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REVERSAL OF STEROID- AND ANTI-LYMPHOCYTE ANTIBODY-RESISTANT REJECTION USING INTRAVENOUS IMMUNOGLOBULIN (IVIG) IN RENAL TRANSPLANT RECIPIENTS

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Background. **Despite the recent advances in immunosuppression, steroid-resistant rejection remains a difficult problem in renal transplant recipients.**

Methods. **We reviewed our experience with i.v. immunoglobulin (IVIG) in the treatment of steroid- and antilymphocyte antibody-resistant rejection in renal transplant patients. Between September 1996 and March 1999, 17 patients were treated with IVIG to reverse steroid- or antilymphocyte antibody-resistant rejection. A total of 2 g/kg of IVIG was administered to patients during each treatment course.**

Results. **With a mean follow-up of 21.5±9.5 months from the time of IVIG administration, patient and graft survival rates were 94% (16/17) and 71% (12/17), respectively. The baseline mean serum creatinine level prior to rejection was 2.2±0.7 mg/dl and peaked at 3.3±1.1 mg/dl at the time of the diagnosis of refractory rejection. IVIG therapy was associated with a fall in the mean creatinine to 2.8±1.1 mg/dl. The most recent serum creatinine in patients with functioning grafts was 2.8±1.6 mg/dl. In 82% of allograft biopsies after IVIG, reversal or reduction in the severity of rejection was demonstrated. In addition, IVIG therapy rescued three of four patients with antilymphocyte antibody-resistant rejection.**

Conclusions. **IVIG rescue therapy for steroid- or antilymphocyte antibody-resistant rejection is associated with resolution or improvement of rejection severity, stable renal function, and reasonable graft survival.**

INTRODUCTION

Although immunosuppression after renal transplantation has become increasingly powerful in recent years, rejection remains an important complication. Steroid-resistant rejection is an especially significant problem, and despite the availability and efficacy of antilymphocyte antibody therapy, remains associated with accelerated rates of renal allograft loss (*1*). Apart from mycophenolate mofetil and tacrolimus rescue therapy, few other treatment modalities have been able to reverse steroid-resistant rejection (*2*, *3*).

In the past, i.v. immunoglobulin (IVIG) has been used to treat autoimmune disorders (*4*), as well as infectious diseases in immunosuppressed patients (*5*). Recently, it has been used in transplantation to prevent graft versus host disease (GVHD) in bone marrow transplant recipients (*6*), and to reduce anti-HLA antibodies in sensitized patients awaiting organ transplantation (*7*). There is also preliminary evidence that it can be used as an induction agent (*8*, *9*), and that it may be able to reverse antibody-mediated rejection in the early posttransplant period (*10*). However, the data regarding the clinical use of IVIG to reverse steroid-resistant rejection are extremely limited (*10–12*), and to date, no report has addressed its use in treatment of antilymphocyte antibody-resistant rejection. In this study, we report the University of Pittsburgh experience with IVIG in reversing steroid- and antilymphocyte antibody-resistant rejection in renal transplant recipients.

PATIENTS AND METHODS

Between September 1996, and March 1999, 25 patients received IVIG for steroid- or antilymphocyte antibody-resistant rejection at the University of Pittsburgh (the preparation used was Sandglobulin, which had been selected by the hospital independently of any input from the transplant service). Eight patients were excluded from analysis because of concurrent administration of antilymphocyte antibody therapy; thus, a total of 17 patients were analyzed (9 male, 8 female). All patients had biopsy-proven rejection, and all had post-IVIG allograft biopsies to document treatment efficacy. Thirteen patients (76%) were treated for steroid-resistant rejection, and 4 patients (24%) were treated for antilymphocyte antibody-resistant rejection. Patient demographics are shown in Table 1.

