Comparison of C4d Immunostaining Methods in Renal Allograft Biopsies

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Immunostaining of renal allograft biopsies for C4d deposition has become an important diagnostic tool in the recognition of humoral-mediated graft rejection. The majority of studies have been performed on frozen tissue sections with one of several commercially available antibody reagents. However, only a single small series that compared reagents or methods, including staining of formalin-fixed, paraffin-embedded tissue, has been published. Two different staining methods in 138 renal allograft biopsies were compared directly: A mAb (Quidel, San Diego, CA) on frozen tissue sections with indirect immunofluorescence (IF) and a polyclonal antibody (Biomedica Gruppe, distributed by ALPCO, Windham, NH) applied to formalin-fixed, paraffin-embedded tissue with immunohistochemical (IHC) detection. An initial data set of 107 consecutive cases showed complete agreement between staining methods in 104 (97%) cases. Overall, nine of 107 cases were positive with one or both methods, representing 8.4% of all allograft biopsies tested, 15% of clinically indicated biopsies, and 24% of biopsies with a histologic diagnosis of acute cellular rejection. A second set of 31 cases included 17 cases that were positive by either method, with concordance in 29 of 31 cases. Combining the two data sets, the overall specificity of the IHC method compared with IF was 98%, and sensitivity was 87.5%. Direct comparison demonstrates that IHC staining of formalin-fixed, paraffin-embedded tissue with anti-C4d polyclonal antibody has acceptable sensitivity and specificity, as compared with IF staining of frozen tissue with the Quidel mAb.

Clin J Am Soc Nephrol 1: 583–591, 2006. doi: 10.2215/CJN.00900805

Rejection of transplanted organs involves complex immunologic pathways that commonly are divided into the predominantly T cell–mediated (cellular) or the predominantly antibody-mediated (humoral) pathways (1). Historically, antibody mediated rejection was best characterized in the setting of hyperacute/accelerated acute rejection (1). However, features of humoral rejection in allografts at later posttransplantation intervals has gained attention in recent years, especially with the characterization of reagents to detect C4d deposition in the capillary bed of renal allografts (2–26).

C4d is a split product of C4, a well-characterized component of the classical (antibody-activated) complement pathway (12,27–29). Unlike C4 or other pathway intermediates, the breakdown product C4d binds covalently to adjacent membrane surfaces upon activation and thereby is amenable to detection using conventional methods (12,27,28). Numerous recent publications have correlated the presence of C4d deposition in peritubular capillaries of renal allograft biopsies with poor graft outcome (13–26). Detection of C4d staining in peritubular capillaries, together with positive posttransplantation cross-match (donor-specific antibodies [DSA]), and evidence of histologic damage have been incorporated into the revised Banff '97 classification system as criteria for diagnosis of humoral rejection in renal allografts (10).

Accordingly, treatments that are targeted at antibody-mediated allograft damage are being developed for suspected cases of acute humoral rejection, including combinations of plasmapheresis, intravenous Ig (IVIg), immunoadsorption, tacrolimus/mycophenolate mofetil rescue, and Rituximab, among others (30–39). These treatments are substantially different from conventional treatment for cellular rejection, such as pulse steroids, and anti–T cell reagents (*e.g.*, anti-thymocyte globulin, OKT3), *etc.* (1). Therefore, consistent and robust staining procedures for detection of C4d deposition in allograft biopsies have become an important part of the diagnostic workup of renal allograft biopsies.

Three different antibody preparations are commercially available for detection of C4d in tissue biopsies: A mAb that is available from Quidel (cat. no. A213; San Diego, CA) (12), a mAb that is available from Biogenesis (clone 10-11; Brentwood, NH) (14), and a polyclonal antiserum from Biomedica Gruppe (developed by Regele *et al.* and distributed by ALPCO Diagnostics, cat. no. 004-BI-RC4D, Windham, NH) (17,20). Whereas all of these are reactive on frozen tissue sections, the Biomedica polyclonal antiserum is reactive on formalin-fixed, paraffin-embedded tissue after standard pretreatment (17). Several studies have demonstrated that the presence of C4d in peritubular capillaries correlates with worse graft outcome regardless of the antibody preparation used to detect it (13–26). However, to our knowledge, only a single small study that directly compared

Received August 31, 2005. Accepted February 9, 2006.

