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Quantification of immunosuppression by flow cytometric measurement of intracellular cytokine synthesis

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Abstract The availibility of a method to measure the effects of drugs on immune reactivity should be helpful in optimizing treatment after organ transplantation. Since cyclosporine A (CSA) interferes with activation of T cells and cytokine synthesis, production of IL-2 and IFN-γ might constitute a marker of this drug's effects. We measured the capacity for mitogen-stimulated production of these cytokines in whole blood by using immunostaining of intracellular and membrane antigens, followed by flow cytometry. The percentage of $CD4^+$ T cells producing IL-2 or IFN-γ was strongly reduced in 20 transplant patients compared with 24 healthy controls. The capacity for IL-2 production of $CD4^+$ and $CD8^+$ cells correlated inversely with CSA blood levels (P values 0.0087 and 0.0396, respectively). IFN-γ production by $CD4^+$ T cells showed a negative correlation with the prednisolone dose ($P = 0.0175$) and, for the $CD8^+$ subset, with CSA trough levels ($P = 0.0023$). These data show that inhibition of T cell cytokine synthesis by CSA and prednisolone can be quantified.

Key words Cytokine synthesis · Flow cytometry · T cells · Cyclosporine A · Whole blood

Introduction

Immunosuppressive agents are administered in fixed amounts, or targeted to standard blood levels. Non-pharmacological factors such as malnutrition, surgical trauma, and coexistent morbid conditions such as diabetes mellitus also affect immunocompetence, yet are not always taken into account when prescribing these drugs. Clinical experience shows that the current "one size fits all" approach results in overtreatment of some patients (as evidenced by severe opportunistic infections), whereas others are undertreated (as manifested by rejection episodes). Unfortunately, simple laboratory tests to individualize treatment are not available. Cyclosporine A (CSA), the cornerstone of most immunosuppressive regimens, inhibits intracellular signal transduction in T lymphocytes, thus suppressing T-cell activation and production of interleukin-2 (IL-2) and other cyto-

kines [7]. We hypothesized that the capacity of T cells to produce IL-2 upon stimulation might constitute a marker for the effects of this drug. Since CSA is rapidly eliminated from T cells upon isolation of mononuclear cells and incubation in culture medium [1], we have used a whole-blood system for stimulation and immunostaining. Here, we show that this method is helpful in quantifying the effects of CSA on the capacity of T cells for IL-2 and interferon-gamma (IFN-γ) production.

Materials and methods

Patients and controls

Intracellular cytokine production was studied in 13 liver and 7 kidney transplant recipients. Patients were studied a median of 16 (7–1500) days after transplantation. None of them had experienced rejection or major opportunistic infections within 1 week before blood sampling, or did so during the week after. Baseline immunosuppressive regimens consisted of prednisolone and CSA with or without azathioprine, as described elsewhere [3, 4]. Six patients received prednisolone in combination with CSA, 2 were on prednisolone and azathioprine, and 12 on prednisolone, CSA, and azathioprine. The median dosage of prednisolone was 0.29 mg/kg (range 0.13–1.72, $n = 20$) and of azathioprine 1.6 mg/kg (range 0.82–2.19, $n = 14$); the median CSA level was 229 μg/l (range 29–563, $n = 18$). Blood was taken at the time of CSA trough level determination and before intake of other immunosuppressive drugs. Twenty-four healthy laboratory workers served as controls.

Monoclonal antibodies (mAbs)

MAbs 5344.111 (anti-IL-2, mouse IgG1, fluoroisothiocyanate-conjugated) and 25723.11 (anti-IFN-γ, mouse IgG2b, fluoroisothiocyanate-conjugated) were obtained from Becton Dickinson (Mountain View, Calif., USA; mAbs B-B11 (anti-CD3, mouse IgG1, Cy-Q-conjugated), B-F5 (anti-CD4, mouse IgG1, phycoerythrin-conjugated), and MCD8 (anti-CD8, mouse IgG1, phycoerythrin-conjugated) were all obtained from Immuno Quality Products (Groningen, The Netherlands).

Flow cytometric cytokine synthesis analysis

Heparinized blood was diluted 1 : 1 with RPMI 1640, and incubated for 4 h at 37 °C, 5 % CO_2, with 25 ng/ml PMA (Sigma, Zwijndrecht, The Netherlands) and 1 ng/ml calcium ionophore A 23187 (Sigma), and in the presence of 2 μg/ml of monensin (Sigma); blood was incubated without mitogens to obtain unstimulated controls. Subsequently, cells were incubated for 20 min at RT in the dark with mAbs directed against cell surface antigens. Expression of CD4 is strongly down-modulated by phorbol esters, precluding the use of CD4 mAbs to delineate this subset [9]. CD8 expression diminishes only slightly after stimulation. We therefore used CD8 mAb to delineate the $CD4^+$ and $CD8^+$ cells within the $CD3^+$ subset, considering $CD3^+$ $CD8^-$ cells to represent $CD4^+$ cells. Erythrocytes were lysed by incubation with FACS lysing solution (Becton Dickinson) for 8 min at RT in the dark, and permeabilized in FACS permeabilizing solution (Becton Dickinson) for 10 min. Cells were washed in a buffer consisting of PBS with 0.5 % BSA and 0.1 % azide, and stained for 30 min at RT with mAbs against cytokines. After washing, cells were fixed by incubation with 1 % paraformaldehyde/PBS and stored at 4 °C until analysis. Five-parameter analysis was performed on a Epics-Elite flow cytometer (Coulter Electronics, Hialeah, Fla., USA). Calibration was done daily using Quantum-R beads (Flow Cytometry Standards Europe, Leiden, The Netherlands). A total of 2000 $CD3^+$ cells were acquired from each sample and analyzed using Winlist software (Verity Software House, Topsham, Me., USA).

