C4d Positivity Is Often Associated with Acute Cellular Rejection in Renal Transplant Biopsies Following Campath-1H (Alemtuzumab) Induction

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Abstract. Peritubular capillary C4d positivity, a marker for antibody-mediated rejection, is observed in approximately 20-50% of indicated renal transplant biopsies and in just 2% of unremarkable protocol biopsies. However, C4d staining has not been evaluated in protocol renal biopsies from patients with Campath-1H induction treatment, and the association between various types of inflammatory cells and acute antibody-mediated rejection is unclear. This study investigated the rates of C4d positivity in unremarkable protocol renal biopsies, biopsies with acute tubular necrosis (ATN), and biopsies with acute cellular rejection (ACR), all following Campath-1H treatment and post-operative immunosuppression. There was low positivity of C4d staining in both the protocol and ATN groups, but the ACR group had a 47.2% rate of positivity (combining focal and diffuse positive cases). Since Campath-1H treatment caused significant depletion of circulating lymphocytes but not circulating monocytes in renal recipients, this study also investigated the role of monocytes in humoral rejection. In ACR cases, CD68 positive monocytes were composed of 59.4 ± 4.69% inflammatory cells, which was significantly higher than CD3 positive lymphocytes (38.9 ± 4.4%). Co-localization of positive C4d staining in endothelium and marginating CD68 positive monocytes was illustrated by double staining. Our data indicate that acute antibody-mediated rejection occurs much more frequently in renal transplants with ACR. Moreover, the high percentage of monocytes observed in ACR cases (due to monocytes being less sensitive to Campath-1H depletion) suggests that monocytes are involved in antibody-mediated rejection.

Keywords: Campath-1H, Alemtuzumab, antibody mediated rejection, renal transplantation

Introduction

C4d is an element of complements and is involved in the classical pathway of complement cascade activation [1]. This marker has been shown to be useful in identifying antibody-mediated rejection in 22-50% of renal transplant biopsies [2-4], since in correlation with circulating anti-donor antibodies, a study reported 95% sensitivity and 96% specificity of C4d staining in peritubular capillaries for the diagnosis of acute antibody-mediated rejection [5]. Antibody-mediated rejection based mainly on C4d positivity has recently been accepted in the Banff criteria [6]. One study demonstrated that 0-2% of protocol transplant biopsies (in the absence of Campath-1H treatment) were positive for C4d, and that C4d positivity in protocol biopsies has no significant impact on allograft survival [7].

Campath-1H (Alemtuzumab) is a humanized monoclonal antibody to human CD52 antigen, which is mainly distributed on the surface of various types of inflammatory cells [8]. Previous flow cytometry studies reported that bolus injection of Campath-1H results in a significant decline in circulating lymphocytes, monocytes, and natural
killer cells in patients who receive bone marrow transplants [9,10]. The CD52 negative fraction of inflammatory cells includes CD3 positive T lymphocytes (19%), CD14 positive monocytes (10%), CD19 positive B lymphocytes (2%), and CD56 positive natural killer cells (7%) [10]. However, no flow cytometry study has investigated CD52 positive inflammatory cells or evaluated C4d staining in protocol biopsies of renal transplant recipients following both Campath-1H treatment and postoperative immunosuppression.

The goals of this study were (1) to determine whether circulating monocytes in renal transplant recipients are less sensitive than lymphocytes to Campath-1H depletion; (2) to compare the rates of C4d positivity in unremarkable protocol renal transplant biopsies, biopsies with acute tubular necrosis (ATN), and biopsies with acute cellular rejection (ACR), following Campath-1H treatment and postoperative immunosuppression; and (3) to investigate the potential role of monocytes in antibody-mediated rejection.