Significantly, these patients were not believed to be at an increased risk for antibody-mediated hyperacute or accelerated rejection. Rejection was not suspected during the first postoperative week in any of the 17 patients, and the time from transplantation to

TABLE 1. Patient demographics

n	17 (100%)
Mean age (yr)	43.9±15.0 yr (range 26–72)
Gender	9 male: 8 female
% PRA (DTT)	12.5±22.3 (range 0–72)
Number of prior rejection episodes with current graft	2.0±1.6 (range 0–6)
Second renal transplant	5 (29%)
Third renal transplant	1 (6%)
Living-related renal transplant	2 (12%)
Previous liver transplant	2 (12%)
Patients failing antilymphocyte therapy	4 (24%)

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commencement of IVIG therapy was 17.5±23.7 months (range 1–84). The mean % PRA was 12.5±22.3 (range 0–22); six patients (35%) had previously received a renal allograft.

Sixteen (94%) patients had received tacrolimus-based immunosuppression. The remaining patient received microemulsion cyclosporine. Six (35%) patients received mycophenolate mofetil maintenance therapy, and six (35%) patients had been weaned off corticosteroids prior to the development of refractory rejection.

A total of 2 g/kg of IVIG was administered over 2 to10 days for each treatment course, according to the fluid balance status of each patient. Four (24%) patients received two courses of IVIG, and three (18%) patients received three or more courses. The mean tacrolimus dose was increased by 1.5±2.4 mg/d as part of the treatment for refractory rejection. The IVIG course was accompanied by a steroid recycle in 10 patients, and in 7 patients, mycophenolate mofetil was added (mean dose 1143±690 mg/day, range 500–2000 mg/day).

RESULTS

The mean follow-up was 39.0±23.1 months from the time of transplantation and 21.5±9.5 months from the time of IVIG therapy. The patient survival rate was 94% (16/17), and the graft survival rate was 71% (12/17). The sole mortality involved a patient who had had a liver transplant 7 years previously. She developed fungal endocarditis 13 months after IVIG therapy and died. Four graft losses (80%) were attributed to chronic rejection, and the remaining graft loss was related to a failure of IVIG rescue therapy.

Renal allograft function was reasonably well maintained after IVIG therapy. The baseline serum creatinine level before development of rejection was 2.2±0.7 mg/dl, and rose to 3.3±1.1 mg/dl at the time of the refractory rejection episode. IVIG therapy was associated with a reduction in the mean serum creatinine 2 weeks after the conclusion of therapy (2.8±1.1 mg/dl). The current serum creatinine in patients with functioning grafts is 2.8±1.6 mg/dl.

Before the initiation of IVIG therapy, 47% (8/17) of patients had Banff IA, 29% (5/17) had Banff IB, and 24% (4/17) had Banff II rejection. After IVIG therapy, 53% of allograft biopsies (9/17) demonstrated complete resolution of rejection, and 29% (5/17) demonstrated reduced rejection severity. Overall, 82% of allograft biopsies had a reduction in rejection severity. Surprisingly, the severity of rejection prior to IVIG therapy did not predict treatment outcome (Fig. 1).

As a number of our patients were treated concomitantly with a steroid recycle or mycophenolate mofetil, it was difficult to analyze the impact of IVIG by itself on refractory rejection. Thus, we analyzed a subset of seven patients who received IVIG without any other adjunctive therapy. The demographics of patients receiving IVIG alone were similar to those in patients receiving mycophenolate mofetil and/or a steroid recycle in addition to IVIG (Table 2). Of these seven patients who received IVIG monotherapy, three (43%) had Banff IA, two (29%) had Banff IB, and two (29%) had Banff II rejection before the initiation of therapy. Six of seven (86%) post-IVIG allograft biopsies in these patients demonstrated a reduction in or resolution of rejection. The mean baseline serum creatinine level in these patients was 2.2±0.9 mg/dl, rose to 3.7±1.2 mg/dl at the time of rejection, and fell to 2.7±1.3 mg/dl 2 weeks after IVIG therapy. The current mean serum creatinine in the six patients with functioning allografts is 3.0±2.1 mg/dl. Thus, IVIG by itself appeared to be able to reverse refractory rejection.

