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Detection of interstitial increase in macrophages, characteristic of acute interstitial rejection, in routinely processed renal allograft biopsies using the monoclonal antibody KP1

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Abstract. Acute interstitial rejection (AIR) of renal allografts is accompanied by a characteristic peritubular increase in macrophages, which can be identified with the CD14 monoclonal antibody (mAb) WT14 in cryostat sections. Since frozen tissue is not always available, we tested whether this increase can also be demonstrated in Bouin-fixed, paraffin-embedded biopsies, using the CD68 anti-macrophage mAb KP1, which can also be applied to paraffin sections. Sections of 16 biopsies with AIR and 11 controls were stained with KP1. In 25 of the 27 biopsies, macrophages were strongly positive for KP1. Two AIR biopsies were completely negative, probably due to prolonged fixation. In the remaining 14 AIR biopsies, the number of KP1-positive cells was significantly higher than in the controls [1184 ± 410 per mm^2 (mean ± SD) vs 112 ± 126 per mm^2]. We conclude that, especially in cases in which frozen tissue is not available, the demonstration of increased numbers of monocytes/macrophages with mAb KP1 can be a helpful adjunct in the histological diagnosis of AIR in routinely processed renal biopsies.

Key words: Kidney transplantation, biopsy – Monoclonal antibody, kidney biopsy – KP1, monoclonal antibody, kidney biopsy

In renal transplantation it can be very difficult to differentiate, either clinically or histologically, acute interstitial rejection (AIR) from other causes of declining graft function. The use of cyclosporin as an immunosuppressive treatment has added to the diagnostic problems since the histological changes caused by cyclosporin nephrotoxicity can overlap with those in AIR [15, 22]. Therefore, in addition to the usual histological criteria [18], immunohistological findings, such as the presence of T lymphocytosis and increased expression of HLA-DR on tubular epithelial cells (TEC), have been used as diagnostic markers for AIR [1, 3, 11]. In difficult cases, however, even these markers are not specific enough to yield a definitive diagnosis. There is an increase in T lymphocytes in most cases of interstitial inflammation, and the upregulation of HLA-DR expression is not specific for AIR either, as it also occurs during viral infections [25] and is not invariably present in AIR [9].

Several authors have shown that AIR is accompanied by an increase in macrophages [2, 10, 24], but until recently this finding did not receive much attention for diagnostic purposes, probably due to difficulties in visualization in histological sections [4]. A new anti-CD14 antibody, WT14, has shown such intensive and characteristic binding to interstitial macrophages in AIR that it could be used as a marker for rejection with better specificity than increased HLA-DR expression on TEC [4]. However, like most monoclonal antibodies (mAbs), WT14 can only be applied to cryostat sections. Since the small size of renal core biopsies does not always allow for separation of part of the material for immunohistology, it would be advantageous to have an antimacrophage antibody at one's disposal that reacts with antigenic structures that are resistant to routine fixation and embedding in paraffin. For routine fixation of renal tissue, either buffered formalin or Bouin's solution are used. Bouin's solution [13, 17] is the optimal fixative for the methenamine silver stain and trichrome stains, necessary for histological examination of renal tissue. This fixative, with a pH of 1.8, has an adverse effect on the preservation of antigenic structures, even more so than neutral-buffered formalin. In the present study, we tested whether the new antimonocyte/macrophage mAb KP1, which can be applied to routinely processed tissues [19], can be used as a marker for the diagnosis of AIR in routinely processed, Bouin-fixed, paraffin-embedded renal allograft biopsies.

Materials and methods

Antibodies

The mAb KP1 (CD68, Dakopatts, Denmark), raised against a lysosomal fraction of human lung macrophages, recognizes a fixation-resistant epitope on monocytes, tissue macrophages, and granulocyte precursors, both in cryostat sections and in tissues fixed in

