The Expression of C4d and HLA–DR in Renal Allografts with the Histologic Features of Antibody–Mediated Rejection

Background: Deposition of C4d along the peritubular capillaries is generally associated with an antibody-mediated response. We evaluated, with performing C4d immunostaining, the diagnostic accuracy of the cases that were previously diagnosed as antibody-mediated rejection (ABMR) when based only on the histologic findings, and we examined possible correlation of C4d with HLA-DR. Methods: Forty-five renal transplantation biopsies, which showed ABMR-like histology, were obtained. The expressions of C4d and HLA-DR in the transplant rejection cases were investigated using immunofluorescent and/or immunohistochemical staining. Results: There were 14 discordant cases among a total of 45 cases when C4d was used as a diagnostic marker and the original slides were reviewed. These total cases consisted of the C4d negative cases in two cases of hyperacute rejection and all the cases of ABMR and ABMR with chronic/sclerosing allograft nephropathy (CAN) and two C4d positive cases (one each of acute cellular rejection (ACR) and CAN according to their original diagnosis) and all these cases were then revised according to Banff 07. The expression of HLA-DR tended to be correlated with the log-transformed duration of grafts until three years after the transplantation. Conclusions: This study demonstrates that C4d together with the histologic findings should be used for making the diagnosis of ABMR. The tubular HLA-DR expression over time should be studied to further understand the mechanism of graft rejection.

Key Words: Graft rejection; Complement C4d; HLA-DR; Kidney
C4d were neutrophils in the peritubular capillaries and glomeruli, fibrinoid necrosis, fibrin thrombi, ATN-like features and vasculitis-like features. Further, the diagnosis of ABMR was made only when the histologic impression was severe enough to probably cause graft dysfunction.

As new standards of ABMR were established, it was necessary to investigate previously diagnosed renal graft biopsy cases with employing C4d. We investigated the cases that had the histologic features suggestive of ABMR with employing C4d to determine the accuracy of making the diagnosis of ABMR. Also included in this study were the previously diagnosed cases of chronic allograft nephropathy (CAN) because some recent reports about CAN suggest the role of antibody regardless of the histologic findings, and some of these cases can now be diagnosed as chronic ABMR while the others would be diagnosed as chronic TCMR.\(^5\)\(^1\)\(^1\)

Together with C4d staining, we also investigated the expression of HLA-DR in the same cohort that was used for C4d staining to examine the time-dependent expression of HLA-DR and its possible relationship with C4d. A HLA-DR expression is limited to macrophages, dendritic cells, B cells and vascular endothelium in nontransplanted kidneys.\(^5\) The expression of HLA-DR in renal tubular cells increases in the cases of acute TCMR. In addition, in cases of acute TCMR, it is uncertain how the tubular HLA-DR expression is induced, and activated T cells may play a role in that mechanism.\(^5\)\(^1\)\(^1\) In our previous study on the tubular expression of HLA-DR, ICAM-1 and VCAM-1 in renal allograft biopsy cases, the tubular HLA-DR expression was constantly increased in acute TCMR.\(^5\)\(^1\)\(^2\) There are few studies on the correlation between the tubular HLA-DR expression and the peritubular C4d expression.\(^5\) We tried to discover any cross reactions between ABMR and cellular rejection on the grounds that C4d is a specific, sensitive marker of ABMR and HLA-DR is also a specific, sensitive marker of cellular rejection.

This study was undertaken to investigate the expression of C4d and HLA-DR in the cases with histologic features suggestive of ABMR, and then we evaluated the relationship between the C4d expression and the HLA-DR expression in the tubular epithelium.

