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Mononuclear cells infiltrating kidney allografts in the absence of rejection

Effect of conversion from cyclosporin to azathioprine therapy

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Abstract. Focal small mononuclear cell infiltrates were found in renal allograft biopsies of 13/14 transplant recipients with a stable function after long-term cyclosporin A (CsA) therapy. Phenotypical analysis of the infiltrating cells using monoclonal antibodies showed a slight preponderance of T cells (56% ± 8%), with only small percentages of B cells (5% ± 2%), NK cells (2% ± 1%), and monocytes (2% ± 1%). Within the T-cell population the median calculated CD4/CD8 ratio was 1:3. Thirty-five percent of the infiltrating mononuclear cells remained unidentified with the monoclonal antibody panel used (silent cells). Three months after immunosuppressive therapy had been changed from CsA to azathioprine (AZA), the size of the infiltrates was significantly increased and there was a marked invasion of mononuclear cells between tubular epithelium despite a significant improvement in creatinine clearance ($P < 0.01$). The phenotypical composition of these infiltrates was dominated by T cells (84% ± 3%), with a median CD4/CD8 ratio of 2:7 due to an increase in CD4+ cells and a decrease in CD8+ cells after conversion ($P < 0.05$). The percentages of B cells, NK cells, and monocytes showed no significant changes after conversion. During AZA therapy nearly all infiltrating mononuclear cells were stained with the monoclonals used, leaving no silent cells postconversion.

Key words: Cyclosporin A - Azathioprine - Kidney transplantation - Mononuclear cell infiltrates - Monoclonal antibodies.

The use of the immunosuppressive agent cyclosporin A (CsA) in organ transplantation and autoimmune diseases is hampered by its nephrotoxic side effects [2, 9]. In an attempt to minimize the adverse effect of CsA on the kidney but preserve the long-term benefits for graft survival, we switched the immunosuppressive regimen from CsA to azathioprine (AZA) 1 year after renal transplantation [14].

Recently, we reported on the histology of biopsies from stable kidney allografts taken after 1 year of continuous CsA therapy [16]. Typical morphological lesions indicative of CsA nephrotoxicity included isometric vacuolization in proximal tubular epithelial cells and arteriolar deposits of IgM and complement factors. In control renal biopsies taken 3 months after conversion we observed a significant regression of the CsA-induced morphological lesions. At the same time, we also noticed a striking increase in the interstitial mononuclear cell infiltrates in combination with tubulitis; although this suggested graft rejection, we found no clinical evidence of it. On the contrary, creatinine clearance markedly improved in all patients and remained stable during an 18-month follow-up period. In the present study we looked in more detail at this phenomenon of mononuclear cells infiltrating the renal allograft in the absence of rejection after conversion from CsA to AZA therapy. Monoclonal antibodies were used for phenotypical characterization and quantification of these cells.

Materials and methods

Twenty recipients of a cadaveric renal allograft were entered in this study. All patients had received CsA and low-dose prednisone (10-15 mg) from the day of transplantation. CsA plasma concentrations were aimed at trough levels of 50-150 ng/ml (polyclonal radioimmunoassay) to avoid toxicity. One year after transplantation renal function was evaluated during a hospital admittance. On the last day a percutaneous Tru-cut needle biopsy of the kidney allograft was performed and CsA was stopped. The next day AZA was started at a mean dose of 2 mg/kg per day. At the time of conversion, all patients had stable kidney function and rejection episodes had not occurred in the previous 6 months. Three months after conversion a biopsy specimen was taken from those patients who had successfully converted. Part of the biopsy spe-

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Table 1. Monoclonal antisera used to assess antigenic determinants

Monoclonal antibody	Specificity
anti-*Leu* 2a	Suppressor/cytotoxic T cells (CD 8+)
anti-*Leu* 3a	Helper/inducer T cells (CD 4+)
anti-*Leu* 4	Pan-T-lymphocytes (CD 3+)
anti-*Leu* 7	Natural killer cells
anti-*Leu* 14	Pan-B cells (CD 22+)
anti-*Leu* M3	Monocytes/macrophages