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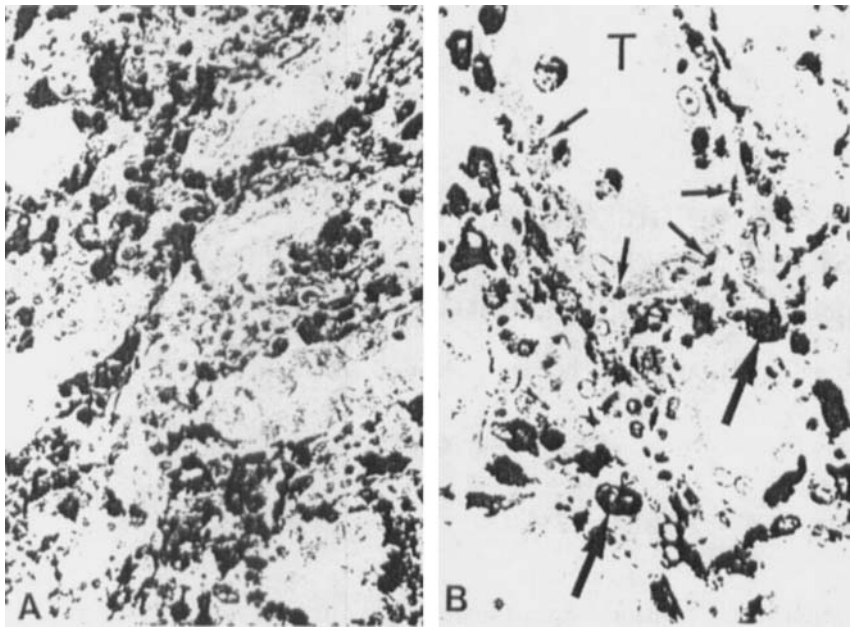

Fig. 1 A, B. KP1 staining in a renal allograft with acute interstitial rejection: **A** interstitial increase in KP1-positive cells. Tubular epithelial cells are negative (×250); **B** detail, showing the difference in staining intensity between large, strongly positive macrophages *(large arrows)* and weakly stained peritubular monocytoid cells (*small arrows*; ×400). *T* Tubule lumen

formalin or Bouin's solution [19]. In macrophages and monocytes, KP1 gives a fine to coarse granular cytoplasmic staining pattern. A weak to negative staining is seen in granulocytes, whereas lymphocytes are negative [19]. The antibodies UCHL1 (CD45RO) and L26 (CD20; both from Dakopatts, Denmark) react in paraffin sections with T cells and B cells, respectively [7, 21]. A polyclonal HLA-DR antibody [16], raised in rabbits and kindly provided by Dr. H. Ploegh, The Netherlands Cancer Institute, Amsterdam, together with a monoclonal antibody against a nonpolymorphic HLA-DR determinant (clone L243, Becton Dickinson) and the CD14 antimonocyte/macrophage monoclonal antibody WT14 [4] were applied to cryostat sections, and the results were compared with those obtained in the fixed material.

Biopsy specimens

Renal allograft biopsies of patients with and without AIR were selected retrospectively from the pathological and clinical files. Sixteen cases with clinically and histologically proven AIR were selected. Criteria for selection were the presence of an interstitial infiltrate with tubular invasion consistent with AIR [18], an increase in WT14-positive cells in cryostat sections with a characteristic peritubular pattern [4], and a favorable response to antirejection therapy. Control cases included 11 renal allograft biopsies without AIR and with diagnoses listed in Table 1. Seven of these control biopsies showed patchy interstitial infiltrates. In all cases the core biopsy contained sufficient cortical tissue to allow for counting of cells in ten high-power fields. All patients had been treated with cyclosporin, which was replaced by azathioprine and low-dose prednisone 12 weeks after transplantation. The time of biopsy varied from 1 to 307 weeks after transplantation.

Histology and immunohistology

Renal core biopsies of all cases were fixed in Bouin's solution (pH 1.8) for 1–3 h, except for three biopsies (two AIR cases and one control) that had been fixed over the weekend for 40–48 h. The tissues were embedded in paraffin and 3-μm sections were routinely stained with methenamine silver, periodic acid-Schiff, and chrome aniline blue stain.