**MATERIALS AND METHODS**

**Patients and tissue processing**

Renal tissues were obtained by percutaneous allograft biopsy or graft nephrectomy from 235 patients (295 cases), with including eight consultation cases, that were seen at our hospital between 1985 and 2005. Histologic findings suggestive of ABMR such as neutrophils in the peritubular capillaries and glomeruli, fibrinoid necrosis, fibrin thrombi, ATN-like features and vasculitis-like features or a previous diagnosis of CAN were the criteria for inclusion, and this allowed us to select 45 allograft biopsies or nephrectomy specimens. The original diagnoses, where the Banff 97 classification had been applied, were classified into 10 cases of hyperacute rejection, 3 cases of acute ABMR, 4 cases of acute ABMR with CAN, 15 cases of acute cellular rejection (ACR) with intimal arteritis and 13 cases of CAN.\(^1\)\(^3\) The clinical data such as age, gender, the timing of transplantation, the type of donor and the serum creatinine level at biopsy were collected from the medical records. Each case was reclassified according to the Banff 07 classification after staining for C4d and revision of diagnosis.\(^1\)\(^1\)

For light microscopy (LM), the renal tissue was fixed in Duboscq-Brasil solution and then embedded in paraffin. Serial sections were cut at 2-4 μm and then stained with hematoxylin and eosin, periodic acid Schiff, Masson’s trichrome and methenamine silver. Specimen adequacy and lesion scoring were reclassified according to the Banff 07 classification.\(^1\)\(^4\)

For the immunofluorescence (IF) study, fragments of the biopsy were snap frozen and cut with a cryostat at 3-4 μm and these were stained with fluoresceinated antiserum that was monospecific for IgG, IgM, IgA, C3, C1, C4, fibrinogen, albumin and HLA-DR (DAKO, Carpenteria, CA, USA), and in 15 cases, frozen tissues were available for using polyclonal antibody for C4d (Biogenesis, Poole, England, UK). Immunofluorescent sections were examined with an Olympus BX51 microscope.

For the electron microscopy, the tissue was diced into 1 mm cube fragments, fixed in chilled 2.5% glutaraldehyde in cacodylate buffer, washed in buffer, postfixed in 1% osmium tetroxide, dehydrated in a graduated series of alcohol solutions and embedded in Epon. Ultrathin sections were stained with lead citrate and these were examined on a Hitachi H-750 or Hitachi H-7600s electron microscope at 75 or 80 kV.

**Immunohistochemistry (IHC)**

Immunohistochemical staining for C4d and HLA-DR was performed by using the avidin-biotin peroxidase complex method. The paraffin-embedded tissue blocks were sectioned at 2 μm thickness. The slides were deparaffinized in xylene and rehydrated in alcohol. Epitope retrieval using a pressure cooker (cit-
rate buffer pH 6.0, 10 min) was performed for C4d (Biogenesis, Poole, England). With 3% H2O2 as well as endogenous biotin by an avidin/biotin-blocking Kit (Vector Laboratories, Burlingame, CA, USA), the endogenous peroxidase was blocked. The polyclonal anti-C4d antibody (Biogenesis, Poole, England) and monoclonal anti-HLA-DR antibody (DAKO) were incubated with the slides at a dilution of 1:50 at room temperature. Subsequently, secondary biotinylated antibody and avidin-biotin complex reagent were applied and the sections were counterstained with Meyer’s hematoxylin for 40 s. A linear staining pattern of C4d along the peritubular capillaries (ptc) was regarded as positive, and the results were recorded as negative (negative C4d staining or incomplete granular staining), minimal (<10% of ‘ptc’ with linear peritubular capillary staining), focal C4d positive (10-50% of ‘ptc’ with linear peritubular capillary staining) and diffuse C4d positive (>50% of ‘ptc’ with linear peritubular capillary staining). A cytoplasmic expression of HLA-DR in the tubular epithelium was regarded as positive, and the proportion of positive tubular cells was estimated as a percentage.

Statistical analysis

All statistical analyses were carried out using SPSS software (SPSS., Ver. 12.0.1, USA). One way ANOVA tests were carried out for the clinical features and the expressions of C4d and HLA-DR among the five diagnosis groups (HAR, acute ABMR, acute ABMR with CAN, ACR and CAN). A linear relationship was assumed between the duration of the graft and the expression of HLA-DR, and a simple regression test was carried out. Differences of the degree of HLA-DR expression between the C4d positive and C4d negative groups were evaluated by the Mann-Whitney U test.