Table 2. Percentages of mononuclear cell and T-cell subsets in kidney allograft biopsies before and after conversion from cyclosporin A (CsA) to azathioprine (AZA) (mean ± SEM). * $P<0.01$; ** $P<0.05$

	CsA (%)	AZA (%)
Leu 4+	56±8	84±3*
Leu 3a+	54±6	73±3**
Leu 2a+	46±6	27±3**
Leu 7+	2±1	2±1
Leu 14+	5±2	10±3
Leu M3+	2±1	3±1

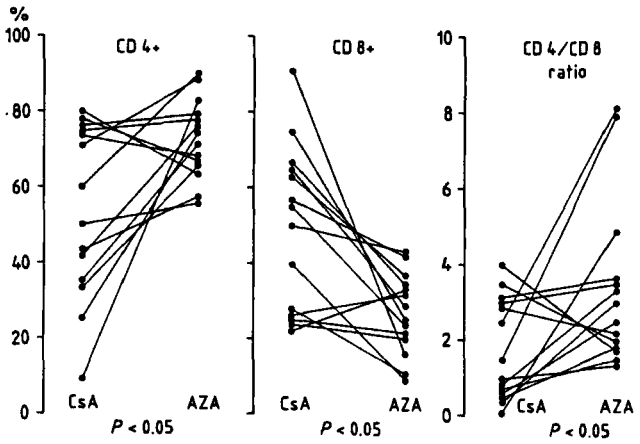

Fig. 1. Percentages of CD4+ (*Leu* 3a+) and CD8+ (*Leu* 2a+) cells and CD4/CD8 ratio in the individual kidney allograft biopsy before and after conversion from cyclosporin A *(CsA)* to azathioprine *(AZA)*

cimen was fixed in 4% formaldehyde and embedded in paraffin wax. For light-microscopic examination serial 2-μm-thick sections were cut from paraffin-embedded tissue and stained with hematoxylin-azafloxin, Jones and Jones azan. Another part of the biopsy specimen was quickly frozen in liquid nitrogen and stored. This biopsy material was used partly for immunomorphological studies with monoclonal antibodies. A panel of monoclonal antisera (Becton-Dickinson, Mountain View, Calif., USA) was applied to assess the antigenic determinants of the mononuclear cells infiltrating the graft tissue (Table 1). Cryostat sections were stained by a standard indirect immunoperoxidase technique. Cells with a plasma membrane distinctly stained brown were considered positive. After all biopsies were coded, light-microscopic examination was performed by two observers who were unaware of the treatment that each patient had received at the moment of biopsy. The following characteristics of the interstitium were assessed and graded according to severity on a semiquantitative scale (absent, mild, moderate, and severe): cellular infiltrates classified as diffuse or focal, consisting of mononuclear or polynuclear (neutrophilic and eosinophilic) cells; presence of tubulitis (mononuclear cells infiltrating the tubular epithelium) and vasculitis (mononuclear cells infiltrating the vessel wall). After immunohistochemical labeling with monoclonal antibodies the percentage of positive-staining cells was estimated semiquantitatively and expressed as a percentage (i.e., 5%, 10%, 20%, 30%, etc.) of the total number of infiltrating mononuclear cells in at least four high-power fields. Repeated scoring of the percentage of positive-staining cells yielded reproducible results. The ratio of CD4+ and CD8+ cells was calculated by dividing the estimated percentages of *Leu*-3a reactive cells by that of the *Leu*-2a reactive cells. Statistical analysis was performed using the Student's *t*-test for paired data, the Mann-Whitney U test, and the Fisher exact test.

Results

Clinical data

In 16 patients elective conversion from CsA to AZA was successfully performed, showing an improvement in kidney function without signs of rejection. In two patients a second biopsy specimen taken at the readmission was omitted because of a prolonged bleeding time. Consequently, in 14 patients with a stable graft function a renal allograft biopsy specimen was obtained before and 3 months after conversion.