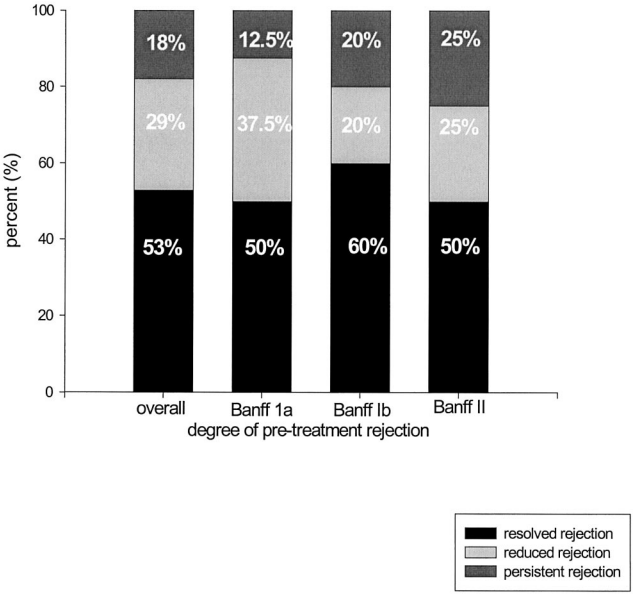

FIGURE 1. Reversal of rejection after administration of IVIG.

Four patients received IVIG to reverse antilymphocyte antibody-resistant rejection. Three patients had failed OKT3 therapy, and one patient had failed anti-thymocyte globulin (ATG) therapy for either steroid-resistant or Banff II rejection. Allograft biopsy at the time of anti-lymphocyte antibody-resistant rejection demonstrated Banff Ia in two patients, Banff Ib in one patient, and Banff II rejection in the remaining patient. IVIG was able to completely reverse rejection in one patient and reduce rejection severity to borderline rejection in two other patients. Overall, IVIG rescued three of four patients with antilymphocyte antibody-resistant rejection. IVIG treatment lowered the mean serum creatinine from 3.0±1.3 mg/dl at the time of refractory rejection to 2.5±1.1 mg/dl 2 weeks after the completion of treatment. The current mean serum creatinine is 2.0±1.2 mg/dl in the three patients with functioning allografts.

Complications after IVIG therapy were rare. Having failed ATG, one patient received IVIG and developed symptomatic cytomegalovirus (CMV) infection 5 months after treatment of refractory rejection. Another patient, status post liver transplantation 7 years earlier, developed fungal endocarditis 13 months after IVIG therapy. It is likely that these infectious complications were related to intensive long-term immunosuppression. IVIG was well tolerated, and no complications could be directly attributed to IVIG therapy.

DISCUSSION

Pooled human gammaglobulin, IVIG, has been used to treat infectious complications in immunosuppressed patients (*5*), autoimmune idiopathic thrombocytopenic purpura (*4*), and vasculitis (*13*) since the early 1980s. Only recently has it been used in bone marrow and solid organ transplantation. IVIG therapy is associated with a lower incidence of GVHD in bone marrow transplant recipients (*6*). Its ability to reduce the levels of anti-HLA antibodies has enabled sensitized patients with prohibitively high PRAs to be transplanted (*7*). IVIG has also been used in induction therapy (*8*, *9*) and has

TABLE 2. Demographics of patients receiving IVIG monotherapy versus IVIG with adjunctive therapy

Category	IVIG alone	IVIG with adjunctive therapy	Significance
No.	7	10	
Adjunctive therapy	None	MMF and/or steroid recycle	
Gender	4 male/3 female	5 males/5 females	NS
Age (yr)	41.3±16.8 (26–72)	45.7±14.1 (30–71)	NS
% PRA	18.1±30.3 (0–82)	7.3±13.3 (0–36)	NS
Time from transplant to IVIG therapy (mo)	17.3±30.3 (1–84)	17.6±19.7 (1–58)	NS
Previous kidney transplant	3 (43%)	3 (30%)	NS
Previous liver transplant	2 (29%)	0 (0%)	NS
Living-related transplant	1 (14%)	1 (10%)	NS
Patients failing antilymphocyte therapy	1 (14%)	3 (30%)	NS

NS, Not significant ($P>0.05$).

demonstrated the ability to reverse antibody-mediated rejection (*10—12*).