RESULTS

Clinical findings

The cohort for this study consisted of 10 cases of hyperacute rejection (HAR), 3 cases of acute ABMR, 4 cases of acute ABMR with CAN, 15 cases of ACR with intimal arteritis, and 13 cases of CAN according to their original diagnosis. After staining for C4d, each case was reclassified according to the Banff 07 classification.14

Males predominated in the HAR cases with only one female patient, and the mean age was 37.3 years (age range: 23 to 56 years). Renal transplantation was performed from two cadaveric donors, six living unrelated donors and two living related donors. One patient underwent a second transplantation from another living related donor. One patient showed aggravated renal function within one week, and the serum creatinine level was 6.8 mg/dL at the time of biopsy. After diagnosis, he was successfully treated with a combination of plasma exchange, polyclonal rabbit anti-thymocyte globulin and rituximab.15

For the three cases of acute ABMR, there were two cases of type IIA and one case of type IIB ACR in addition to the histologic features of ABMR. All the patients were male and their mean age was 42.7 years.

For the four cases of acute ABMR with CAN, three underwent graftectomy and the mean age was 36.5 years.

The 15 cases of ACR included 10 cases of type IIA, 2 cases of type IIB and 3 cases of type III. Males predominated for the cases with ACR with five female patients, and the mean age was 33.7 years (age range: 20 to 53 years). Living related donors predominated, with one cadaveric donor and four living unrelated donors.

Among the 13 cases of CAN, 8 cases were superimposed on varying degrees of ACR and 5 cases were diagnosed as CAN alone. One case was CAN grade I, three cases were CAN grade IIa, one case was CAN grade IIb, three cases were CAN grade IIIa, and 5 cases were CAN grade IIIb. In this group, the male-to-female ratio was 8:5, and the mean age was 39.6 years. There were 2 cadaveric donors, 7 living unrelated donors, and 4 living related donors.

While biopsy was carried out immediately after kidney transplantation for almost all the HAR cases, the mean duration from transplantation to biopsy was 2.5 months for the cases of acute ABMR, 1.2 years for the cases of acute ABMR with CAN, 5 months for the cases of ACR and 5.5 years for the CAN group. The serum creatinine level at the time of biopsy averaged 5.13 mg/dL for the cases of acute ABMR, 9.85 mg/dL for the cases of acute ABMR with CAN, 5 mg/dL for the cases of ACR and 6.60 mg/dL in the CAN group (Table 1).

Histopathologic findings

For the 10 cases with HAR, the histologic findings of all the cases were very similar. Margination of neutrophils and platelets occurred along the damaged endothelium of the small arteries, arterioles, glomeruli and peritubular capillaries (Fig. 1A), and the lumina of the capillaries were filled with red cells and fib-
Table 1. Summary of clinicopathologic data and immunohistochemical findings about C4d and HLA-DR in renal allograft

<table>
<thead>
<tr>
<th>Type of rejection</th>
<th>HAR (N=10)</th>
<th>AABMR (N=3)</th>
<th>AABMR with CAN (N=4)</th>
<th>ACR (N=15)</th>
<th>CAN (N=13)</th>
<th>p-value*</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>37.3±12.5</td>
<td>42.7±9.3</td>
<td>36.5±7.9</td>
<td>33.7±10.3</td>
<td>39.6±8.1</td>
<td>0.50</td>
</tr>
<tr>
<td>Duration (day)</td>
<td>133.3±80.8</td>
<td>5.13±2.78</td>
<td>9.85±4.32</td>
<td>4.99±2.54</td>
<td>6.60±6.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>-</td>
<td>-</td>
<td>Interstitial and tubular mononuclear infiltration, intimal to necrotizing vasculitis</td>
<td>Tubular atrophy, interstitial fibrosis, sclerosing glomeruli, obliterator vasculopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils in vessels and glomeruli, microthrombi, fibrinoid necrosis</td>
<td>Neutrophils in peritubular capillary, glomeruli and/or interstitium, fibrin thrombi, necrosis, vasculitis, superimposed AR-like features</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4d</td>
<td>Diffuse: 5, focal: 3</td>
<td>All negative</td>
<td>All negative</td>
<td>Focal: 2</td>
<td>Focal: 2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HLA-DR (%)</td>
<td>11.0±15.2</td>
<td>35.0±32.8</td>
<td>12.5±11.9</td>
<td>25.3±23.1</td>
<td>21.2±16.6</td>
<td>0.26</td>
</tr>
<tr>
<td>Accuracy%</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>86.7</td>
<td>84.6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*, One-way ANOVA test; \(^{\circ}\), Accuracy is the sum of the true positive and true negative cases divided by the whole cases (sum of true positive, true negative, false positive and false negative cases) determined by C4d staining and original slide review. HAR, hyperacute rejection; AABMR, acute antibody-mediated rejection; ACR, acute cellular rejection; CAN, chronic/sclerosing allograft nephropathy.