In all these patients, CsA plasma trough levels before conversion were within the therapeutic range (101 ± 22 ng/ml, mean ± SEM). Median serum creatinine before conversion was 165 (range 103-255) μmol/l and fell to 131 (95-168) μmol/l 3 months after conversion ($P<0.01$) with a concomitant increase in median creatinine clearance from 44 (range 31-72) ml/min to 59 (50-100) ml/min ($P<0.01$). Thereafter, these parameters remained stable during a follow-up period of 18 months.

Morphological data

Sufficient biopsy material for light-microscopic examination was obtained from all 14 patients, and satisfactory indirect immunoperoxidase staining was achieved with all monoclonal antibodies.

Before conversion focal cellular infiltrates were present in 13 of 14 renal allograft biopsies. Their intensity ranged from mild to moderate. Mononuclear cells predominated over a variable but small admixture of polynuclear cells. A minimal infiltration of mononuclear cells between the epithelial tubular cells was found in only two biopsies. No relationship could be found between graft function or CsA trough level and extent or intensity of infiltration. Phenotypical characterization of the mononuclear cells showed 56% ± 8% *Leu*-4+ (pan-T) cells. The percentages of *Leu*-14+ (B) cells, *Leu*-7+ (NK) cells, and *Leu*-M3+ (monocytic) cells were respectively 5% ± 2%, 2% ± 1%, and 2% ± 1% (Table 2).

Within the T-cell population the percentage of *Leu*-3a+ (CD4) cells was 54%±6% and of *Leu*-2a+ (CD8) cells 46%±6%. The median calculated CD4/CD8 ratio was 1:3 (range 0.1-4.0). With the monoclonal antibody panel used, 35% of the infiltrating cells could not be identified. The percentages of unstained cells were not correlated with the CsA trough levels.

Three months after conversion of immunosuppressive therapy from CsA to AZA the light-microscopic appearance of the interstitium showed a significant increase in the intensity of the cellular infiltrates ranging from moderate to severe ($P<0.01$). The location was still focal in 11 but had become diffuse in three patients. These infiltrates consisted mainly in mononuclear cells. In nine biopsies a marked increase in mononuclear cells between the epithelial tubular cells (tubulitis) was found ($P<0.01$). A focal infiltration of mononuclear cells within the arteriolar wall was present in one patient. Phenotyping of the mononuclear cells within the infiltrates showed 84%±3% *Leu*-4+ cells versus 56%±8% before conversion ($P<0.01$; Table 2). The other mononuclear cell subsets tested (B-cells, NK-cells, and monocytes) showed no significant changes after conversion. T-cell subsets showed a rise in *Leu*-3a+ cells from 54%±6% to 73%±3% ($P<0.05$), while the percentage of *Leu*-2a+ cells decreased from 46%±6% to 27%±3% ($P<0.05$; Fig. 1). The median calculated CD4/CD8 ratio after conversion was 2:7 (range 1.3-8.1) compared with 1:3 (range 0.1-4.0) during CsA ($P<0.05$). In all postconversion biopsies the CD4/CD8 ratio exceeded 1:0. No relationship could be established between morphological or phenotypical changes and improvement in kidney allograft function.

Discussion

In the present study we have tried to characterize the phenotype of the mononuclear cells invading renal allografts in the absence of rejection after conversion from CsA to AZA therapy. Before conversion we found a slight preponderance (56%) of T-lymphocytes (CD3+ cells) in small focal interstitial infiltrates. Within the T-cell population, the CD4+ cells slightly dominated the CD8+ cells, resulting in a median CD4/CD8 ratio of 1:3. The contribution of B cells, NK cells, and monocytes was only minor [10]. Surprisingly, 35% of the cells in the infiltrates could not be identified within the limits of the monoclonal antibody panel used.

The presence of neutrophilic and eosinophilic polymorphonuclear cells, or even of noninflammatory cells, might be of relevance to the unstained cell population. However, on light-microscopic examination only a very small admixture of these cells was found in the interstitial infiltrates. This agrees with recent reports showing that infiltrating polynuclear cells are rarely found in stable allografts but are associated with irreversible rejections [6, 18].