Published online ahead of print. Publication date available at www.cjasn.org.

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ISSN: 1555-9041/103-0583

Table 1. Patient and biopsy characteristics: Stanford data set ($n = 107$)

Biopsy Population	*n* (%)	C4d+ (%)
Pediatric transplant biopsies	71 (66%)	6 (8.4%)
Living donor graft biopsies	75 (70%)	3 (9.4%)
Female recipient graft biopsies	48 (45%)	7 (14.5%)[a]
High PRA[b]	20/101 (20%)	2 (10%)
Blood transfusion[c]	37/65 (53%)	
Nonprotocol biopsies	60 (56%)	9 (15%)
Biopsies with cellular rejection	33 (31%)	8 (24%)
	Average	Range
Time posttransplantation	2.67 yr	1 wk–10 yr

[a]Six of seven female patients were pediatric; no pregnancies.
[b]Biopsies from recipients with panel reactive antibodies (PRA) >20% before or after transplantation (class I or class II) by Luminex beads; data available on 101 patients.
[c]Biopsies from recipients with blood transfusion before, during, or after transplantation; data available on 65 patients, only two of whom were C4d$^+$.

reagents has been published (40). We compared C4d staining results with the Quidel mAb on frozen tissue using indirect immunofluorescence (IF) detection, with C4d staining results with the Biomedica polyclonal antisera on formalin-fixed, paraffin-embedded tissue using immunohistochemical (IHC) detection.

Materials and Methods

Case Selection

This study was approved by the Institutional Review Boards of Stanford University Medical Center and Oregon Health and Science University (OHSU). A database search of the Department of Pathology at Stanford University Medical Center from September 2002 through September 2003 revealed that 240 renal transplant biopsies were performed at Stanford University Medical Center. Of these, 126 cases had both formalin-fixed and frozen tissue collected for the Renal Pathology laboratory, and 107 cases were available for further study. Protocol biopsies that were taken at predetermined intervals posttransplantation without evidence of graft dysfunction (3, 6, 9, 12, 24, and 36 mo) were included, as were biopsies that were performed for clinical indication, most commonly elevation of serum creatinine. However, biopsies that were taken at time of engraftment were excluded. A significant proportion of the Stanford transplant population is pediatric, and the majority of patients received living-donor grafts. Forty-five biopsies were from parent-to-child living donations; no child-to-parent graft biopsies were performed during the study period. Transplantation across ABO-incompatibility or positive cross-match is not performed at Stanford. Additional details of the initial biopsy study population are listed in Table 1. Hematoxylin and eosin– and periodic acid-Schiff–stained sections were reviewed by at least two renal pathologists, and a biopsy diagnosis was rendered according to the Banff '97 classification system (41); discrepancies were resolved by conference at a multiheaded microscope. Histopathologic features that were associated with humoral rejection were assessed semiquantitatively, including peritubular capillary dilation; peritubular capillary inflammatory cells (each as absent [−], any present [+], moderate [++], or abundant [+++]); or features of acute tubular injury, interstitial edema, and lymphoid aggregates (each as present or absent).

Given the relatively low number of C4d-positive cases in the aforementioned data set, additional cases were obtained from OHSU. A database search was conducted for cases in which any C4d peritubular capillary staining was noted, regardless of intensity or the degree. The OHSU cases include small numbers of patients who were treated before transplantation for positive cross-match or high degree of sensitization and a single blood group A2 kidney grafted into a blood group O recipient. A retrospective survey of all available transplant biopsies at OHSU was not undertaken. Unfortunately, during the study period, patient serum was not routinely sent for DSA analysis at either institution; we have DSA data from only nine OHSU biopsy time points and no Stanford cases. These did not include IF/IHC discordant cases and are not analyzed further.

Indirect IF Staining

Frozen sections of tissue that were transported in saline (Stanford data set) or Michels medium (OHSU data set) were stained using standard procedures with monoclonal anti-C4d (cat. no. A213; Quidel) at 1:100 dilution (28). Secondary anti-mouse FITC-conjungated antibody was applied at a concentration of 1:20 (cat. no. 55521; Cappel/MP Biomedicals, Irvine, CA). Quantification of staining was recorded as described below.