Fig. 1. Representative photographs of hyperacute rejection according to its original diagnosis. Note margination of neutrophils and mononuclear cells in congested peritubular capillaries (ptc) (A). Linear ptc staining for C4d is demonstrated by immunohistochemistry (B) and immunofluorescence microscopy (C). One hyperacute rejection case shows tubular expression of HLA-DR (D).
rin thrombi. The endothelium was stripped off the underlying basal lamina, and the interstitium was edematous and hemorrhagic. Widespread microthrombi were usually found in the vascular lumina. Some small arteries showed fibrinoid necrosis. Interstitial or tubular mononuclear infiltrates were sparse. One case showed sparse neutrophils in the peritubular capillaries without infiltration of inflammatory cells in the tubules, interstitium or glomeruli.

The histology was variable for the 15 cases with ACR. Interstitial and tubular mononuclear cell infiltration and edema as well as mild interstitial hemorrhage were consistent findings in almost all the cases (Fig. 2A). Arteries or arterioles were involved to variable degrees from slight endothelial swelling with varying degrees of intimal arteritis (Fig. 2B) to transmural arteritis and necrosis of the arterial wall.

Among the 13 cases with CAN, obliterative intimal fibrosis was seen in 7 cases and this was associated with a mild to severe degree of tubular atrophy and interstitial fibrosis (Fig. 3A). Focal segmental or global sclerosis of glomeruli was present in some cases. Superimposed ACR, ranging from IA to III, was noted in 12 cases and there was a mild to severe degree of tubulitis and interstitial mononuclear cell infiltration and increased neutrophils and mononuclear cells in the peritubular capillaries (Fig. 3B) with intimal arteritis to transmural necrotizing arteritis.

Three cases of acute ABMR and four cases of acute ABMR with CAN showed a moderate to severe degree of neutrophilic infiltration in the peritubular capillaries, glomeruli and/or interstitium, with occasionally noted fibrinoid necrosis and/or fibrin thrombi. These findings were mixed with a variable degree of mononuclear infiltration in the interstitium and vasculitis-like features that are seen in cases of acute rejection (AR). For the acute ABMR with CAN cases, there were also areas with variable degree of interstitial fibrosis and tubular atrophy, obliterative arteries and sclerosing glomeruli.

Fig. 2. Light microscopic and immunohistochemical findings of acute rejection according to its original diagnosis. Note mild lymphocytic tubulitis and lymphocytic infiltration and focal hemorrhage in interstitium in acute rejection (A) (Methenamine silver). Acute rejection, type IIb (t1, i2, v2) shows features of acute rejection with moderate intimal arteritis (B) (PAS) and focal linear peritubular C4d expression (C). Strong HLA-DR expression is present in about 70% of tubular epithelial cells (D).
Peritubular capillary C4d deposition

C4d deposition along the ptc, as examined by IHC, was found in 12 cases (26.7%) among a total of 45 cases, with diffuse staining in 5 cases and focal staining in 7 cases. In the 15 cases for which frozen tissues were available, we also performed IF staining for the same C4d and no discrepant cases were found between the methods of IF and IHC.

For the cases with HAR, C4d deposition along the ptc was diffuse (Fig. 1B, C) in 5 cases (50%) and focal in 3 cases (30%). For the one case with diffuse C4d staining, the diagnosis was corrected to acute ABMR from an original diagnosis of HAR because the onset of renal dysfunction was later than is typically noted for HAR and there were sparse neutrophils in the peritubular capillaries without infiltration of inflammatory cells in the tubules, interstitium or glomeruli. For the cases with ACR, 2 cases showed focal C4d deposition along the ptc, and both cases were diagnosed as AR with one case each of type IIA and III (Fig. 2C).

