Transpl Int (1999) 12: 10–17

ORIGINAL ARTICLE

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P-glycoprotein expression is not a useful predictor of acute or chronic kidney graft rejection

Received: 9 June 1998
Accepted: 25 September 1998

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Abstract Because of the role of P-glycoprotein (P-gp) in multidrug resistance (MDR), it has been suggested that P-gp might play a role in acute and chronic rejection after organ transplantation. The purpose of the present work was to investigate a possible relationship between graft outcome and P-gp expression on peripheral mononuclear cells of renal transplant recipients. We determined P-gp expression in 27 patients with long-term, stable graft function (ST) and in 15 patients with chronic deterioration of graft function (CR). In addition, 40 patients were studied prior to, and at intervals after, transplantation with 21 healthy individuals serving as controls. P-gp values were highest in healthy controls and in ST patients. We found no correlation between P-gp values and acute rejection. CR patients tended to have lower levels of P-gp expression. Our results contradict the opinion that an overexpression of P-gp induces acute or chronic rejection by inhibiting the efficacy of immunosuppressive treatment.

Key words Renal transplantation · P-glycoprotein expression rejection · Rejection, P-glycoprotein expression, renal transplantation · P-glycoprotein expression, rejection, renal transplantation

Introduction

Cyclosporin A (CyA), the drug most widely used for the immunosuppressive treatment of renal transplant recipients, has been shown to be a substrate of P-glycoprotein (P-gp) [1, 6, 12]. P-gp is a transmembrane glycoprotein that functions as a metabolically active efflux pump for a variety of substances ranging from ions to peptides [7]. Overexpression has been implicated in multidrug resistance (MDR) of cancer cells [11]. More recently, it was shown that P-gp is found less frequently in healthy controls and in patients with long-term, stable graft function than in patients experiencing rejection [8, 9, 13, 14]. It was suggested that immunocompetent cells, particularly T cells, might escape from CyA-mediated immunosuppression via the acquisition of drug resistance mediated by the expression of P-gp.

Chaudhary et al. [2] and others [3, 4, 10] showed that all lymphocytes express P-gp. However, for unclear reasons, studies dealing with the phenomenon of MDR in transplant recipients did not confirm the presence of MDR-1 mRNA in healthy controls [9, 14]. An additional difficulty with the interpretation of published data is that several studies were only concerned with P-gp expression or MDR-1 mRNA on isolated mononuclear cells and not with expression on defined lymphocyte subpopulations [8, 9, 13, 14]. We therefore decided to examine P-gp expression on subpopulations of peripheral blood lymphocytes and monocytes of renal transplant recipients and healthy controls. Furthermore, we evaluated whether patients with an overexpression of P-gp are prone to develop a higher frequency of acute rejection episodes. Since it is known that the development of chronic rejection is significantly associated with the fre-