Although IVIG has been shown to be able to block anti-HLA antibodies via antiidiotype antibodies (*14*), the mechanism by which it reverses established non-HLA antibody-related rejection is unclear. Several theories have been proposed. Marchalonis et al. proposed that antiidiotype antibodies from IVIG can bind to the hypervariable region of the T cell receptor, and thereby inhibit T cell-mediated rejection (*15*). IVIG has also been thought to provide anti-CD4 activity (*16*) and block cytokine receptors (*17*). Other in vitro studies have shown that IVIG has been shown to down-regulate both T and B cell activation and antibody production (*18*), as well as suppress tumor necrosis factor (TNF) production (*17*). Recent in vivo studies have demonstrated the ability of IVIG to prolong xenograft survival (*19*).

Casadei et al. presented data suggesting that IVIG can rescue up to 82% of grafts with steroid-resistant rejection (*11*, *12*). Our data support their findings. With a follow-up of 21.5 months, both graft and patient survival were relatively good, with maintenance of stable renal allograft function. For the first time, we also demonstrated that IVIG was able to reverse anti-lymphocyte antibody-resistant rejection.

A number of patients in our study were treated concomitantly with a steroid recycle and initiation of mycophenolate mofetil, confounding our ability to assess the efficacy of IVIG in reversing rejection. However, in a subgroup of patients who did not receive additional concurrent antirejection therapy, rejection was completely eliminated or markedly reduced in 6 of 7 patients. Therefore, IVIG therapy by itself appears to be able to reverse steroid-resistant rejection.

We included the patients that received steroid recycles and/or mycophenolate mofetil in conjunction with IVIG therapy in our analysis to determine whether the addition of adjunctive therapy was associated with improved or reduced treatment efficacy, and whether this combination was associated with an unacceptably high complication rate. Eight of 10 patients (80%) who were treated with adjunctive therapy demonstrated a reduction in rejection severity, compared to 6 of 7 patients (86%) in the IVIG monotherapy group. The complication rates in both groups were similar. One of 7 patients (14%) in the IVIG monotherapy group developed fungal endocarditis 13 months post-IVIG therapy, and 1 of 10 patients (10%) in the adjunctive group developed CMV 5 months post-IVIG. Again, neither of these complications could be attributed directly to IVIG, and were likely secondary to intensive immunosuppressive therapy both before and after IVIG therapy.

It appears that the efficacy of IVIG therapy in the treatment of steroid-resistant rejection may be similar to that of OKT3 or ATG. The main advantage of IVIG over antilymphocyte therapy is the relative paucity of side effects. In addition, its inherent antiviral properties make IVIG an attractive agent in treating rejection in patients who are at high risk for immunosuppression-related viral infections, such as CMV. However, despite these antiviral properties, one patient developed CMV enteritis 5 months post-IVIG therapy. We attributed this complication to intensive immunosuppressive therapy that included a course of ATG before the use of IVIG. It appears that IVIG does not confer complete prophylaxis against CMV when given shortly after the conclusion of ATG therapy.

The cost of IVIG therapy is an important issue. Although one course of IVIG is currently more expensive than antilymphocyte antibody therapy, IVIG can be given in an outpatient setting, without the need for continuous monitoring or central venous access. Ultimately, prospective, randomized studies will be required to evaluate the efficacy, optimal therapeutic dose, and relative cost of IVIG in the treatment of organ transplant rejection.

In conclusion, our data suggest that IVIG rescue therapy for steroid-resistant rejection is associated with histological resolution or improvement of rejection severity, maintenance of renal function, and long-term graft survival. In addition, it seems that IVIG is capable of reversing antilymphocyte antibody-resistant rejection.