For the cases with CAN, C4d immunohistochemical staining was focally positive in 2 cases, one with superimposed AR (Fig. 3C) and the other with CAN. There was no duplication or ‘double contours’ in glomerular basement membranes in these cases. For the cases of acute ABMR with or without CAN, no C4d staining in the ptc was found. Linear C4d staining is more common in the cases of HAR (8/10, 80%) than in the other forms of rejection (p<0.01) (Table 1).

Discrepancy with original diagnosis in terms of the C4d expression

There were 14 discordant cases among a total of 45 cases for...
which C4d was used as a diagnostic marker and the original slides were reviewed (Table 2). There were two C4d negative cases among the HAR cases and all the cases of acute ABMR and acute ABMR with CAN were negative for C4d while two C4d positive cases were found among the ACR and CAN cases, respectively. For one case of C4d positive HAR, the diagnosis was changed from HAR to acute ABMR as was previously mentioned. We defined the accuracy as a sum of the true positive and negative cases divided by the entire number of cases belonging to each class of rejection, which was determined by C4d or the original slide review (Table 1). The accuracy of diagnosing HAR, acute ABMR, acute ABMR with CAN, AR and CAN were 70%, 0%, 0%, 86.7%, and 84.6%, respectively. Each case was reclassified according to Banff 07 with considering the C4d staining and the histological findings (Table 2). No distinct histologic or clinical findings were found between the discrepant and consistent cases and this was also true between the C4d positive and C4d negative cases.

### Tubular expression of HLA-DR

The expression of HLA-DR in the tubular epithelium was variable, ranging from a negative expression to 80% (Fig. 1D, 2D, 3D). For the cases with HAR, the HLA-DR expression in the tubular cells was negative in 4 cases and it was very low, ranging from 5% to 15%, except for one case that showed a HLA-DR expression of over 25% (Fig. 1D). The expression of HLA-DR in glomeruli, ptc, or the endothelium of arteries or arterioles was negligible. Inflammatory infiltrates in the ptc were negative.

Most cases with acute ABMR, acute ABMR with CAN, ACR and CAN showed a tubular expression of HLA-DR, ranging from 5 to 80% of the tubular epithelial cells. The glomeruli, endothelial cells of arteries, arterioles or capillaries had various degree of a HLA-DR expression. Interstitial infiltrates were usually positive for HLA-DR. Some atrophic tubules, the intima or media of hyalinized vessels and the mononuclear infiltrates in the tubules or interstitium were occasionally positive for HLA-DR.

We then analyzed the change of the expression of HLA-DR over time (days) regardless of the diagnosis category. When a simple regression test was carried out for all the cases (45 cases), no linear relationship was found. But when we excluded the cases with a duration of a graft over 1,000 days (9 cases), the linear relationship between the expression of HLA-DR and the log-transformed duration was observed (R square=0.11, p=0.049) (Fig. 4).

The expression of HLA-DR showed no difference among the HAR, acute ABMR, acute ABMR with CAN, AR, and CAN...
cases (Table 1). There were also no significant relationships between the C4d expression and the HLA-DR expression (Fig. 5).

**DISCUSSION**

Diagnosing ABMR before the introduction of C4d was one of the most challenging decisions, even for experienced pathologists. One study reported that C4d deposition in the ptc, as assessed by immunofluorescence microscopy showed a sensitivity and specificity of 95% and 96%, respectively. C4d staining in ptc is highly correlated with the presence of circulating donor antibodies. In one series, 90% of the C4d-positive AR patients had circulating donor-specific antibodies and at the same time, only 2% of the C4d-negative AR patients were positive for circulating donor specific antibodies. For ABO-incompatible transplants, 53% of the recipients showed C4d deposition in their graft biopsies. The widely accepted diagnostic criteria for ABMR are now C4d deposition in peritubular capillaries and circulating donor-specific antibodies, together with at least one of the following three histologic findings: 1) neutrophils in the peritubular capillaries, 2) arterial fibrinoid necrosis and 3) acute tubular injury.