For immunohistology, 3-μm sections were deparaffinized with xylene for 10 min, followed by hydration in ethanol and rinsing in running tap water. Endogenous peroxidase activity was inhibited by pretreatment with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min. Nonspecific binding sites were blocked by preincubation with 40% normal swine serum in PBS for 15 min. For staining with KP1, we used the avidin-biotin technique [12]. A first incubation with KP1 (dilution 1:50) for 60 min was followed by a second incubation with a biotin-conjugated horse anti-mouse immunoglobulin (dilution 1:100) for 30 min and a third incubation with the avidin-biotin peroxidase complex (Vector Laboratories, USA) for 45 min. An indirect immunoperoxidase procedure was used for staining with UCHL1 and L26 [8], consisting of a first incubation with UCHL1 (dilution 1:40) and L26 (dilution 1:100) for 60 min and a second incubation with peroxidase-labeled rabbit anti-mouse immunoglobulin (dilution 1:80, Dakopatts). For staining of paraffin sections with the polyclonal rabbit anti-HLA-DR, a peroxidase-antiperoxidase technique was used [8]: incubation of the primary antibody (dilution 1:80) was followed by a swine anti-rabbit immunoglobulin (dilution 1:20) and a third incubation with peroxidase-antiperoxidase complex (dilution 1:800, Dakopatts).

Staining of cryostat sections with WT14 and the monoclonal and polyclonal antibodies against HLA-DR was performed as described previously [4]. Briefly, after a first incubation with specific antibody for 60 min, the sections were incubated with peroxidase-labeled rabbit anti-mouse immunoglobulin and swine anti-rabbit immunoglobulin, respectively. All incubations were carried out at room temperature and each incubation step was followed by washing in PBS. In all procedures diaminobenzidine was used as the chromogen and sections were counterstained with hematoxylin for 2 min.

Scoring of KP1, WT14, and HLA-DR positivity

Using an ocular grid divided into to 25 fields, we counted the KP1-positive mononuclear cells in AIR and in control biopsies in ten cortical high-power fields (magnification × 400) that were representative for the whole cortical area. Positive cells located in the tubules and in the vessels were included in the counts. Glomeruli were not included in the selected fields. In frozen sections, the diffusion of the diaminobenzidine product makes counting of separate WT14-positive cells unreliable. Therefore, WT14 positivity in the frozen material was expressed semiquantitatively as the extent of peritubular positivity in the section area [4, 5]. We distinguished five

Table 1. KP1 staining of interstitial monocytes/macrophages in Bouin-fixed renal allograft biopsies with acute interstitial rejection and controls. Comparison with staining patterns of polyclonal HLA-DR antibody in serial sections and with monoclonal HLA-DR and WT14 antibody in cryostat sections

Histological diagnosis	*n*	Paraffin sections		Corresponding cryostat sections	
		KP1-positive cells per mm² [a] (range)	HLA-DR positivity of TEC [b]	HLA-DR positivity of TEC [c]	WT14 pattern [d]
Acute interstitial rejection	14	1184 ± 410* (429–1922)	+ (10) ± (3) − (1)	+ (11) ± (3) − (0)	A(14)
Controls	11	112 ± 126 (3–367)	+ (1) ± (6) − (4)	+ (4) ± (5) − (2)	
Cyclosporin nephrotoxicity	3	3,172,367	−, −, ±	±, −, ±	D,NA,B
Acute tubular necrosis	2	37,305	−, ±	−, +	D,C
IgA nephropathy	2	27,46	±, ±	±, +	C,B
Membranous nephropathy	1	16	±	±	E
Focal glomerulosclerosis	1	27	+	±	D
Renal artery stenosis	1	51	−	±	F
No pathological changes	1	175	±	+	C

* $P < 0.001$ as compared to controls (Wilcoxon rank sum test)

[a] Expressed as mean ± SD for AIR and control groups, and given as individual counts for the 11 control biopsies

[b] Expressed as number of biopsies (in parentheses) with positive (+), weakly positive (±), or negative (−) staining with polyclonal HLA-DR antibody

[c] Expressed as number of biopsies (in parentheses) with positive (+), weakly positive (±), or negative (−) staining with monoclonal HLA-DR antibody

[d] Extent of peritubular increase in WT14-positive cells expressed as A: diffuse, over more than 80% of the section area, B: patchy, over 50%–80% of the section area; C: irregular staining of low density over 20%–50% of the section area; D: slightly increased staining over less than 20% of the section area; E: dispersed positive cells, as in normal kidneys; F: inadequate because of necrosis; NA: no frozen tissue available

patterns: A, diffuse, over more than 80% of the section area; B, patchy, over 50%–80% of the section area; C, irregular staining of low density over 20%–50% of the section area; D, slightly increased staining over less than 20% of the section area; and E, dispersed positive cells, as in normal kidneys. HLA-DR staining of the tubular epithelial cells was graded semiquantitatively as positive, weakly positive, or negative, in both routinely processed and frozen sections. The staining intensities of the peritubular capillaries and interstitial cells were used as an internal reference.