Table 2. Banff grading of acute cellular rejection in C4d positive cases[a]

Banff Grade	*n*	C4d$^+$
No evidence of rejection	76	9
Inadequate[b]	18	2
Borderline	14	4
IA	18	6
IB	7	3
IIA	5	2
Total	138	26

[a]Data include both Stanford and Oregon Health and Science University (OHSU) data sets.
[b]None had evidence of rejection.

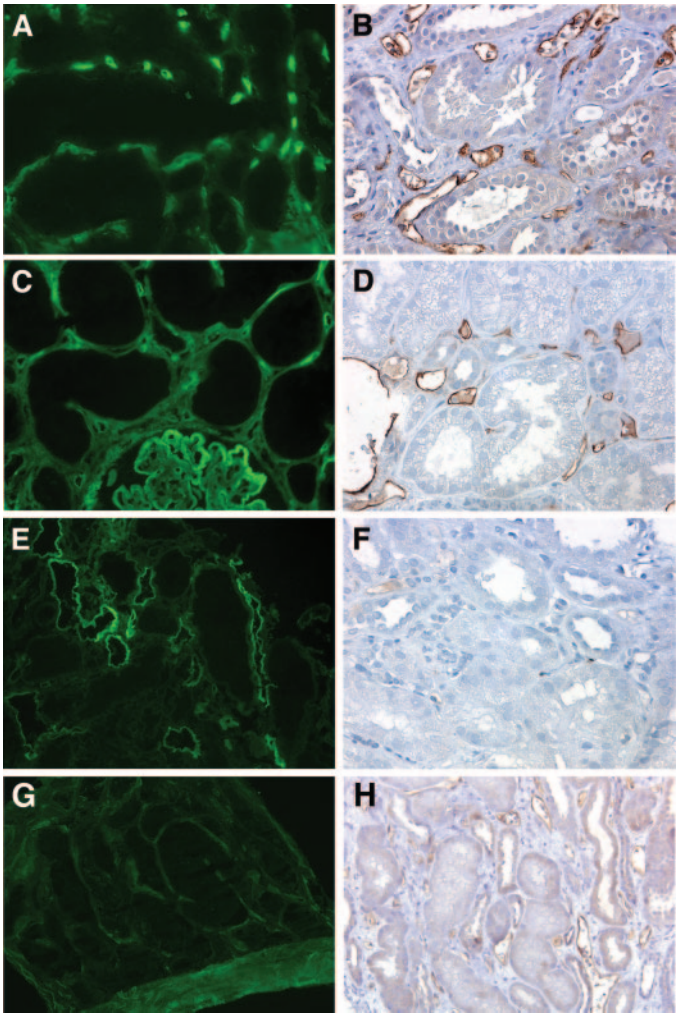

Figure 1. Comparison of immunofluorescence (IF) and immunohistochemical (IHC) C4d staining. (A and B) Positive C4d staining in peritubular capillaries by both IF (A) and IHC (B; case S-C). (C and D) Case S-F showing focal IF positivity on repeat staining (equivocal, see Results, Peritubular Capillary Staining for C4d) and positive IHC staining. (E and F) Case S-I, positive IF staining and negative IHC staining, with trace IHC C4d labeling of a rare capillary. (G and H) Case O-27 with negative IF staining and positive, weak IHC staining. (A, C, E, and G) IF staining of frozen tissue sections. (B, D, F, and H) IHC staining of formalin-fixed, paraffin-embedded tissue sections. Magnifications: ×250 in A, C, E, and G; ×200 to 400 in B, D, F, and H.

IHC Staining

Formalin-fixed, paraffin-embedded tissue was sectioned at 4 μm and stained with polyclonal antiserum to C4d (Biomedica Gruppe Austria; distributed by ALPCO, Windham, NH). The majority of Stanford cases were stained by hand using routine protocols, including pretreatment for 15 min in boiling citrate (pH 8.0), a primary antibody concentration of 1:20 or 1:40 (titered by antibody lot), and secondary goat anti-rabbit IgG antibody (cat. no. 111-065-045; Jackson ImmunoResearch, West Grove, PA) at 1:360 dilution. Detection was performed with streptavidin/horseradish peroxidase (Jackson ImmunoResearch) and developed with Stable DAB (Dako, Carpenteria, CA). Fourteen of the Stanford cases and all OHSU cases were stained on a Ventana Benchmark automated stainer, using "mild" conditions and a primary antibody concentration of 1:20. Subsequently, all positive or discordant Stanford cases were repeated with this method. As demonstrated by repeat staining of positive cases and control tissue (sections of graft nephrectomy with histopathologic features of humoral rejection and known C4d deposition), these methods give equivalent staining in our hands. Intensity of staining in peritubular capillaries was scored semiquantitatively (negative, trace, 1+, 2+, 3+), and the percentage of positive peritubular capillary staining in nonatrophic areas also was estimated, including cortex and medulla. Staining of glomeruli and larger vessels was scored similarly.