Materials and Methods

Data were collected from 101 patients at our institution who received renal transplants from living or deceased donors, from May 2003 to August 2005. All of these patients received intra-operative induction with Campath-1H (Alemtuzumab, Berlex Inc., Seattle, WA). The pretreatment regimen included 1 g of iv methyprednisolone, 650 mg of oral acetylsalicyclic acid, 50 mg of iv diphenhydramine, and 30 mg of iv Campath-1H every 2 hr (given before unclamping of the transplanted kidney vessels). Post-operative immunosuppression included either FK506 (target level of 10 ng/ml from day 1) or mycophenolate mofetil (1 g bid from day 1). All patients received prednisone, starting at 20 mg/day, with subsequent weaning of 2.5 mg/day each week to discontinuation over 8 weeks. The patients received Valgan-cylovir (450-900 mg daily) for cytomegalovirus (CMV) prophylaxis from the first week post-transplant, with the dosage and duration dependent on renal function, pre-transplant donor status, and recipient CMV status. Testing for CMV antigenemia was done weekly for the first 3 mo, biweekly from mo 3 to 9, and monthly thereafter. Patients were monitored weekly by complete blood counts and assays of serum electrolytes and creatinine.

Flow cytometry. For the flow cytometry study, we obtained blood samples from 14 patients with renal transplants (one week to 3 mo following Campath-1H treatment) and from 10 non-transplant patients as controls. Blood lymphocytes and monocytes were measured in red cell-lysed whole blood (Optilise-C; Beckman-Coulter Corp, Miami, FL) by flow cytometry. Immunofluorescence labeled markers including CD3 (representing T lymphocytes), CD19 (representing B lymphocytes), CD14 (representing dendritic monocytes), and CD52 were purchased from Dako Cytomation (Carpinteria, CA). Counting events were acquired with a model FC-500 flow cytometer (Beckman-Coulter) equipped with a 488 nm argon laser. The absolute numbers of circulating lymphocytes and monocytes were calculated from the total white blood count and were obtained from our hematology laboratory.

Enzyme-linked immunosorbent assay (ELISA). To evaluate levels of cytokines after Campath-1H treatment, we measured interleukin-12 (IL-12), a major cytokine in immune reactivity, in non-transplant controls and in patients with renal transplants and Campath-1H treatment, as described for the flow cytometry study. IL-12 is a pleiotropic cytokine produced primarily by antigen-presenting cells (B lymphocytes, monocytes, and dendritic cells) and has multiple effects on T lymphocytes and natural killer (NK) cells, including the ability to stimulate cytotoxicity, proliferation, cytokine production, and Th1 subset development [11,12]. This cytokine is a disulfide-linked 70 kDa heterodimeric glycoprotein, composed of a 40 kDa (p40) and a 35 kDa (p35) subunit. The IL-12 p40 ELISA mainly determines the free mass of p40 in the plasma. IL-12 ELISA kits were purchased from R and D Systems (Minneapolis, MN). The IL-12 concentrations in plasma samples (100 µl) were measured by spectrophotometry according to the manufacturer’s instructions.

Histologic staining and evaluation of renal biopsies. Renal biopsies were fixed in formalin and embedded in paraffin. Tissue blocks were cut into 3 µm sections for routine staining (3 sections for hematoxylin-eosin stain, 3 sections for periodic acid-Schiff stain, and 1 section for Masson’s trichrome stain). Sections were de-waxed in xylene and rehydrated with graded ethanols to water. To exclude antibody-mediated rejection in the biopsies, cases were retrospectively collected. Paraffin embedded renal sections were stained using rabbit polyclonal C4d antibody (Alenco Diagnostic, Salem, NH) in protocol renal transplant biopsies (negative for any significant changes) (n = 54), renal transplant biopsies with ATN (n = 28), and renal transplant biopsies with ACR (n = 26). Slides were stained immunohistochemically for C4d using a Dako Autostainer (model E172566, Dako Cytomation, Carpinteria, CA). C4d staining was graded as follows: <25% of peritubular capillary (PTC) staining = negative; 25% PTC staining = focal positive; and >50% PTC staining = diffuse positive. CD68 (clone PGM, a specific marker for monocyte/macrophages) and CD3 antibodies, a marker of T lymphocytes, (Dako Cytomation) were also stained using the Dako Autostainer. Monocytes were stained pink with alkaline phosphatase, in order to distinguish them from C4d positive cells on the same slide, which were stained brown with horseradish peroxidase. After the brown C4d stain was denatured using denaturing solution (Biocare Medical, Concord, CA) and washed with Tris-buffered saline, diluted CD68 primary antibody was applied manually, followed by secondary antibody and alkaline phosphatase, according to the manufacturer’s protocol (Biocare Medical). ACR was...
Biopsies with features of dilated tubules, diminished brush borders, and sloughed epithelial cells in the lumina were considered to have acute ATN when no features of acute rejection were present. Incidence results were expressed as percentages of the total number of cases.