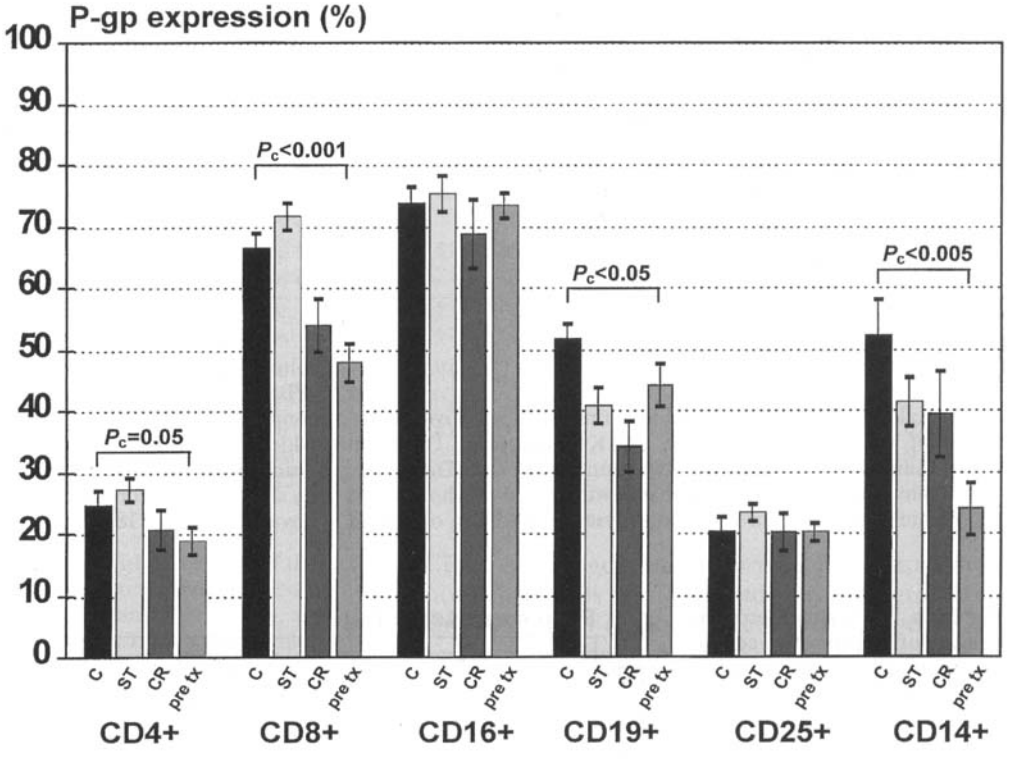


Fig. 1 P-gp expression (percentage of P-gp-positive cells) on mononuclear cells of 21 healthy control individuals (*C*), 27 ST patients (*ST*), 15 CR patients (*CR*), and all 40 patients prior to renal transplantation (*pre tx*). Data are shown as mean ± SEM. **P*-values were obtained from the Kruskal-Wallis test for the comparison of the four groups

quency and severity of acute rejection, we also attempted to evaluate whether chronic rejection might be associated with a higher frequency of P-gp expressing cells.

Materials and methods

Renal transplant recipients

Patients examined pre- and post-transplantation

Forty patients were tested for P-gp expression prior to transplantation. Of these, 31 had been on chronic hemodialysis and 9 on continuous ambulatory peritoneal dialysis. All patients underwent renal transplantation between August 1996 and May 1997 at the Department of Urology, University of Heidelberg, with 33 patients receiving a first graft and 7 a second graft. Immunosuppressive treatment consisted of CyA (serum trough levels of 150–250 ng/ml initially and 100–200 ng/ml after 3 months), methylprednisolone (MP; 250 mg/day initially, tapered to a maintenance dose of 7.5 mg/day after 45 days), and mycophenolate mofetil (2 g/day). Prophylactic antithymocyte globulin (Fresenius, Oberursel, Germany) was administered to five patients. Eight patients experienced a total of 12 acute rejection episodes. Acute rejection was diagnosed by typical clinical signs, together with an increase in serum creatinine levels, and by Doppler sonography and renal scintigraphy. Graft biopsies were performed in all but one case. In 35 of the 40 patients, between 5 and 12 samples per patient were tested for P-gp expression within 3 months after transplantation.

Patients with stable graft function or chronic deterioration of function

In addition to the abovementioned patients, we studied 27 patients with long-term, stable graft function (ST) and 15 patients with chronic deterioration of graft function (CR). In these patients, only one sample per patient was tested for the presence of P-gp. The patients underwent transplantation at least 28 months prior to testing (range 28–198 months). Seven of the CR patients suffered from histologically proven chronic rejection and eight were diagnosed as suffering from chronic rejection because of a progressive deterioration in graft function and increasing plasma creatinine concentrations. ST and CR patients were not significantly different with respect to age (ST patients 51 ± 13 years; CR patients 45 ± 16 years; $P = 0.19$), time after transplantation (ST patients 87 ± 52 months; CR patients 88 ± 48 months; $P = 0.99$), or the number of HLA-A, -B, -DR mismatches (ST patients 2.2 ± 1.3; CR patients 1.4 ± 1.1; $P = 0.07$). As expected, serum creatinine levels were higher in CR patients (ST patients 1.2 ± 0.4 mg/dl; CR patients 4.1 ± 1.6 mg/dl; $P < 0.0001$). Immunosuppressive treatment consisted of CyA (serum trough levels: ST patients 163 ± 61 ng/ml; CR patients 168 ± 65 ng/ml) and either MP (3.1 ± 1.4 mg/day) in 33 patients (ST $n = 25$; CR $n = 8$) or a combination of MP (3.8 ± 1.6 mg/day) and azathioprine (Aza; 59.4 ± 40.0 mg/day) in 9 patients (ST $n = 2$; CR $n = 7$). Patients with chronic rejection did not receive any special treatment; however, a greater percentage of CR patients (47% vs 7%) were treated with CyA/Aza/MP.

Controls

Twenty-one healthy individuals, not treated with any kind of immunosuppressive agent, served as controls.

Table 1 P-gp expression (percentage of P-gp-positive cells) on mononuclear cells of 40 patients before renal transplantation in relation to the occurrence of acute rejection episodes after transplantation. Data are given as mean ± SEM

	Patients without acute rejection episodes after transplantation ($n = 32$)	Patients with acute rejection episodes after transplantation ($n = 8$)	P value
P-gp+/CD4+	18.03 ± 1.78	23.31 ± 5.84	0.61
P-gp+/CD8+	47.22 ± 3.17	51.82 ± 4.36	0.57
P-gp+/CD16+	73.26 ± 2.11	74.17 ± 3.27	0.87
P-gp+/CD19+	44.52 ± 3.49	42.35 ± 9.59	0.85
P-gp+/CD25+	19.28 ± 1.12	24.30 ± 4.09	0.30
P-gp+/CD14+	24.28 ± 4.36	23.38 ± 11.19	0.66