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MEASUREMENT OF MYCOPHENOLATE MOFETIL EFFECT IN TRANSPLANT RECIPIENTS[1]

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Background. **Immunosuppression involves the nature of the immunosuppressive agents and individual differences in patient factors. We investigated whether the effect of mycophenolate mofetil (MMF) is measurable using an in vitro measure of immunocompetence and related its effects to cyclosporine (CsA) in vitro.**

Methods. **Liver or kidney transplant recipients receiving prednisone; CsA or tacrolimus; and MMF, azathioprine (AZA), or neither, were studied. Immunocompetence was assessed by one-way mixed lymphocyte culture using patients' peripheral blood leukocytes (PBL) and three validated stimulators. The effect of immunosuppressive agents added in vitro on normal PBL stimulation by *Staphylococcus* enterotoxin B was determined by the carboxyfluorescein diacetate succinimidyl ester measurement of division.**

Results. **Patients receiving MMF had an average immunocompetence level of 12±23, compared with 39.7±65 and 25.5±42 for those receiving AZA or neither AZA nor MMF, respectively. Thus, there was an approximately 80% suppression of the response in the MMF group. Assessment of normal cell division revealed that CsA allowed multiple cell generations but suppressed the numbers of cells in each, whereas MMF blocked proliferation into subsequent generations. Addition of clinically relevant levels of mycophenolic acid, the active agent for MMF, added to more moderate levels of CsA, was required to achieve greater than 80% suppression, consistent with the degree of immunocompetence depression measured in patients.**

Conclusions. **These data provide the novel finding that the effect of MMF treatment on patients is measurable in their PBL as decreased immunocompetence in vitro. The effect of MMF on normal PBL approximates the 80% inhibition that we found in patients. Moreover, the effect of MMF on cell division provides**

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a rationale for the superior effectiveness of regimens including MMF.

INTRODUCTION

The increasing number of more selective immunosuppressive agents available for organ transplantation has the potential to be of great benefit. Typically, newer agents are used as adjuncts in a regimen based on corticosteroids and either cyclosporine (CsA) or tacrolimus (FK). The initial trials of agents such as mycophenolate mofetil (MMF) and rapamycin showed clinical efficacy when used as adjuncts (*1*, *2*). However, it is difficult to optimize the regimens in general or for specific subgroups using large clinical trials with outcome endpoints for each of the many possible combinations. In particular, it is unclear whether it is preferable to maximize the dose of one agent (e.g. a calcineurin inhibitor), or to add a second agent (e.g. MMF or sirolimus) to a moderate dose of the first agent to optimize immunosuppression. This is important because lower doses of multiple agents may minimize side effects associated with any single agent. A surrogate method of measuring immunosuppressive effects in patients is desirable to determine the response to new agents and to prevent the complications of over- or under-immunosuppression.

The use of blood levels to adjust dosages for immunophilin-binding drugs is very helpful. The use of blood levels to monitor MMF has been assessed (*3*, *4*) but has not been generally employed for clinical management because the pharmacokinetics are not amenable (rapid peak, then nearly unmeasurable trough) (*5*, *6*), and because of the relatively wide therapeutic index of MMF. However, even the measurement of the levels of all agents would not take into account the biologic variability among patients to the net effect of the measured blood levels (*7*). Using an approach designed to address these issues, we report the novel finding that a mixed leukocyte culture (MLC) test-based immunocompetence assay can detect the net effect of MMF treatment in a group of patients receiving MMF in combination with other immunosuppressive agents. Additionally, we found a difference in the in vitro effects of these drugs that may help determine optimal regimens that include MMF.

METHODS AND MATERIALS

Subjects. An additional blood sample was drawn at the time of routine blood drawing from liver (156 assays) and kidney (269 assays) transplant recipients. Samples were obtained at the trough immediately before the next dose of immunosuppressants, under a human studies committee-approved protocol. The patients were on routine immunosuppression with prednisone and a calcineurin inhibitor, optimized per clinical indications. Increasing numbers of patients routinely received MMF after MMF became available, when clinically appropriate. Assignment to a particular regimen was made based on clinical criteria and patients were not randomized. Review was performed retrospectively. In the majority of cases, one or two assays were performed per patient. In a few cases, blood from the same patient was assayed three to five times. In cases of multiple assays, the patient's results were assigned to the immunosuppression category in use at the time the blood was drawn. The patients did not differ by age, gender, or race. Trough blood levels of FK were determined using the Tacrolimus II IMX System (Abbott, Abbott Park, IL), and CsA by Cyclo-TRACsp (DiaSorin, Stillwater, MN). Blood was obtained from normal volunteers or from hemapheresis leukopaks for testing effects of drugs added in vitro.