**Statistics.** Values were expressed as mean ± SD. The unpaired t test was used to compare groups of data. In addition, the Chi-square test was used to compare the rates of C4d positivity among the protocol biopsy, ATN, and ACR groups. Values of p <0.05 were considered statistically significant.

**Results**

**Campath-1H treatment significantly depleted lymphocytes but not monocytes.** Plasma levels of interleukin-12 in renal recipients who received Campath-1H treatment were significantly lower than in controls (Table 1). Based on blood cell counting, the total leukocyte counts and percentages of monocytes were similar in Campath-1H treated renal recipients and controls (Table 1). Compared to controls, the percentages of lymphocytes were markedly depleted after Campath-1H treatment, whereas the percentages of neutrophils were significantly higher (Table 1). Flow cytometric patterns of a control case and a renal recipient case following Campath-1H treatment are shown in Fig. 1. The flow cytometry study demonstrated that Campath-1H significantly depleted CD3 positive T lymphocytes and CD19 positive B lymphocytes in renal recipients, when compared to the control group (Table 1). Overall, CD52 positive cells were also significantly reduced after Campath-1H treatment. However, CD14 positive monocytes and CD14/CD52 positive monocytes were not significantly different in the Campath-1H treated group vs the control group.

C4d positivity was low in unremarkable renal protocol biopsies and indicated biopsies with ATN, but was much higher in indicated biopsies with ACR. There was low positivity of C4d staining in the protocol group and in the ATN group. But the ACR group had a 47.2% positive rate (combining focal and diffuse positive cases) (Table 2). Indicated biopsies (combining ATN and ACR cases) had...
Fig. 1. The upper 2 panels (above dashed line) show flow cytometry patterns from a control case (CD3: 56.38%, CD19: 7.48%, CD14: 4.92%, CD52: 87.72%, CD14/CD52: 4.82%). CD3 positive T lymphocytes and CD19 positive B lymphocytes were the dominant cells positive for CD52. Only a small percentage of CD52 positive cells were CD14 positive monocytes. The lower 2 panels (below dashed line) show flow cytometry patterns from a renal transplant recipient who received Campath-1H treatment 1 week after the procedure; CD3: 1.47%, CD19: 1.83%, CD14: 41.44%, CD52: 23.58%, CD14/CD52: 39.38%). CD3 positive T lymphocytes and CD19 positive B lymphocytes were largely depleted by the Campath-1H treatment. The majority of CD52 positive cells were CD14 positive monocytes, since monocytes appeared to be relatively resistant to Campath-1H treatment, despite their carrying CD52 antigen.

Fig. 2. The percentage of CD68 positive monocytes was significantly higher than that of CD3 positive T lymphocytes in renal transplant biopsies with acute cellular rejection.

