0.5% citraconic anhydride in the water bath. Glomerular(A2) positive staining served as internal control. Peritubular capillary (B1) positive staining (2+) using 10 mM citrate buffer, pH6 in the microwave. Positive internal control (B2). Peritubular capillary (C1) positive staining (3+) using proteinase K digestion. Positive internal control (C2).

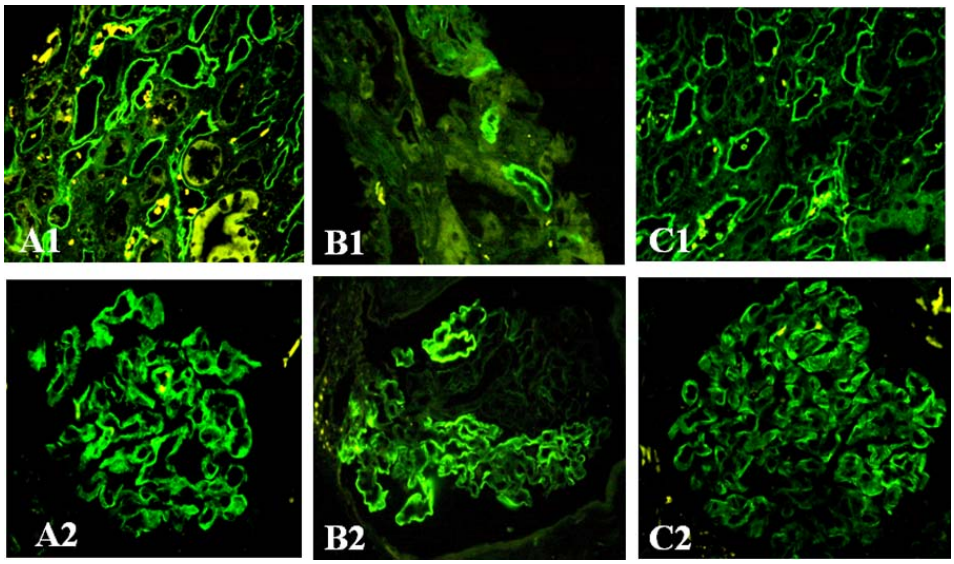

Fig. 2 Representative micrographs showing a comparison between IF staining of formalin-fixed, paraffin-embedded kidney allograft tissue. Peritubular capillary (A1) positive staining using 0.5% citraconic anhydride in the water bath plus proteinase K digestion. Glomerular (A2) positive staining served as internal control. Peritubular capillary (B1) positive staining using 10 mM citrate buffer, pH6 in microwave plus proteinase K digestion. Positive internal control (B2). Peritubular capillary (C1) positive staining using proteinase K digestion. Positive internal control (C2).

combined action of heat-induced and enzyme digestion[23]. The formulations of AR solutions and enzymatic digestion protocols used for AR method are available. Using 10 mM citrate buffer, pH6 in the microwave treatment is a conventional method for unmasking antigens embedded in paraffin[26]. In the case of antigens deposited in glomeruli and detected by using IF method, however, microwave treatment plus enzyme digestion is more effective in unmasking than microwave alone[10]. Enzymatic digestion can have an activity combined with heat-induced treatment to produce enhanced demonstration and localization of a number of antigens including the immunoglobulin and complement[24]. Enzymatic digestion has been speculated to break the protein cross-links and the introduction of heat-induced antigen retrieval was a breakthrough, greatly enhancing tissue antigenicity and reproducibility of staining[24]. Recently, some studies have been reported that a citraconic anhydride solution, a reversible protein cross-linking agent, and heat under an optimal conditions was able to satisfactorily unmask a wide variety of antigens for IHC and this new protocol has advantages including superior morphological preservation, greater reproducibility, and more intense staining after retrieval[23]. In present study, citraconic anhydride solution in a water bath was compared with two conventional methods, 10 mM citrate buffer, pH6 in the microwave treatment and enzymatic digestion to detect C4d deposition in peritubular capillaries of renal allograft biopsies. The results have shown that heat-induced treatment plus enzymatic digestion unmarked antigen in paraffin-embedded renal allograft tissue by using IF whereas heat induction only was not enough for unmasking this antigen. The deposit of C4d, however, was clearly detected when using both heat induction and enzymatic digestion in the IP method.

Either 0.05% citraconic anhydride in the water bath only or plus enzymatic digestion revealed the best results for IP and IF methods. In addition, the degree of intensity and number of positive cases were higher than using other AR methods. In these results, it was found that 0.05% citraconic anhydride buffer pH7.4 was the most appropriate AR solution. Results of the present study agreed with previous reports[23,25]. Namimatsu and co-workers have described that this method provides an efficient antigen retrieval for successful immunostaining of a wide variety of antigens under optimal conditions[23].