Cell preparation and MLC. Stimulator leukocytes were prepared (*8*) from hemapheresis donor packs, separated on a Ficoll-Hypaque (SIGMA Chemical Corporation, St. Louis, MO) gradient, washed, and suspended in tissue culture media (TCM), RPMI 1640 with glutamine and penicillin/streptomycin. The cells were irradiated (5000 R), aliquoted, and frozen in dimethyl sulfoxide.

The peripheral blood leukocytes (PBL) from patients to be tested were purified on Ficoll-Hypaque, washed, and cultured (TCM with 10% pooled human AB serum) (SIGMA Chemical Corporation) in triplicate, using 10^6 responders and stimulators per well, with each of three stimulator donors, and with phytohemagglutinin (PHA) (GIBCO, Grand Island, NY) as a positive control. Tritiated thymidine (New England Nuclear, Cambridge, MA) was added on day 5, and 18 hr later the cells were harvested and assayed by scintillation counting. When the PHA stimulation was less than four times the TCM control, the experiment was discarded as technically inadequate; this occurred rarely and no patient was documented to be PHA-unresponsive. The result for each stimulator was calculated as the average of the counts in the three wells divided by the average of the TCM controls without stimulator cells.

Validation and interpretation of immunocompetence results. Stimulator cells were subjected to internal validation. In addition to the stimulator populations used to provide the results, two or three additional newly prepared stimulators were tested several times; occasionally (approximately 5% of the time) these preliminary populations were not appropriately stimulatory, as compared with the validated stimulators. These preliminary stimulators were discarded.

To evaluate the maximum degree of responsiveness, the highest value of the three validated stimulators tested for that patient for that day was designated as the ImmunoCompetence (IC) value. Lower IC values reflect lower T cell proliferations, i.e., diminished immune responsiveness. We have previously shown that this approach provides a valid simplification of the original technique (*9*). Normal PBL have an IC value above 40 (95% confidence interval: 40 to 80). Values between 20 and 40 may reflect inadequate immunosuppression, and values below 3, may reflect over-immunosuppression, based on past experience.

Experiments were assessed with ANOVA, and groups were compared with Welch's t test; in cases where individual comparisons were significant at $P<0.05$, as indicated, F tests revealed $P\leq0.0028$ for the overall data.

In vitro drug dose-response curves. The carboxyfluorescein diacetate succinimidyl ester (CFSE) technique was used to measure proliferation (*10*) for these studies, because it provides a measure of overall proliferation and may distinguish between different drug mechanisms. Cultures of 2×10^6 PBL/ml from normal volunteers were prepared as described above, but with the inclusion of 5 μM CFSE (Molecular Probes, Inc., Eugene, OR).

Staphylococcus enterotoxin B (SEB) (SIGMA Chemical Corporation), chosen as more physiologic than PHA, was added at 1 μg/ml as a polyclonal MHC-based superantigen stimulus, and titrations of CsA or mycophenolic acid (MPA) immunosuppressive agents were added in indicated dosages. Immunosuppressive drug doses were chosen to bracket and exceed clinically relevant levels, recognizing that the steady-state level in tissue culture may not be directly related to the effects resulting from in vivo pharmacokinetics. After 4 days of culture, the cells were harvested and the number of cells in each division generation, containing one-half of the CFSE in the previous generation, was assessed by flow cytometry. The division index (D.I.) was calculated by summing the numbers of cells corrected for their generation. A D.I. of 1 corresponds to an average of one division per cell.