Results

Two of the 16 cases of AIR were completely negative for KP1. In the other 14 biopsies, KP1 staining was strongly positive in interstitial cells that had the cytological appearance of macrophages. In the sections of patients with AIR, these cells were located not only in the interstitium but also between TEC, in the tubular lumina, and in the vessels (Fig. 1). A weaker staining was seen in smaller mononuclear cells, with oval nuclei and a moderate amount of clear cytoplasm, consistent with the cytological appearance of small histiocytic cells or monocytes. These cells were only present in biopsies with interstitial infiltrates due to AIR and were located directly around the tubular basement membranes, in a pattern similar to the one we previously observed in cryostat sections using mAb WT14 [4] (Fig. 1B). In the control biopsies these peritubular cells were not seen. In five AIR biopsies, plasma cells and polymorphonuclear granulocytes also showed weak positivity for KP1, but both cell types could easily be differentiated from cells of monocyte/macrophage lineage by their typical nuclear features. There was a moderate variation in staining intensity between different biopsies. As shown in Table 1, the number of KP1-positive cells was significantly higher in AIR than in the controls: 1184 ± 410 per mm² versus 112 ± 126 per mm² (mean ± SD; $P < 0.001$, Wilcoxon rank sum test).

Table 1 also shows the results of the HLA-DR staining in serial paraffin sections and of WT14 and HLA-DR staining in the frozen part of the biopsy. Comparison of KP1 with WT14 staining on cryostat sections of the frozen part of the biopsies is only valid in the control group since, in the AIR group, a diffuse increase in WT14-positive cells (pattern A) had been used as a selection criterion.

Binding of the polyclonal HLA-DR antibody to the HLA-DR antigens on interstitial cells, peritubular capillaries, and TEC was not abolished by the use of Bouin's solution. A variable staining of TEC was found in both rejection cases and controls (Table 1). Ten of 14 rejection biopsy specimens demonstrated a positive HLA-DR staining, three a weakly positive staining, and in one case the staining was completely negative. In the control group, TEC was positive for HLA-DR in one biopsy, weakly positive in six specimens, and completely negative in four others. The staining results of polyclonal anti-HLA-DR in paraffin sections were largely comparable with those of monoclonal anti-HLA-DR in the cryostat sections (Table 1).

UCHL1, generally used for staining T cells in formalin-fixed tissues, did not stain the T cells in our Bouin-fixed material. Similarly, L26 staining was negative in all biopsies, except in one case of AIR.

Discussion

Our results show that macrophages can be immunohistologically identified with mAb KP1 in routinely processed renal biopsies and that as in our earlier findings using mAb WT14 in frozen material, a significant increase in interstitial macrophages can be demonstrated in cases with AIR.

The moderate variation in staining intensity between different biopsies was probably caused by differences in fixation times, since the two biopsies that were completely negative proved, in retrospect, to have been fixed in Bouin's solution for 40 and 48 h (over the weekend). Since a few KP1-positive cells are always present, even in normal kidneys, a completely negative KP1 staining can be considered not evaluable. Therefore, both biopsies were excluded from the count. One could argue that the deleterious effect of prolonged fixation in Bouin's solution could have been prevented by the use of buffered formalin as the routine fixative for graft biopsies. The advantage of Bouin's solution, however, is that it gives superior results for the methenamine silver stain and trichrome stains in particular, which are essential for optimal study of glomerular pathology [17].

Although glomerular lesions are, in general, not a hallmark in the diagnosis of AIR, accurate study of the glomerular basement membrane is still indicated in renal graft biopsies, not only for the diagnosis of transplant glomerulopathy, but also in cases of recurrence of the original renal disease in the graft [14] and for de novo developing glomerular diseases [6]. Optimal visualization of the glomerular basement membrane in the paraffin sections is even more essential in cases in which no frozen tissue is available for immunofluorescence. For these reasons, and for the practical reason that we prefer a uniform fixation method for graft biopsies and nongraft biopsies, we have thus far routinely used Bouin's solution for renal tissue in our laboratory. After our findings with KP1 in the Bouin-fixed material, we compared the results with those of histological and immunohistological stainings in a small series of graft biopsies fixed in buffered formalin. To our surprise, the KP1 staining in formalin-fixed tissue was not better, and specifically not more intense, than that in the Bouin-fixed material with a normal (1–3 h) fixation time. The staining of TEC with polyclonal anti-HLA-DR was, in general, more intense in the formalin-fixed biopsies, whereas the staining with methenamine silver and trichrome were, as expected, less satisfactory.