Sample preparation

Peripheral blood was drawn into heparinized tubes. Five micrograms of the first step antibody MRK16 (Kamiya, Thousand Oaks, Calif., USA) or IgG2a isotype control (Becton Dickinson, Sunnyvale, Calif., USA) was incubated with 100 µl of whole blood for 30 min at 4 °C. Erythrocytes were lysed by addition of NH_4Cl solution for 10 min and washing with phosphate-buffered saline (PBS; Gibco, Eggenstein, Germany). Fifty microliters of FITC-conjugated F(ab')2 fragment (Medac, Hamburg, Germany) was added at a 1:40 dilution. Following a 30-min incubation, a second lysing and washing step was carried out. To block any free reactivity sites on the secondary antibody, 1 µg of mouse-γ-globulin (Dianova, Hamburg, Germany) was added and incubated for 20 min.

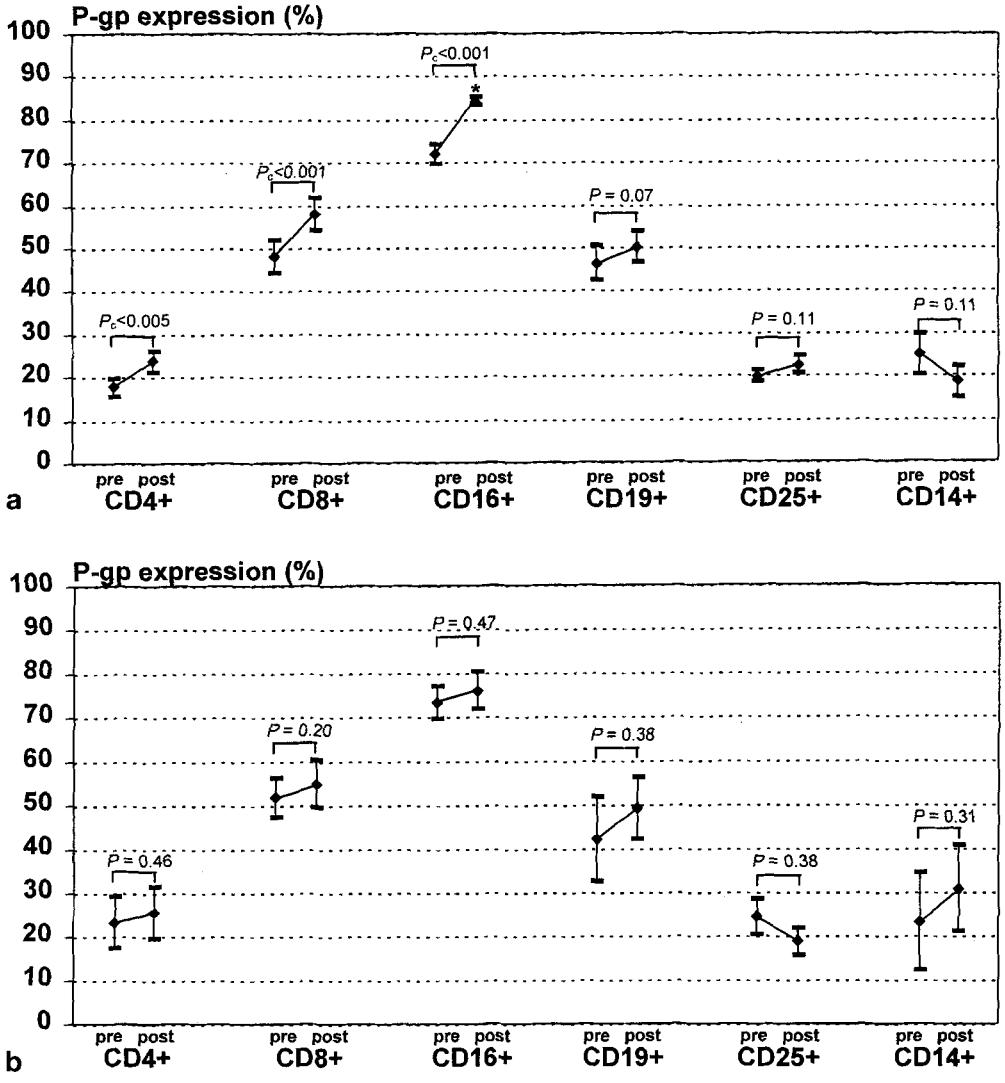


Fig. 2a, b P-gp expression (percentage of P-gp-positive cells) on mononuclear cells of patients before (*pre*) and within 5 days after (*post*) renal transplantation: **a** patients without acute rejection episodes ($n = 27$); **b** patients with acute rejection episodes ($n = 8$). Data are shown as mean ± SEM

Table 2 P-gp expression (percentage of P-gp-positive cells) on mononuclear cells of 35 patients before (pre) and within 5 days after (post) transplantation in relation to onset of graft function. Data are given as mean ± SEM