Although any well-defined diagnostic criteria for ABMR were not established before the introduction of C4d, we have attempted to diagnose and/or exclude ABMR based on the histologic findings of the renal allografts at this institution. Our criteria for ABMR were neutrophils in the peritubular capillaries and glomeruli, fibrinoid necrosis, fibrin thrombi, ATN-like features and vasculitis-like features, but these criteria were not directly applied when we diagnosed ABMR. The diagnosis of ABMR was made only when the histologic changes were severe enough to likely cause graft dysfunction. After C4d was introduced at our institution, we believed that a validation study was necessary for validating our previous diagnosis of renal allografts together with using HLA-DR expression, which is known as a useful marker for AR.

Although all the available renal allografts were not included in the study, to minimize the possible presence of unrecognized ABMR, we used broad inclusion criteria: 1) the presence of any histologic findings among neutrophils in the peritubular capillaries and glomeruli, fibrinoid necrosis, fibrin thrombi, ATN-like features and vasculitis-like features, 2) any cases of CAN because some recent reports about CAN have suggested the role of antibody regardless of the histologic findings. We found 14 discordant cases among 45 renal grafts. By reviewing the allograft biopsy slides, we found that one case, whose original diagnosis was HAR, was overestimated and the clinical and histologic features were closer to acute ABMR. The other 13 revised cases could be either ABMR or TCMR, except for two cases. In these two cases, whose original diagnosis was HAR, C4d was not stained at all while the clinical and histologic findings were diagnostic of HAR. These might be caused by a loss of antigenicity due to the long duration of storing the
tissue (over 4 years), but there have been no studies about whether C4d antigen is still stable after a long duration of storage. C4d was not stained in any of the cases that we had diagnosed as ABMR, with or without CAN. This means that diagnosing of ABMR by using only the histologic finding risks misdiagnosis and so C4d staining is necessary for all the renal allograft cases. We should also consider the possibility that the results might have been different if C4d from another supply company had been used because the sensitivity and specificity of C4d were reported to be different according to the producer.18,19

We investigated the tubular expression of HLA-DR in one cohort. We found that the tubular expression of HLA-DR tended to increase with the duration of the allografts. This relationship was significant when the log transformed duration was correlated with the tubular expression of HLA-DR for the cases whose duration of transplantation was less than 1,000 days (R square=0.11, p=0.049). These findings show that the expression of HLA-DR increases more rapidly in the early posttransplantation period and then it leads to a plateau, and then the expression is variable at around three years after renal transplantation. The expression of HLA-DR in the tubular epithelium of a grafted kidney may be caused by the activated T cells of the recipient which acquired the donor’s DR antigen.17 Then the tubular epithelium expressing HLA-DR, in turn, recruits more T cells and a vicious cycle begins. This explains why the expression of HLA-DR increases rapidly in the early posttransplantation period. However, the slow change later on cannot be explained in this way. Immune tolerance and/or the influence of therapy could be possible answers to this problem, but more study on this is necessary. One of the limitations of applying the results of this study about the time-dependent expression of HLA-DR to a general renal transplantation population is that the cohort of this study was designed for identifying the false positive or negative cases of ABMR instead of using the whole transplant recipient population at this institution.

A recent study showed that the tubular MHC class II (HLA-DR) expression was significantly correlated with C4d deposition.1 The difference of that study from our study, with regard to the method of evaluation, was that we measured the expression rate of HLA-DR in the tubular epithelium. In our study, any significant relationship was not found between the expression of HLA-DR and C4d. We thought that it was more relevant that the expression of HLA-DR and C4d should be independent considering that HLA-DR had been regarded as a marker for TCMR and C4d as a marker for ABMR because TCMR and ABMR appear to be very different processes.12,20

In conclusion, we found 14 discordant cases among the cases of renal allografts at this institution mainly by applying C4d staining. Tubular HLA-DR expression is increased from the onset of transplantation with a special pattern and in a time-dependent manner. There is no significant correlation between C4d deposition and the tubular HLA-DR expression. Further studies are necessary to determine and evaluate reliable markers for making the precise diagnosis of renal allograft rejection.

REFERENCES

12. Kim WS, Lee WM, Park CH, Kang CM, Kwak JY, Park MH. Pre-