As shown in Table 1, there was a good correlation between a low extent of WT14 staining (patterns D and E) and low numbers of KP1-positive cells. In the WT14 categories B and C, both low and high numbers of KP1-positive cells were found. For WT14 category C, such a variation can be expected. However, in WT14 category B, with a high extent of WT14 positivity, the finding of a low number of KP1-positive cells, as in one of our control cases (Table 1), is unexpected. The cryostat section of this biopsy showed, apart from a few patchy infiltrates, a peritubular increase in WT14-positive cells in 60 % of the tissue area. In this case the discrepancy between WT14 and KP1 staining could be due to sampling error, but loss of antigenic structures seems more likely since this biopsy had been fixed in Bouin's solution over the weekend. The HLA-DR expression on tubular epithelial cells was found to be weakly positive in the paraffin section, whereas it was scored as positive in the parallel cryostat section, which is also an argument for loss of antigenicity due to prolonged fixation in Bouin's solution.

Obviously, apart from the different scoring systems used for quantitation of KP1 and WT14 staining, the number of control biopsies is too low to allow for statistical comparison of the results between or within the subgroups. Moreover, the number of biopsies, in both the AIR and control groups, is not sufficient to recommend routine use of the number of KP1-positive cells as a quantitative indication of the probability or severity of AIR. Apart from this, the time-consuming procedure of counting cells per mm^2 is not feasible for routine diagnosis. We have used the counting of KP1-positive cells as a means of illustrating a quantitative increase in interstitial monocytes/macrophages in cases of AIR and to test whether KP1 staining may be helpful in cases in which frozen tissue is not available or not representative. When a frozen fragment of renal cortex is available, WT14 staining is preferable: the sections can be cut immediately, the staining is easy to perform, and the extent of positivity, graded as patterns A to E as previously published, can be estimated in a few seconds. Another disadvantage of KP1 staining is that the peritubular localization of the macrophages, which is so characteristic of WT14 staining in frozen sections of biopsies with AIR [4, 5], is not very prominent in the paraffin sections. This is due to the relatively weak staining intensity of the monocytoid cells that are located directly around the tubular basement membranes (Fig. 1B). In cases in which these weakly staining cells are not observed, it may be difficult or impossible to differentiate the interstitial increase in macrophages from an increase due to interstitial inflammation other than AIR. A mAb like WT14, with a high affinity for these monocytoid cells, would be preferable, but thus far we have not found a CD14 antibody that gives a satisfactory staining in routinely fixed tissue. In frozen sections, KP1 staining resulted in a stronger positivity of monocytes/macrophages than in paraffin sections but was accompanied by a variable and sometimes considerable background staining that obscured the peritubular pattern present in serial sections stained with WT14. For similar reasons, staining of frozen sections with the polyclonal HLA-DR antibody had no advantage over staining with the monoclonal antibody.

The variability in HLA-DR staining of TEC in rejection cases and controls (Table 1) is in accordance with our previous findings in cryostat sections [4] and with the results of other studies [20, 23]. Since these variable results were also seen in the parallel frozen sections (Table 1), a fixation artifact as cause for this variation seems unlikely. In paraffin sections the staining results with the polyclonal

antibody were, both in Bouin's solution and in formalin-fixed material, less intense than those obtained with the monoclonal HLA-DR antibody in cryostat sections. Still, since grading was performed with the use of internal controls, the blindly scored, semiquantitative scales corresponded fairly well with those in the frozen sections (Table 1).

The absence of positive staining of the infiltrates in AIR with UCHL1 and L26 was probably due to the low pH of Bouin's solution, since the staining was positive in the formalin-fixed biopsies.

In conclusion, we found that mAb KP1 can be used for staining of mononuclear cells of monocyte/macrophage lineage in routinely processed renal allograft biopsies even after Bouin's solution, provided fixation times are kept within the recommended limits of 1–3 h. Staining of routinely fixed allograft biopsies with KP1 can thus serve as an adjunct in the diagnosis of AIR, especially in cases for which frozen material is not available for staining with anti-CD14 monoclonal antibody.

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