		Immediate onset of graft function ($n = 14$)	Delayed onset of graft function ($n = 21$)	P value
P-gp+/CD4+	pre	19.92 ± 3.03	18.26 ± 2.69	0.50
	post	27.68 ± 3.37	21.79 ± 3.10	0.08
P-gp+/CD8+	pre	54.90 ± 4.54	43.86 ± 3.77	0.06
	post	64.30 ± 4.36	52.33 ± 4.03	0.05
P-gp+/CD16+	pre	74.51 ± 3.00	71.10 ± 2.60	0.47
	post	82.74 ± 2.22	82.41 ± 1.63	0.65
P-gp+/CD19+	pre	49.13 ± 4.96	43.03 ± 5.31	0.39
	post	47.57 ± 5.06	51.57 ± 4.29	0.48
P-gp+/CD25+	pre	18.91 ± 1.22	22.21 ± 2.15	0.28
	post	21.62 ± 2.57	21.70 ± 2.32	0.89
P-gp+/CD14+	pre	23.87 ± 7.05	24.92 ± 5.59	0.57
	post	21.99 ± 5.85	20.81 ± 4.54	0.97

The cells were washed with PBS and labeled with 10 or 20 µl of the appropriate PE-conjugated, subset-specific monoclonal antibody: α-CD4 (helper/inducer subset; Ortho, Raritan, N.J., USA), α-CD8 (cytotoxic/suppressor subset; Ortho), α-CD16 (natural killer cells; Becton Dickinson), α-CD19 (B-cells; Ortho), α-CD25 (activated T- and B-cell subsets; Becton Dickinson), α-CD14 (monocytes; Becton Dickinson), or isotype control (IgG1, IgG2a; Becton Dickinson). After 30 min of incubation, the cells were washed again with PBS and subjected to flow cytometric measurement using a FACScan flow cytometer (Becton Dickinson). Using isotype controls, background staining was assessed to be less than 2% and subtracted from the specific fluorescence. The range for P-gp values was 2%–61% for CD4+, 13%–87% for CD8+, 24%–94% for CD16+, 1%–91% for CD19+, 5%–56% for CD25+, and 0%–93% for CD14+ cells, respectively.

Statistical analysis

Group comparisons were done using the Wilcoxon rank sum test and the Kruskal-Wallis test. Statistical analysis of sequential testing was carried out for the total period with the Friedman test, and for two subsequent dates with the matched-pairs signed rank test. Results are given as mean ± SD if not noted otherwise. In order to reduce the likelihood of reporting spurious associations, P values that reached significance ($P < 0.05$) were corrected by multiplying the uncorrected values by the total number of comparisons (P_c).

Results

Control individuals

The incidence and the pattern of distribution of P-gp-expressing cells in the 21 healthy controls corresponded to data reported in the literature [2, 10]. The highest P-gp levels were found on CD16+ and CD8+ cells (74% and 66%, respectively). CD19+ and CD14+ cells had intermediate levels (52% and 52%, respectively), and CD4+ and CD25+ cells had the lowest levels of P-gp expression (25% and 20%, respectively; Fig. 1). This typical general pattern of P-gp expression on mononuclear cells was also found in both groups of transplant recipients (ST and CR patients) and in patients before transplantation (Fig. 1).