In the present study, it was found that the

Table 2. Comparison the positive cases and staining patterns of PTC C4d staining by the IF method with different antigen retrieval conditions (n = 25)

Conditions of antigen retrieval	Positive cases	PTC staining pattern	
		Diffuse	Focal
0.05% citraconic anhydride in water bath plus digestion	17 (68%)	6 (24%)	11 (44%)
10mM citrate buffer in microwave oven plus digestion	14 (56%)	1 (4%)	13 (52%)
Proteinase K digestion	13 (52%)	3 (12%)	10 (40%)

Table 3. Comparisons of the intensity of PTC C4d staining by the IF and IP methods with the most appropriate AR method (n = 25)

IP method		IF method				Total
		0	+1	+2	+3	
	0	8	0	0	0	8
	+1	0	2	1	0	3
	+2	0	1	8	0	9
	+3	0	1	3	1	5
Total		8	4	12	1	25

sensitivity of IP staining was equivocal to indirect IF staining in formalin-fixed, paraffin-embedded tissue. A previous report demonstrated that monoclonal C4d antibodies were suitable for IF staining in frozen tissue when polyclonal C4d antibodies were applied to IP staining in paraffin-embedded tissue[7,20,21]. The present study is the first report to demonstrate the capability of polyclonal C4d antibody applied to both IP and IF staining in paraffin-embedded renal allograft tissue with the modified antigen unmarking procedure.

Conclusion

It is now possible to conclude that the solution of 0.05% citraconic anhydride in a water bath is useful for unmasking C4d deposition in peritubular capillaries of renal allografts by the IP method and when combined with proteinase K digestion for an indirect IF method without any architectural damage in formalin-fixed, paraffin-embedded tissue. In the IP method it is recommended to use 20% (v/v) egg white for blocking nonspecific staining after incubating with 5% normal horse serum to reduce the non-specific background. Application of the present study is to help a laboratory that has limited types of antibody but needs to apply for both IP and IF methods.

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การศึกษาเปรียบเทียบการตรวจหา C4d ในเนื้อเยื่อปลูกถ่ายเอกพันธ์ของไตที่ฝังในพาราฟิน ด้วยเทคนิคอิมมูโนฮิสโตเคมี

เอมอร พนมศรี, นิษณา นามวาท, รุ่งเพ็ชร ตั้งรัศมีประเสริฐ, ชาญ ชาญธีระประวัติ, อนุชา พัวไพโรจน์

ภูมิหลัง: การเกาะตัวของ C4d complement ที่ peritubular capillary เป็นหนึ่งในเกณฑ์ที่ใช้วินิจฉัยภาวะ humoral rejection แบบเฉียบพลันของการปลูกถ่ายไต ยังมีข้อมูลอยู่น้อยในการแสดงเทคนิคที่มีประสิทธิผลในการย้อมโปรตีน C4d

วัตถุประสงค์: เพื่อเปรียบเทียบผลการย้อม C4d โดยใช้เทคนิค indirect immunofluorescence และ immunoperoxidase ใน renal allograft ที่ตรึงด้วยฟอร์มาลินและฝังในพาราฟิน

วัสดุและวิธีการ: ดูการย้อมโปรตีน C4d ใน renal allograft ด้วยเทคนิค indirect fluorescence และได้ใช้วิธีการเปิดแอนติเจนด้วย 3 วิธี ได้แก่ (i) 0.05%citraconic anhydride ใน water bath ร่วมกับการย่อยด้วย proteinase K (ii) 10mM citrate buffer ใน microwave และ (iii) ย่อยด้วย proteinase K. เปรียบเทียบกับการย้อมด้วยเทคนิค Immunoperoxidase ในชิ้นเนื้อที่ตรึงด้วยฟอร์มาลินและฝังในพาราฟิน

ผลการศึกษา: จากการทดลองพบว่าน้ำยาชนิดที่ประกอบด้วย 0.05% citraconic anhydride pH7.4 ใน water bath ช่วยให้การย้อมด้วยเทคนิค IP ดีขึ้น ขณะที่น้ำยาชนิดที่ประกอบด้วย 0.05% citraconic anhydride pH7.4 ใน water bath ร่วมกับการย่อยด้วยเอนไซม์ proteinase K ช่วยให้การย้อมด้วยเทคนิค IF ดีเพิ่มขึ้น เมื่อเปรียบเทียบผลการย้อมระหว่าง IP กับ IF ในตัวอย่างทั้งหมดพบ C4d บริเวณ peritubular capillaries จำนวนเท่ากันคือ 68% (17 ราย/ 25 ราย)

สรุป: 0.05% citraconic anhydride ใน water bath เหมาะต่อการเปิดแอนติเจน C4d บริเวณ peritubular capillary ในชิ้นเนื้อไตของผู้ป่วยเปลี่ยนไต เมื่อย้อมด้วยเทคนิค IP และเหมาะต่อการย้อมด้วยเทคนิค IF เมื่อใช้ร่วมกับการย่อยด้วย proteinase K