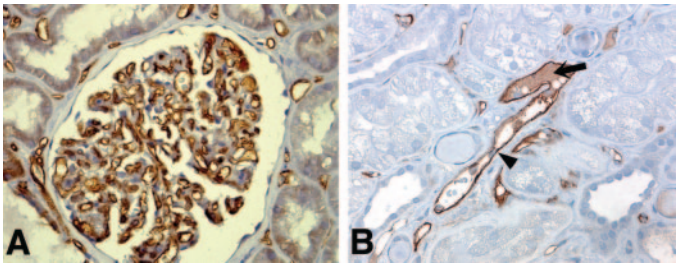

Figure 2. C4d IHC staining characteristics. (A) Strong C4d staining of glomerular endothelial cells, as well as surrounding peritubular capillaries (case O-8). (B) Distinction between C4d labeling of peritubular capillary endothelial cells (arrowhead) and intracapillary serum (arrow; case S-F). Magnification, ×400.

Results

Biopsy Characteristics: Stanford Data Set

A total of 107 renal allograft biopsies from Stanford University Medical Center were stained with antisera to C4d using both indirect IF and IHC methods. Forty-seven biopsies were performed at predetermined intervals in grafts with normal function (Table 1), whereas 60 biopsies were performed in grafts with clinical evidence of dysfunction, most commonly elevated creatinine. Alternatively, patients presented with proteinuria, anuria, hematuria, or as follow-up for previously treated acute rejection. Of 107 biopsies, 59 showed no histologic evidence of rejection, whereas 33 biopsies had some degree of acute cellular rejection, including Banff Borderline to Banff IIA (Table 2). No Banff IIB or III rejections were identified during this period. Fifteen biopsies did not meet Banff guidelines for biopsy adequacy (fewer than two cores and/or fewer than 10 glomeruli); however, there was no evidence of rejection in the material provided. Therefore, these specimens are grouped with the no rejection biopsies for further analysis.

Peritubular Capillary Staining for C4d

Nine cases (Stanford group 1) showed strong positive C4d staining that met the revised Banff '97 guidelines for C4d positivity by either IHC or IF (Tables 2 and 3), defined as "bright linear staining along capillary basement membranes typically involving over half of the sampled capillaries" (10). Only nonatrophic areas were evaluated (4,39,42). Of biopsies that were done for clinical cause, nine (15%) of 60 were C4d positive. Eight (24%) of 33 acute cellular rejection cases were positive. Data from the IF staining technique yielded eight of

Table 3. Staining characteristics of positive cases[a]

Case	IF			IHC			PTC		Cell Type
	Score	Intensity	(%)	Score	Intensity	(%)	Dilate	Cell	
Stanford									
S-A	+	2 to 3+	100	+	2+	80	++	++	M P
S-B	+	2 to 3+	100	+	2+	70	+	+++	M P
S-C	+	3+	100	+	3+	90	++	++	M
S-D	+	2+	100	−	1+	<10	+++	+++	P M
S-E	+	3+	100	+	3+	100	++	+	M
S-F	eq	1+	30	+	2 to 3+	70	−	++	N
S-G	+	3+	>50	+	3+	80	+	+	M
S-H	+	2 to 3+	100	+	2+	60	+	+	M N
S-I	+	2 to 3+	>50	−	1+	10	+++	+	M
OHSU									
O-5	+	2 to 3+	>90	+	2 to 3+	>90	+++	++	M P
O-6	+	3+	>90	+	3+	>90	+	+++	N M
O-7	+	2+	90	+	3+	>90	−	+	N
O-8	+	2 to 3+	100	+	3+	100	++	+	N M
O-10	+	3+	100	+	3+	>95	−	+	M N
O-11	+	2 to 3+	80	+	3+	80	+++	++	N M P
O-12	+	3+	80	+	2 to 3+	90	+	+	M
O-16	+	2 to 3+	90	+	3+	90	++	++	N M P
O-18	+	2 to 3+	90	+	3+	>95	+	+	N M P
O-22	+	2 to 3+	80	+	1 to 2+	50	++	+	M
O-23	+	1 to 2+	60	−	1+	<5	+	+	M
O-25	+	2+	60	+	1 to 2+	60	++	+	M
O-27	−	0	0	+	1 to 2+	50	+++	+	P M
O-29	+	2+	60	+	1 to 2+	70	+	+	M N
O-30	+	2+	70	+	1 to 2+	70	+	++	M N P
O-35	+	2+	50	+	2+	>80	++	+	M N
O-36	+	3+	100	+	2+	>90	++	+++	M N P