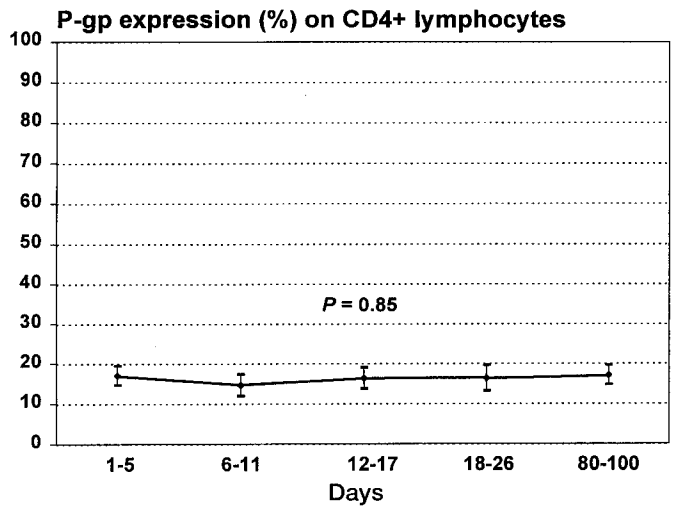
CR and ST patients

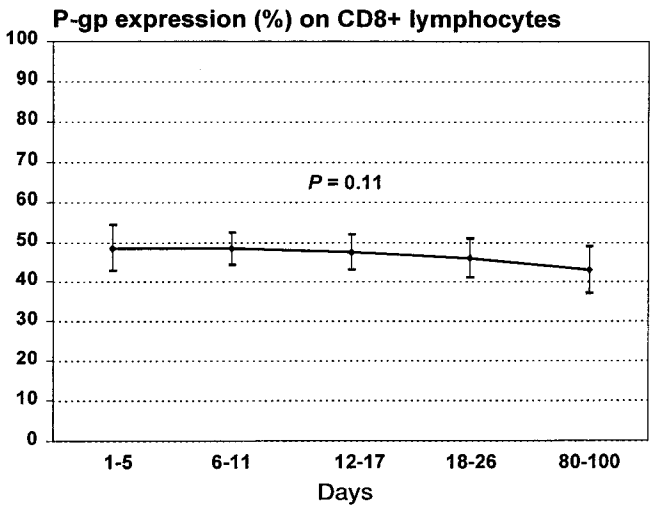
CR patients tended to have lower levels of P-gp expression than healthy controls, except for the CD25+ lymphocyte subset (Fig. 1). ST patients showed higher levels of P-gp expression than CR patients; however, the difference was statistically significant only for CD8+ lymphocytes ($P_c < 0.05$, Wilcoxon rank sum test). For several cell populations, namely, the CD4+, CD8+, CD16+, and CD25+ lymphocyte subsets, P-gp expression levels in ST patients reached those of healthy controls (Fig. 1).

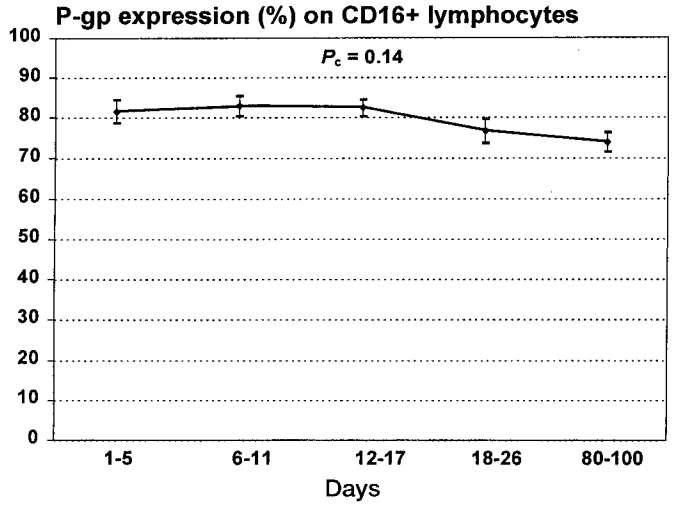
Patients prior to transplantation

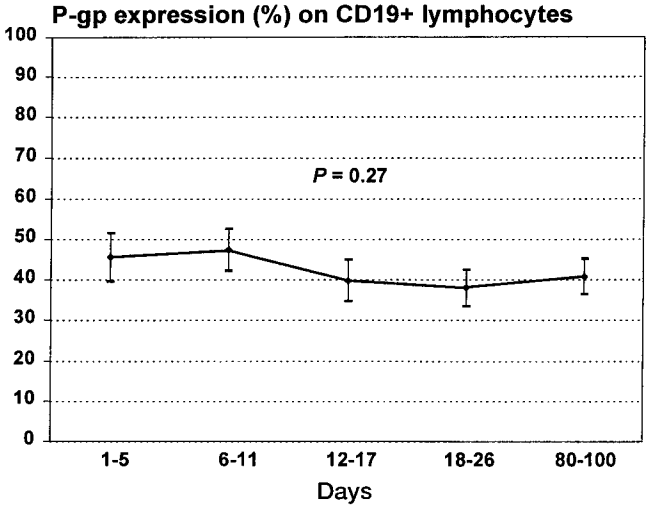
Patients examined prior to transplantation exhibited generally lower levels of P-gp-expressing cells than healthy controls. The difference was statistically significant for CD8+ and CD14+ cells (CD8+: $P_c < 0.005$; CD14+: $P_c < 0.01$ Wilcoxon rank sum test; Fig. 1). Eight of the 40 patients experienced acute rejection episodes after transplantation. There was no association between P-gp values determined prior to transplantation and the occurrence of acute rejections (Table 1).

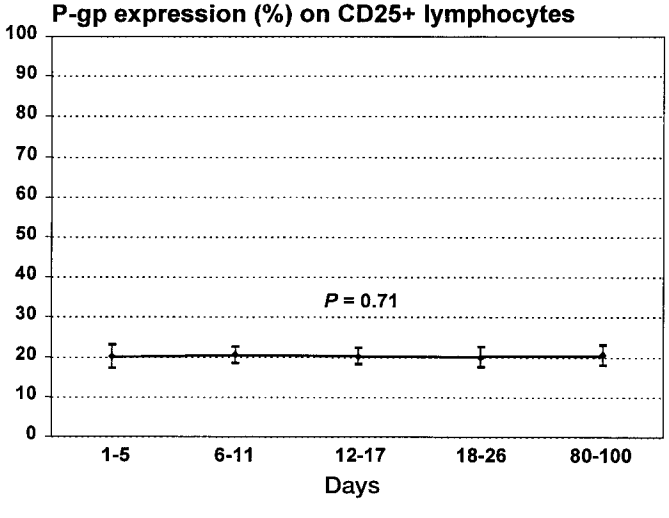
Fig. 3 P-gp expression (percentage of P-gp-positive cells) on mononuclear cells of 15 patients after renal transplantation. Data are shown as mean ± SEM