CSA levels

Whole-blood trough levels of CSA were determined by TDx.

Statistical analysis

Group data are represented as median (range). Groups were compared using the Mann-Whitney U-test, and correlations were analyzed by the Spearman rank correlation test. Univariate regression analysis was done using the Statmost32 4.1 statistical package. P values of 0.05 or less were considered to indicate statistical significance.

Results

Capacity for production of IL-2 and IFN-γ is strongly reduced in $CD4^+$, but not in $CD8^+$ T cells of transplant recipients

No cytokine production could be detected in unstimulated lymphocytes. The percentage of $CD4^+$ lymphocytes that produced IL-2 was strongly reduced in patients compared with healthy controls (Table 1). In the subgroup of patients with CSA levels in the therapeutic range of 100–400 ng/ml, only 15 % (4–48) of all $CD4^+$ T cells produced IL-2. Mitogen-stimulated production of IFN-γ by $CD4^+$ T cells was also significantly lower in

Table 1 Capacity for IL-2 and IFN-γ production of T cells in transplant patients and in healthy controls. Results are given as median (range) percentages of cytokine-positive cells within each subset. P values were calculated using the Mann-Whitney U test

Subset, cytokine	Patients (n)	Controls (n)	P value
$CD4^+$, IL-2	26 (4–68) (20)	59 (40–72) (24)	0.0001
$CD4^+$, IFN-γ	14 (6–64) (12)	27 (14–39) (16)	0.0083
$CD8^+$, IL-2	17 (8–34) (16)	22 (10–41) (16)	0.2206
$CD8^+$, IFN-γ	58 (27–90) (16)	45 (21–85) (17)	0.4281

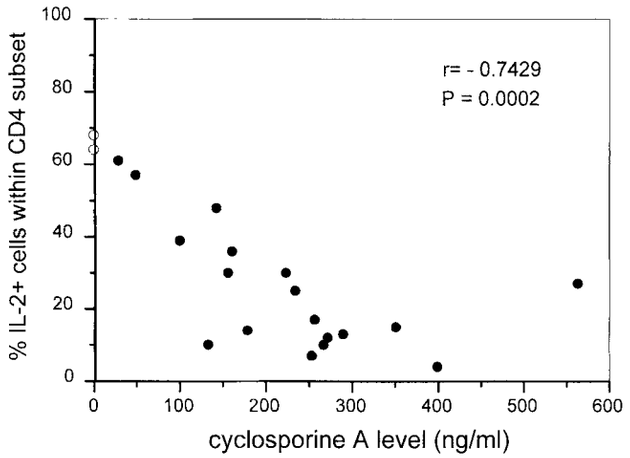

Fig. 1 Capacity for IL-2 production by $CD4^+$ T cells in relation to cyclosporine A (CSA) trough levels. Patients on CSA-based regimens are represented by ●, and patients on prednisolone/azathioprine by ○

transplant recipients. The capacity for IL-2 production by $CD8^+$ T cells was lower in transplant patients, whereas more cells in this subset produced IFN-γ. However, due to substantial overlap, these differences failed to reach statistical significance.

Capacity for cytokine production in relation to immunosuppressive drugs

Univariate analysis showed negative correlations between the percentages of cells producing IL-2 within $CD4^+$ and $CD8^+$ subsets and CSA trough levels (P values 0.0087 and 0.0396, respectively). No statistically significant correlations were found between IL-2 production in either subset and the dosages of prednisolone and azathioprine. Patients with CSA levels within the therapeutic range (100–400 ng/ml) showed considerable interindividual variation in the capacity for IL-2 synthesis by $CD4^+$ cells, suggesting different degrees of susceptibility to the inhibitory effects of the drug (Fig. 1). The capacity for IFN-γ production by $CD4^+$ T cells showed a negative correlation with the prednisolone dose ($P = 0.0175$) and, for the $CD8^+$ subset, with CSA trough levels ($P = 0.0023$). The remaining correlations between individual immunosuppressive agents and cytokine production failed to reach statistical significance.