[a]Discordant cases highlighted in bold. eq, equivocal; IF, immunofluorescence; IHC, immunohistochemical; M, mononuclear cells (lymphocytes, monocytes); N, neutrophil; O, OHSU cases; P, plasma cells; PTC, peritubular capillary; S, Stanford cases; −, negative; +, present, scant; ++, moderate; +++, abundant.

107 positive cases, whereas the IHC method showed seven of 107 positive cases. Six cases were positive by both IHC and IF techniques; the majority showed 2+ to 3+ staining in >70% of capillaries and were unequivocally positive (Figure 1, A and B; Table 3). C4d-positive cases also demonstrated histopathologic features that were described previously in association with humoral rejection, including dilation of peritubular capillaries that contain mono- or polymorphonuclear cells (Table 3) (14,21,41). As noted in Materials and Methods, DSA testing was not available for these biopsies.

Three cases had some discrepancy in staining results between the two methods, including two cases that were positive by IF but negative by IHC (cases S-D and S-I, Figure 1, E and F, Table 3) and one case that was positive by IHC and considered equivocal by IF (case S-F, Figure 1, C and D; initial IF staining was completely negative, whereas repeat showed weak IF staining in a subset of capillaries, and the estimated 30% staining is likely not an accurate estimate because of the background atrophy; therefore, we chose to report this case as equivocal IF for study purposes). Staining results on these cases were confirmed by repeating both IF and IHC studies. Clinical follow-up information for these and other positive cases is provided in Table 4, and staining is shown in Figure 1.

There were five additional cases (Stanford group 2) that showed scant staining of weak intensity by one or both methods (scored as trace to 1+ intensity in <30% of nonatrophic cortex). The staining characteristics of these cases were distinct, both in intensity and in percentage of capillaries labeled, from those scored as positive and did not meet Banff criteria for C4d positivity, as defined above. However, one case from group 2 had interesting follow-up biopsies (received outside the study period). The study biopsy showed C4d staining of 1+ intensity in 30% of peritubular capillaries by IHC and C4d-positive glomeruli; IF was scored as 1+ staining in focal capillaries. Five weeks later, repeat biopsy demonstrated weak (1+) C4d staining of 15% of peritubular capillaries by IHC; 8 wk after the initial biopsy, a repeat biopsy was positive for C4d with strong staining (3+) in >50% of peritubular capillaries by IHC. Eleven

Table 4. Clinical data and follow-up on 24 patients with C4d-positive stain on biopsy[a]