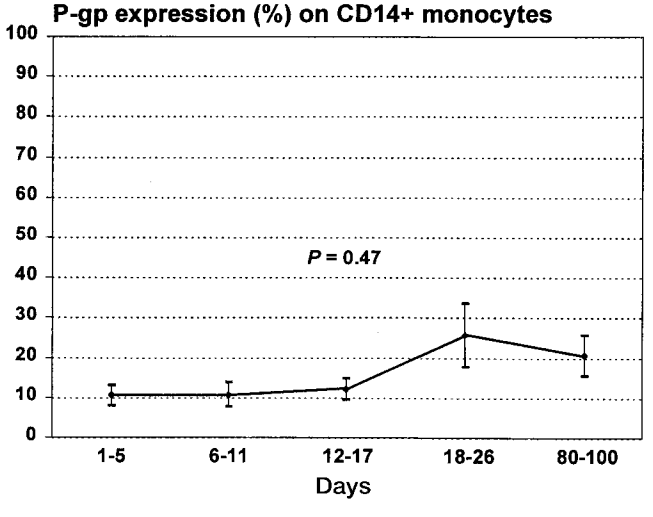

P-gp expression (%) on CD4+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
P = 0.85
1-5
6-11
12-17
18-26
80-100
Days

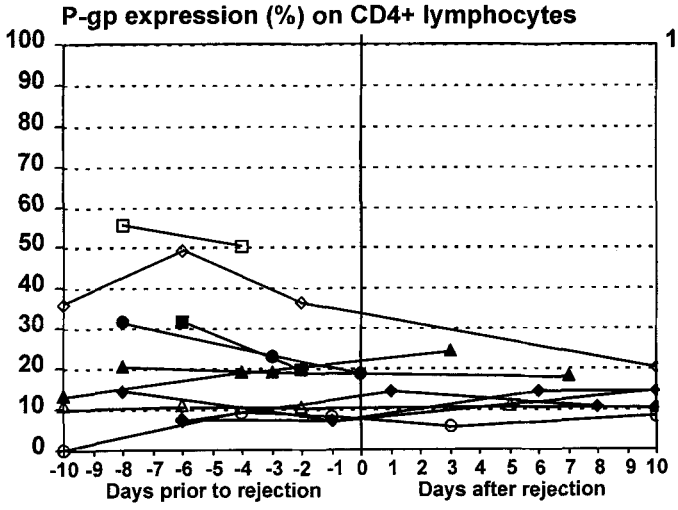

P-gp expression (%) on CD8+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
P = 0.11
1-5
6-11
12-17
18-26
80-100
Days

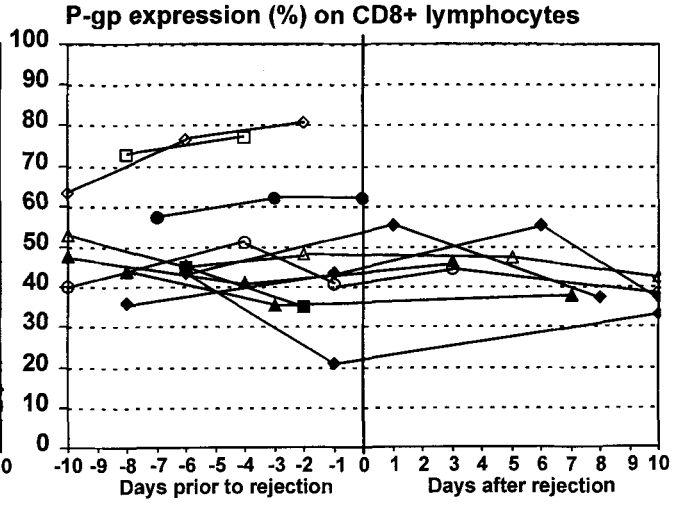

P-gp expression (%) on CD16+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
Pc = 0.14
1-5
6-11
12-17
18-26
80-100
Days

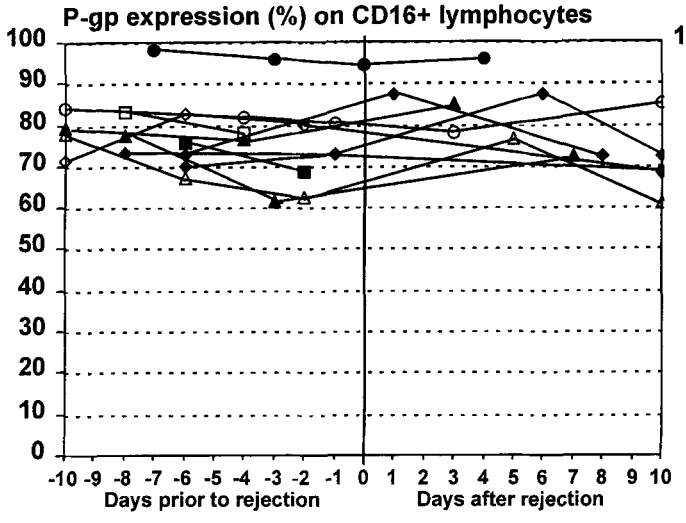

P-gp expression (%) on CD19+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
P = 0.27
1-5
6-11
12-17
18-26
80-100
Days