Fig. 3. Positive C4d staining along endothelium of peritubular capillaries, which was co-localized with marginated monocytes (cells with pink colored cytoplasm) and lymphocytes (cells with scant cytoplasm) (magnification x 600). T = renal tubule; C = capillary.
total 25.9% focal and diffuse positive rate for C4d staining. In ACR cases (n = 26), CD68 positive monocytes were composed of 59.4 ± 4.69% of inflammatory cells, which were significantly higher than CD3 positive lymphocytes (38.9 ± 4.4%, p < 0.0025) (Fig. 2). C4d positivity was seen in peritubular capillaries (Fig. 3, brown stain), which is consistent with antibody-mediated rejection. Monocytes (cells with pink cytoplasm) and lymphocytes (cells with scant cytoplasm) were dominant inflammatory cells showing margination, with concurrent presence of C4d positivity (Fig. 3).

**Discussion**

Kirk et al [14] reported that in response to Campath-1H treatment in 7 patients with renal transplants, the monocyte count appeared resilient, whereas the lymphocyte count remained very low [14]. In the present study, 7.9% of monocytes were CD14+/CD52- in controls, similar to results of a previous study [10]. However, we observed a dramatic reduction of lymphocyte count following Campath-1H treatment. Significant reduction in IL-12 levels after Campath-1H treatment was consistent with the fact that Campath-1H caused a sharp depletion of lymphocytes. Therefore there was a significant decline in a major cytokine that stimulates differentiation and proliferation of T lymphocytes.

However, Campath-1H was not effective in depleting circulating monocytes in patients with renal transplants; only 2 of them showed more than 90% of CD14+/CD52- fraction after Campath-1H treatment, indicating that Campath-1H caused lysis of the majority of CD14+/CD52+ monocytes in these 2 patients. But the CD14+/CD52+ monocytes of the remaining 12 renal recipients were insensitive to Campath-1H treatment, since the majority of CD14 positive cells were also positive for CD52 after the treatment (Table 1). This finding suggests that some CD52 positive monocytes could not interact with Campath-1H to cause monocyte lysis in most of our patients. As CD14 positive monocytes are predominantly dendritic monocytes [15,16], the insensitive nature of some CD14 positive cells to Campath-1H may be a factor to compromise the induction treatment for transplantation, despite the strong depleting effects of Campath-1H on lymphocytes and IL-12 in the circulation.

Antibody-mediated rejection was seen in renal transplant patients following Campath-1H treatment in our study and other studies [17,18]. Only one of 54 unremarkable protocol biopsies was found to have focal positivity of C4d staining in the present investigation, similar to a previous study that found a low C4d positive rate in protocol biopsies in absence of Campath-1H treatment [7]. We also saw a low rate of positive C4d staining in indicated biopsies with ATN, whereas the majority of C4d positive staining was seen in the cases with ACR. This incidence of antibody-mediated rejection indicates that Campath-1H cannot protect renal allografts from the attack of antibody-mediated rejection. Although peritubular capillary C4d deposition has been proven to be a marker indicating antibody-mediated rejection in renal transplants [2,3], the mechanism whereby antibody-mediated rejection causes renal damage remains unclear. In our study, the presence of large numbers of monocytes marginating the vessels positive for C4d staining (as seen in Fig. 2) led us to speculate that monocytes may mediate C4d associated antibody-mediated rejection in our clinical scenario.

Recent advances indicate that monocytes are involved in all stages of acute and chronic transplant rejection [19]. After Campath-1H treatment, monocytes appear to damage renal tissue via mediated cytokines during an attack of ACR, rather than by attacking renal tubules as T lymphocytes do [14]. Neutrophils were not obvious in our cases with antibody-mediated rejection. Our data raise the question whether antibody-mediated rejection is an independent entity or a component of ACR.

In summary, we found that C4d positivity occurred much more frequently in renal transplants with ACR, in comparison to renal transplant cases negative for ACR (protocol biopsies or ATN cases). Since we also observed that monocytes were relatively resistant to Campath-1H depletion, the high percentage of monocytes detected in ACR cases suggests that monocytes may be involved in the process whereby antibody-mediated rejection causes renal damage.
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References