Case	Time Post-transplantation (mo)	Age at Biopsy (yr)	SCr at Biopsy (mg/dl)	Banff Grade	Treatment	SCr 1 Mo after Biopsy	Follow-Up SCr (mg/dl)	Follow-Up Time (mo)
O-23[b]	12	37	0.8[S]	NER	No acute treatment	0.8[c]	0.8	12
O-7	0.25	39	3.7	NER	No acute treatment	1.3[c]	1.5	29
O-10	0.25	41	1.3[S]	NER	No acute treatment	1.0[c]	1.0	15
O-29	0.25	49	4.7[S]	NER[d]	No acute treatment	1.0[c]	1.4	7
S-A	7	58	1.0	Bord	No acute treatment	1.1[c]	1.2	21
O-22	78	28	3.2	NER	Change to rapamycin, increase Cellcept	3.1	3.2	11
O-16	156	72	3.2	NER	No acute treatment	3.4	Dialysis	5
S-F[b]	96	60	3.4	NER	No acute treatment	Dialysis	Dialysis	1
O-12	0.3	11	2.1	Bord	IV steroids	0.8[c]	0.7	23
S-C	120	13	1.34	Bord	IV steroids	0.94	0.9	17
O-5	36	32	3.5	Ia	IV steroids	1.6[c]	1.7	29
S-H	36	20	3.8	Ia	IV steroids and increased rapamycin	2.73	5.4	6
O-35	84	29	2.3	Ia	IV steroids and increased Imuran	2.3	2.2	2
O-25	12	18	1.8	Ia	IV steroids	1.9	5.0[N]	11
O-36	19	19	2.7	Ib	IV steroids	3	5.0[N]	4
O-18	108	52	12	Bord	IV steroids and dialysis	Dialysis	Dialysis	1
S-B	120	13	1.17	Ia	Thymoglobulin and IV steroids	0.95	0.9	18
S-I[b]	120	13	0.97	Ib	Thymoglobulin	0.88	0.9	20
O-6	0.25	66	2.8	NER	IVIg, rituximab, plasmapheresis	1.2	0.9	13
S-E	0.75	26	8.3	IIa	Thymoglobulin	1.3[c]	1.0	33
S-D[b]	36	11	1.3	Ib	Thymoglobulin, IV steroids, increase Prograf	1.08	1.1	12
O-27[b]	32	40	2.1	IIa	Thymoglobulin	1.4–1.8	1.8	2
O-8	0.25	64	2.2	NER	IVIg, rituximab, plasmapheresis, IV steroids	2.0[c]	2.9	15
S-G	36	20	3.2	Bord	Thymoglobulin, IV steroids	2.13	5.4	5

[a]Includes Stanford (S-) and OHSU (O-) data sets. Clinical data and follow-up not available for two C4d-positive cases. Bord, Borderline per Banff criteria; IV, intravenous; [N], patient noncompliance resulting in graft loss; NER, no evidence of rejection; [S], surveillance biopsy; SCr, serum creatinine.
[b]Discordant IHC/IF immunostaining results.
[c]Returns to within 20% of baseline SCr.
[d]Inadequate by Banff criteria.

months after the initial biopsy, the patient had an allograft nephrectomy that demonstrated focal segmental glomerulosclerosis as well as acute and chronic vascular rejection, despite aggressive treatment that included IVIg and rituximab.

Additional C4d-Positive Cases: OHSU Data Set

Given the relatively low absolute number of C4d-positive cases in the initial data set, additional cases were sought from the files of OHSU. The pathology database was searched for cases with nonzero C4d staining. This yielded 31 cases between 2002 and March 2005 in which both residual frozen and formalin-fixed, paraffin-embedded tissue was available. Of these, 17 cases were positive by one or both methods, with 16 cases positive by IF and 16 cases positive by IHC. One case was IF+/IHC−, and one case was IF−/IHC+ (Figure 1, G and H; Table 3). For the OHSU data set, the specificity is calculated as 93% and specificity is 94%. Combining the cases from both the Stanford and OHSU data sets yields an overall specificity of 98% and sensitivity of 87.5%.

Clinical follow-up was obtained for 24 of 26 positive cases from the combined data sets, including the IF/IHC discordant cases (Table 4). Eight patients who had C4d-positive staining without concurrent acute cellular rejection were not acutely treated at either center; these generally were biopsies soon after transplantation, with nearly normal serum creatinine. Eight rejection episodes were treated with pulse steroids, and eight were steroid resistant and required more aggressive treatment. C4d-discordant cases (IF+/IHC− and IF−/IHC+) were seen in both the no treatment and aggressive treatment groups. However, the numbers of cases are relatively small, and follow-up time and treatment are variable.