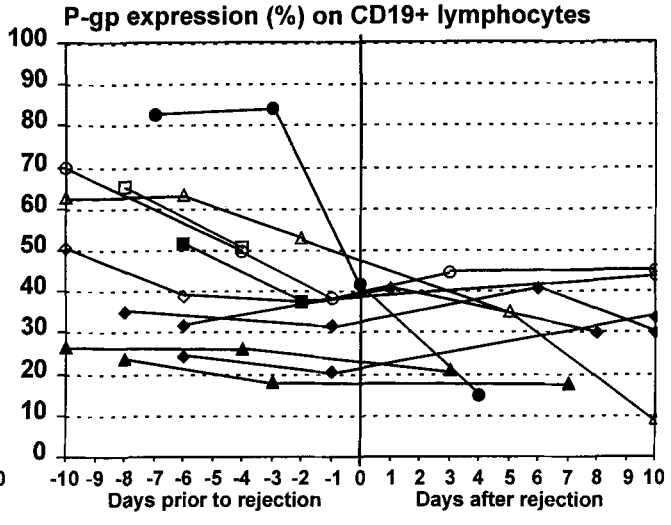

P-gp expression (%) on CD25+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
P = 0.71
1-5
6-11
12-17
18-26
80-100
Days

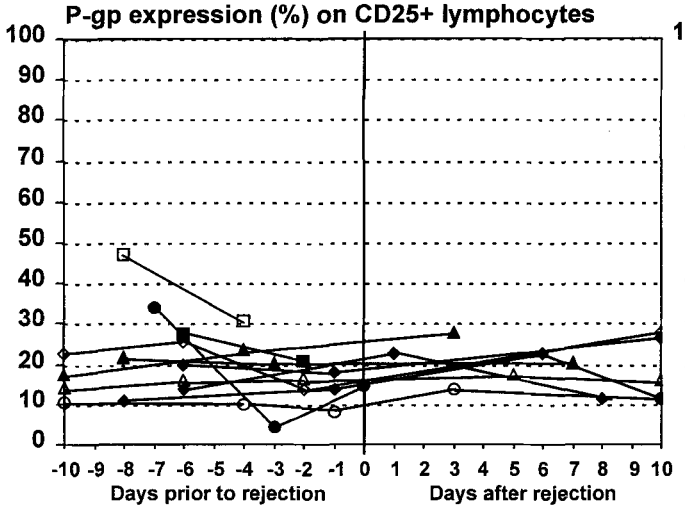

P-gp expression (%) on CD14+ monocytes
100
90
80
70
60
50
40
30
20
10
0
P = 0.47
1-5
6-11
12-17
18-26
80-100
Days

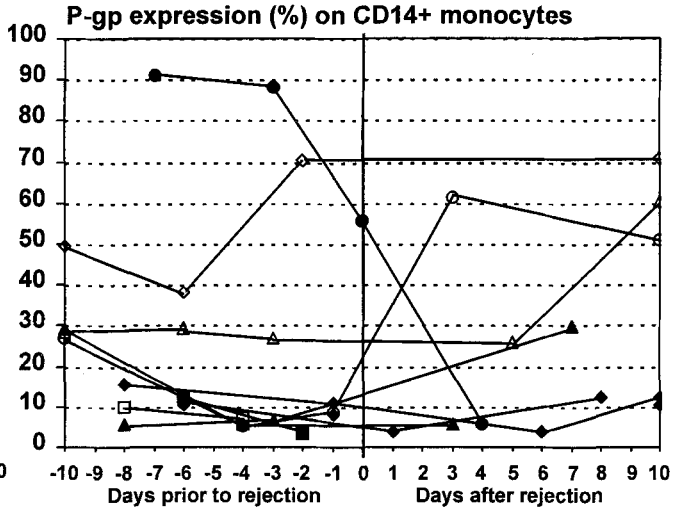

P-gp expression (%) on CD4+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days prior to rejection
Days after rejection


P-gp expression (%) on CD8+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days prior to rejection
Days after rejection


P-gp expression (%) on CD16+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days prior to rejection
Days after rejection


P-gp expression (%) on CD19+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days prior to rejection
Days after rejection


P-gp expression (%) on CD25+ lymphocytes
100
90
80
70
60
50
40
30
20
10
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days prior to rejection
Days after rejection


P-gp expression (%) on CD14+ monocytes
100
90
80
70
60
50
40
30
20
10
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days prior to rejection
Days after rejection