Discussion

These data show that the suppressive effects of CSA and prednisolone on the capacity for cytokine synthesis can be measured in a quantitative way, using short-term stimulation in whole blood and multiparameter flow cytometric analysis. In agreement with their established mechanism of action, CSA and prednisolone, but not azathioprine, were associated with a dose-dependent suppression of cytokine production. IL-2 was inhibited to a greater extent than IFN-γ, and $CD4^+$ lymphocytes appeared to be more sensitive to suppression than $CD8^+$ cells. These observations suggest that this system may be useful for monitoring the effects of CSA and prednisolone on immune reactivity in vivo. The assay has a short turnaround time of about 8 h, which is important for clinical application; it can be adapted to provide information about drug effects on any lymphocyte subset desired or on any functional marker available.

Although the remaining capacity of T cells for IL-2 production showed a strong, inverse correlation with CSA blood levels, functional measurement offers several advantages over the therapeutic drug level monitoring. CSA is highly lipophilic and has a complex biodistribution. At physiological temperatures only 4% of CSA in whole blood is unbound, while most of the drug is bound to erythrocytes, lipoproteins, and other plasma proteins [6]. Hemoglobin and albumin levels may show considerable changes in transplant patients, which undoubtly will affect the biologically active fraction of the drug without being reflected in the whole-blood levels. CSA undergoes extensive hepatic metabolism, and a variety of metabolites are formed, some of which are active [10]. Clearance of metabolites may be reduced especially in cases of hepatic dysfunction [8]. HPLC generally does not measure these metabolites, and thus underestimates the biological activity of CSA, whereas other assays include these metabolites to some extent, and thus may overestimate "true" CSA levels. Of equal importance is the possibility that the susceptibility for the inhibition of calcineurin may show interindividual differences, since patients with comparable CSA blood levels and on equal steroid dosages differed considerably with respect to their capacity for IL-2 production. We included only patients with an uncomplicated clinical course, who might be expected to be sensitive to the drugs administered. It would not be surprising if patients who reject despite adequate dosages of immunosuppressive agents are less sensitive to drug inhibitory effects. Production of IL-2 was only partially suppressed in these immunosuppressed patients. Even in patients with CSA levels in the therapeutic range (100–400 ng/ml), the number of IL-2 producing $CD4^+$ T cells was reduced to only 25% of that in healthy controls. This reduction is somewhat more pronounced than the 50% reduction by CSA of calcineurin activity as reported by Batiuk et al. in renal transplant patients with comparable blood CSA levels [2]. Figure 1 suggests that CSA levels of at least 300–350 μg/l are required for near-complete inhibition of IL-2 production. Knowledge of in vivo dose-response relationships might be particularly useful in lung

and small intestine transplantation, where rejection still constitutes a great problem. IFN-γ synthesis was reduced in both lymphocyte subsets after transplantation. This is in agreement with the observation that the IFN-γ promotor has at least one calcium-inducible, CSA-sensitive element that is a potential NFAT binding site [5].

Prospective studies are necessary to determine to what extent inhibition of cytokine synthesis by immunosuppressive agents correlates with clinical events, and thus truely provides insight into the degree of immunosuppression induced by these agents. These studies will also tell whether monitoring of drug effects is more useful than measurement of drug levels.

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References

1. Batiuk TD, Pazderka F, Enns J, Decastro L, Halloran PF (1995) Cyclosporine inhibition of calcineurin activity in human leukocytes in vivo is rapidly reversible. J Clin Invest 96: 1254–1260
2. Batiuk TD, Pazderka F, Halloran PF (1995) Calcineurin activity is only partially inhibited in leukocytes of cyclosporine-treated patients. Transplantation 59: 1400–1404
3. Berg AP van den , Bij W van der, Son WJ van, Anema J, Giessen M van der, Schirm J, Tegzess AM, The TH (1989) Cytomegalovirus antigenemia as a useful marker of symptomatic cytomegalovirus disease after renal transplantation: a report of 130 consecutive patients. Transplantation 48: 991–995
4. Berg van den AP, Klompmaker IJ, Haagsma EB, Scholten-Sampson A, Bijleveld CMA, Schirm J, Giessen M van der, Slooff MJ, The TH (1991) Antigenemia in the diagnosis and monitoring of active cytomegalovirus disease after liver transplantation. J Infect Dis 164: 265–270
5. Campbell PM, Pimm J, Ramassar V, Halloran PF (1996) Identification of a calcium-inducible, cyclosporine-sensitive element in the interferon-gamma promoter that is a potential NFAT binding site. Transplantation 61: 933–939
6. Lemaire M, Tillement JP (1982) Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood. J Pharm Pharmacol 34: 715–718
7. Liu J, Farmer JDJ, Lane WS, Friedman J, Weissman I, Schreiber SL (1991) Calcineurin is a common target of immunophyllin-cyclosporin A and FKBP-FK506 complexes. Cell 66: 807–815
8. Maurer G, Lemaire M (1986) Biotransformation and distribution in blood of cyclosporine and its metabolites. Transplant Proc 18: 25–34
9. Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC (1995) Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood 86: 1408–1419
10. Vine W, Bowers L (1987) Cyclosporine: structure, pharmacokinetics, and therapeutic drug monitoring. Crit Rev Clin Lab Sci 25: 275–311