C4d Glomerular Staining: Combined Data Sets

In characterizing the polyclonal C4d antiserum, Regele *et al.* (17) previously observed C4d staining of normal glomeruli in a mesangial pattern in frozen section preparations but not with formalin-fixed, paraffin-embedded tissue. Using the IF method on frozen sections, we observed positive control mesangial staining of glomeruli in the majority of sections that contained glomeruli (data not shown). In the case of IHC staining of formalin-fixed tissue, glomeruli showed C4d staining in an endothelial distribution along glomerular capillary walls in 25 cases (Figure 2A). These included 19 cases with positive C4d peritubular capillary staining and six cases with focal weak C4d staining in peritubular capillaries (negative by Banff criteria). Cases with weak peritubular capillary staining also had weaker, patchy glomerular endothelial staining. We did not identify any cases that had glomerular endothelial labeling without some degree of peritubular capillary staining. In two biopsies, positive glomerular C4d staining could be associated

with a coexisting disease process; recurrent membranous glomerulonephritis (C4d IHC staining in a granular capillary wall pattern) or focal segmental glomerulosclerosis. Five of seven cases that showed histologic features of allograft glomerulopathy were C4d positive. However, other cases with C4d-positive glomeruli were morphologically unremarkable.

Comparison of Staining Characteristics: Combined Data Set

IHC generally had higher background staining but better morphologic preservation. In our hands, background IHC staining was derived from two sources. Because we did not use a biotin-free detection system, there was occasional labeling of renal tubular epithelium. However, tubules were readily distinguished from peritubular capillaries on sections, and this did not prove problematic in interpretation. In addition, occasional cases demonstrated light brown labeling of serum protein within peritubular capillaries (Figure 2B). Again, the stain quality and the precise localization of serum labeling were distinct and did not complicate the assessment of endothelial cell staining. Morphologic analysis of frozen tissue sections is not optimal, and strong tubular basement membrane labeling occasionally hampered evaluation of peritubular capillary staining with the IF method. Although it was possible, with experience, to distinguish areas of atrophy by virtue of faint tubular basement membrane IF staining, this proved considerably more difficult than in the IHC stained samples.

Discussion

Positive C4d immunostaining in peritubular capillaries of renal transplant biopsies has been incorporated into the criteria for the diagnosis of humoral rejection, and data are being collected on C4d staining in heart and other solid-organ allograft biopsies (39). Several antibody reagents for analysis of C4d in tissue are available, including reagents that require frozen sections and reagents that react with C4d epitopes in formalin-fixed, paraffin-embedded tissue sections. Differences in staining properties are multifactorial, including tissue preparation (*e.g.*, transport media, fresh frozen *versus* formalin-fixed, paraffin-embedded), primary antibody, secondary antibody, and detection method. We compared the Quidel monoclonal C4d antibody on frozen sections with IF detection with to the Biomedica polyclonal antiserum on paraffin-embedded tissue sections with IHC detection. We found comparable results with the two methods. There was complete concordance in 133 (96.4%) of 138 cases in the combined data set. IF stained 24 of 26 total positive cases, and IHC stained 23 of 26 total positive cases. Unfortunately, DSA data are not available to characterize these cases further. Using IF as the "gold standard" for the combined data set, IHC specificity was 98% and sensitivity was 87.5%. However, two cases that were positive by IHC but negative by IF also may indicate a low rate of false-negative IF. Our data confirm and extend the recent study by Nadasdy *et al.* (40) in which comparable results were seen on 30 renal biopsies that were tested with the Biogenesis and/or Quidel antibodies on frozen tissue and with the polyclonal antibody on frozen and formalin-fixed, paraffin-embedded tissue.

From the Stanford data set, we found 8.4% of cases C4d positive. This compares with 20 to 51% of cases that were reported to be C4d positive in initial large studies of historical renal biopsy samples (13,15–25). However, the subset of our nonprotocol biopsies affords a more valid comparison (15% positive), because biopsies that were tested in previous studies essentially all were prompted by clinical suspicion of rejection. Our C4d data are similar to more contemporary data, although center-specific differences in patient population, HLA-typing method, immunosuppressive regimen, and biopsy practices remain confounding variables. Lorenz *et al.* (26) presented a single-center experience in which 16.7% of 665 biopsies that were performed for clinical cause were found to be C4d positive. Furthermore, Mengel *et al.* (43) presented a multicenter study (Hannover, Barcelona, and Antwerp) and reported a C4d-positive rate of 2.0% of protocol biopsies and 12.2% of biopsies that were performed for clinical indication (43). Our data showing 24% of biopsies with histopathologic evidence of cellular rejection (Banff Borderline through IIA) positive for C4d also compares favorably with the Cedars Sinai experience, presented by Hever *et al.* (44), showing 30.4% of early acute rejection and 31.3% of late acute rejection cases positive for C4d. A recent conference Work Group report cited statistics of 5 to 7% of patients and 12 to 37% of biopsies (that were taken for acute rejection) as having antibody-mediated rejection (39). Whether the patients with C4d staining of peritubular capillaries in our series have worse graft outcome could not be established, because the follow-up period was relatively short in all cases, given a study that was designed primarily to compare reagents and staining methods.