Follow-up examinations

P-gp expression in 35 of the 40 patients was studied again after transplantation. Figure 2 shows the levels of P-gp-expressing mononuclear cells before and within 5 days after transplantation. Results are shown for patients without (Fig. 2a) and with rejection episodes (Fig. 2b). Patients without rejection episodes had an increase in P-gp values within 5 days after transplantation for all lymphocyte subpopulations except for monocytes, for which P-gp values decreased. For three cell populations (CD4 +, CD8 + and CD16 + lymphocytes), the increase reached statistical significance. Patients who experienced rejection episodes (Fig. 2b) showed smaller increases for P-gp values on CD4 +, CD8 +, CD16 +, and CD19 + lymphocytes, whereas P-gp decreased on CD25 + lymphocytes and increased on monocytes after transplantation. However, these differences did not reach statistical significance.

There was no difference in P-gp expression between patients whose graft functioned immediately after transplantation and those whose graft did not (Table 2).

Fifteen patients were investigated for a 3-month period after transplantation (Fig. 3). P-gp expression remained stable on CD4 + and CD25 + lymphocytes, whereas P-gp on CD8 +, CD16 +, and CD19 + cells began to decrease slightly after 12 days. The evolution of P-gp-expressing cells was similar, whether or not patients experienced acute rejection episodes.

Figure 4 shows P-gp expression on the different cell types before and after acute rejection. We could not find a consistent pattern, and the data provide no evidence that an increase in P-gp is followed by an acute rejection episode.

Discussion

Previous studies dealing with P-gp or MDR-1 expression in transplant recipients were based on the assumption that P-gp is not constitutively expressed on all lymphocytes. However, Chaudhary et al. [2] and others [3, 4, 10] have shown that P-gp is expressed on all lymphocytes and in a variable manner also on monocytes. A special pattern of distribution was described for lymphocyte subsets. Our data obtained form 21 healthy individuals confirm the values for P-gp expression on different lymphocyte subpopulations reported by these authors. In addition, we found an expression level of approximately 20% on CD25 + lymphocytes. To our knowledge, there has been no previous report in which P-gp expression on CD25 + lymphocytes was shown.

The present report is the first documentation of P-gp expression on lymphocyte subpopulations and monocytes in patients before and after kidney transplantation. We found that P-gp expression was highest in healthy controls and, except for B lymphocytes and monocytes, in ST patients. CR patients, rather than showing an increase, showed a tendency towards lower levels of P-gp-expressing cells. There was no correlation between P-gp values before transplantation and acute rejection episodes after transplantation. Furthermore, we did not observe a significant increase directly before episodes of acute rejection. These results indicate that P-gp cannot be used as a predictive marker for acute rejection. Furthermore, they contradict the notion that an overexpression of P-gp induces acute or chronic graft rejection by inhibiting the efficacy of immunosuppressive treatment with CyA [9, 13, 14].

The lowest levels of P-gp expression were found in patients prior to transplantation and in CR patients, suggesting that low values might be associated with uremia. This was corroborated by increased P-gp levels immediately after transplantation, when renal function usually improves. However, we could not find any difference in P-gp expression between patients with immediate or with delayed onset of graft function. If uremia affected P-gp expression, one would expect lower values for P-gp in patients with delayed onset of graft function due to acute tubular necrosis.

A reason for the generally increased P-gp expression observed during the first days after transplantation could have been the commencement of immunosuppressive treatment. There is evidence that one of the mechanisms by which lymphocytes recover from CyA is P-gp-dependent [1]. It would, therefore, be intriguing to speculate that the cells that survive the initial phase of immunosuppressive treatment might be those that are able to clear CyA from their cytoplasm. If this were true, we would expect that during the subsequent period, the absolute counts of cell populations with a high P-gp expression should not decrease as much as those with a low P-gp expression. However, that was not the case. We found that the absolute cell counts of CD4 +, CD8 +, CD16 +, and CD25 + lymphocytes decreased by about the same percentage, whereas those of CD19 + lymphocytes increased slightly. It is, therefore, likely that the observed increases in P-gp-expressing cells are due to a higher expression of P-gp and not to a higher probability of survival of P-gp-expressing cells. Based on the currently available data, it is impossible to say whether high P-gp expression is induced by immunosuppressive treatment, as suggested by some authors [5], or whether it is simply a result of cell activation due to contact with alloantigen.

Fig. 4 P-gp expression (percentage of P-gp-positive cells) on mononuclear cells after renal transplantation of 8 patients before the occurrence of rejection and after 11 episodes of acute rejection

An unexpected finding that we cannot explain is that all patient groups exhibited decreased P-gp expression on B lymphocytes (CD19 +) and monocytes (CD14 +), as compared with healthy controls. Both B cells and monocytes present HLA class II antigens on their surface. Since little is known about the physiological role of P-gp, this observation should encourage further attempts to explain the significance of this protein.

Acknowledgement This study was supported by a grant from Forschungsschwerpunkt Transplantation, Heidelberg, Germany.

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