A mononuclear cell population not expressing surface markers or silent cells has not been described before in kidney allograft biopsies. Recently, McWhinnie et al. reported on the phenotypic composition of cellular infiltrates in biopsies of CsA-treated patients who had stable graft function 3 months after transplantation [8]. Forty percent of the leukocyte infiltrate was composed of CD3+ cells, while the other cells were mainly macrophages. An almost equal percentage of CD4+ and CD8+ cells was found, but no mention of the presence or absence of nonstaining mononuclear cells was made. In contrast, nonstaining infiltrating mononuclear cells have been reported in CsA-treated cardiac allografts in the absence of rejection [12]. Although this finding was not discussed in any detail, Pomerance and Stovin tried to identify the nature of these endocardial infiltrates, and they suggested that these cells are of histiocytic origin [11]. We found no evidence of the presence of macrophages in our material using monoclonal antibodies, but the timing of biopsies after organ transplantation is obviously important and might explain the discrepancies between the various studies. For that matter, we are not aware of infiltrate analysis in biopsies of stable kidney or cardiac allografts in patients treated for 1 year with CsA. During the process of engraftment, suppression of immunological activity with loss or modulation of cell surface markers can be imagined, as has been described for major histocompatibility complex (MHC) products in preclinical studies [1, 7].

In fine-needle aspiration biopsies during periods of quiescence, 50% of the infiltrating cells were defined as T cells but no mention was made of nonstaining cells [15, 19]. Three months after conversion from CsA to AZA a significant increase in the number of cells infiltrating the interstitium was detected. Invasion of the tubular epithelium and even of the arteriolar wall was found. Phenotypical analysis showed that the majority of these cells (84%) were CD3+ cells (pan-T-lymphocytes). Under AZA treatment the relative proportion of CD4+ cells increased from 54% to 73%, while that of the CD8+ cells decreased. The number of B cells, NK cells, and monocytes was not affected by the change in immunosuppressive regimen. It should be noted that, in contrast to the situation under CsA therapy,

all infiltrating mononuclear cells could be phenotypically analyzed after conversion to AZA.

Our finding that the majority of the cells infiltrating the graft in AZA-treated renal allograft recipients with stable function are T-lymphocytes is in agreement with the findings of Burdick et al. [3]. We have now shown that these T-lymphocytes can be classified predominantly as CD4+ cells, and that both their absolute and relative numbers increase after conversion from CsA to AZA, resulting in a rise in the CD4/CD8 ratio. While the mononuclear invasion between tubular epithelial cells might represent rejection, we found no clinical evidence of graft-function loss. This is in line with the reports of Hancock et al., Waltzer et al., and Sako et al., who observed during acute rejection an increase in CD8+ but not in CD4+ cells (thus, a fall in the CD4/CD8 ratio) accompanied by an increase in monocytes and NK cells [5, 13, 17]. In contrast, other investigators have reported a preponderance of CD4+ cells during rejection [4]. These differences are difficult to explain. It may be that the interpretation of staining is a source of discrepancy, especially if staining is weak or poor, or that the technique used influences the outcome. Also, the methods of assessing the cellular infiltrates may be of relevance. Not only a semiquantitative scoring technique but even morphometric analysis are potential sources of error in assessing the infiltrates.

However, the CD4+ cell predominance after conversion could be due to the expression of surface markers on the silent mononuclear cells that were present under the CsA therapy. CsA restores interleucine-2 production capacity, and the permanent antigenic stimulus of renal allograft tissue will activate resting (silent) T cells, resulting in generation of more T cells and leading to an increased size of the infiltrates and invasion of the tubular epithelium. Our finding that this phenomenon did not lead to rejection might reflect the formation of a new immunological balance between host and transplanted organ after a change of the immunosuppressive regimen.

In conclusion, after successful conversion from CsA to AZA we found a striking increase in the interstitial mononuclear cell infiltrates in the absence of rejection. Phenotypical analysis revealed that these cells were predominantly CD4+ lymphocytes. The disappearance of cells not expressing surface markers, which are present during treatment with CsA but not with AZA, suggests that the CD4+ cells under AZA are derived from a "silent" T-cell population under CsA.

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