We also confirmed Regele's initial observation (17) that normal glomeruli show mesangial C4d staining with an IF detection method applied to frozen tissue but not with the polyclonal antibody applied to formalin-fixed, paraffin-embedded tissue (IHC method). Cases with strong and diffusely positive glomerular endothelial C4d staining represent the majority of cases with positive peritubular capillary C4d staining. Five of 7 cases with allograft glomerulopathy demonstrated C4d-positive glomeruli. Subsequently, we observed additional allograft glomerulopathy cases with both positive and negative glomerular capillary C4d staining. Again, longer follow-up will be necessary to determine whether patients with glomerular endothelial C4d staining are prone to chronic allograft glomerulopathy, as suggested in the literature (18,20,34,35,45–48).

The modified Banff criteria require C4d staining in 50% of peritubular capillaries to score a biopsy as C4d positive (10). Further studies are needed for clinical validation of this threshold, because previous studies used different definitions of C4d positivity. In the majority of cases from both of our data sets, distinction between positive and negative staining was clear-cut. Cases that were interpreted as being positive had strong staining in the overwhelming majority of peritubular capillaries, whereas cases that were interpreted as being negative showed weak staining in a minority of capillaries and generally would have been scored as negative even with a lower threshold of 25%. Other studies have noted that focal staining may be seen as C4d accumulates, with subsequent biopsies showing

diffuse staining, or in the setting of C4d degradation (16). Likewise, one of our Stanford group 2 patients had markedly increased C4d staining in subsequent biopsies outside the study period, which suggests that focal or weak staining also may be of some significance.

Conclusion

The sensitivity and the specificity of the IHC method is acceptable in comparison with the IF method. Although at the present time IF is considered the "gold standard," IF/IHC-discordant cases were seen in both no treatment and aggressive therapy groups (Table 4); the follow-up graft function in these cases does not show any trend to suggest superiority of positive IF or IHC staining. However, the positive case numbers are too small to draw conclusions of clinical utility. In our renal biopsy practice, IHC offers several advantages. IHC was performed on serial levels of the same tissue that was cut for light microscopy; therefore, IHC offered a generous tissue sample for analysis, with good preservation of histologic features, which is important for scoring nonatrophic areas. Exclusion of atrophic areas of cortex in scoring IF samples was significantly more difficult. Automated IHC staining of levels cut in parallel with tissue for light microscopy permits saving of labor and tissue and results in a permanent archival slide record. Although sources of IHC background staining must be taken into account, the occasional tubular epithelial or serum labeling did not make interpretation of IHC stained slides difficult. Furthermore, as reported in protocols that described IHC localization of glomerular immune complexes in medical renal biopsies, thorough washing of tissue cores before processing may help to alleviate serum staining (49). Our study shows that a small percentage of cases may be falsely negative by either staining method; therefore, our results provide further data in support of the multifactorial criteria for assessment of humoral rejection, as established in the revised Banff classification system (10). Careful correlation of C4d results, donor-specific serum testing, and histologic features should be performed in each case and further correlated with clinical history.

Acknowledgments

A subset of these data were presented in abstract form at the 2005 United States and Canadian Academy of Pathology meeting; March 1, 2005, San Antonio, Texas; and in abstract form at the 8th Banff Conference on Allograft Pathology; July 18, 2005; Edmonton, Alberta, Canada.

We thank Veronica Mason, Marc Visitacion, Lulu Villanueva, and Edward Gilbert as well as Anita Reynon, Palmira Makeef, Shirley Kwok, and Linda Anderson for expert technical help with IHC and IF staining, respectively. We acknowledge the assistance of Sheryl Shah, Mary Ann Head, Dr. Anuja Mittalhenkle, and Dr. Douglas Norman with clinical follow-up data. Dr. Don Houghton provided critical discussion and access to additional material. We thank Caroline Tudor for expert preparation of figures.

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