RESULTS

Table 1 shows that, on average, patients with transplants treated with corticosteroids and CsA or FK had IC values

TABLE 1. Immunocompetence effect of MMF on liver and kidney transplant patients[a]

Patient Group	Immunosuppressive comparison agent					
	MMF		AZA		Neither	
	n	IC±SD	n	IC±SD	n	IC±SD
All recipients	149	12.0±23	86	39.7±65	190	25.5±42[b]
Kidney recipients	120	12.6±21	82	40.7±58	67	21.7±42[c]
Liver recipients	29	9.51±11	4	19.2±2	123	21.7±40[d]

[a] All patients were on prednisone and a calcinerin inhibitor, with or without an additional comparison agent, as indicated.
[b] MMF vs. AZA, $P=0.0002$; MMF vs. neither, $P=0.0002$; AZA vs. neither, $P=0.07$.
[c] MMF vs. AZA, $P=0.00006$; MMF vs. neither, $P=0.0067$; AZA vs. neither, $P=0.09$.
[d] MMF vs. neither, $P=0.0033$.

that were lower than normal, reflecting their immunosuppression. Patients receiving CsA or FK, with or without AZA, had relatively moderate depressions of immunocompetence. However, the average IC levels were significantly lower for patients receiving MMF in addition to CsA or FK. This was true for transplant recipients overall, for kidney transplant recipients, and for the MMF versus neither CsA nor FK comparison for liver transplant recipients. A trend was present in the MMF versus AZA comparison for liver transplants, but not statistically significant because of the small number of liver transplant patients in the AZA group. Moreover, the decreased IC value caused by MMF was a general effect, found both for patients on CsA and on FK, as shown in Table 2. The calcineurin inhibitor levels were similar for all comparison groups (Table 3) except for the very small liver transplant recipients on AZA group.

The positive control data with PHA stimulation were compared for the kidney patients and were not found to be different among the three groups (MMF: 27,786; AZA: 24,197; neither: 28,371; no significant differences).

The effects of CsA and MPA were analyzed for SEB stimulation of normal volunteer PBL at doses bracketing those producing intermediate suppression for each drug. As shown on the CFSE cell division histogram in Figure 1, for CsA there was general suppression of the numbers of cells in several sequential division generations, whereas increased levels of MPA increasingly prevented division beyond the first generation. These mechanisms produce similar division index values, but via different effects.

Figure 2 shows the individual and combined effects of CsA versus MPA on the division index of stimulated normal PBL. In the range approximating therapeutic serum drug levels, both CsA and MPA are immunosuppressive. However, the suppression is more complete with MPA alone than with CsA alone. Note that the levels of CsA in TCM correspond to higher unbound concentrations of drug than for levels measured clinically in blood (see *Discussion*). Also note the strong additive effect of CsA and MPA.

Five experiments measuring the effects of combining CsA and MPA on SEB stimulation of normal PBL are summarized in Figure 3. Note that increasing levels of CsA, even up to 500 μg/ml, which far exceeds clinically safe blood levels, fail to suppress the response beyond 70% inhibition (800 μg/ml produces suppression greater than 70%, data not shown). However, moderate levels of MPA produce suppression that is more complete.

DISCUSSION

This first demonstration of an effect of MMF on the response of patients' lymphocytes in an in vitro analysis of stimulation is novel and important because the effect of MPA on mitogen responsiveness is transitory. After an oral dose, MMF is quickly converted to the active agent MPA, which is rapidly inactivated by glucuronidation. Serum drug levels rise rapidly to a narrow peak within 2 hours and then fall to a minimal trough level for hours before the next dose (*5*, *6*). Furthermore, the drug is easily washed out of lymphocytes (*11*), apparently ruling out persistence in the cultures of PBL from patients. Previous work, with mitogen rather than MLC stimulation, showed that when washed and tested, lymphocytes obtained after drug administration had levels of inosine monophosphate dehydrogenase (the target enzyme of the drug) that were restored to normal (*11*, *12*). Our results with PHA in patients confirm the previous reports that PHA stimulation is not affected by previous MMF treatment, without MPA added to the TCM.

There are several possible mechanisms to explain the effect on lymphocytes from treated patients on this in vitro test without MPA added to the culture. A secondary effect of the drug, such as interference with glycosylation of proteins (*13*) could provide a more long-lasting result, which would explain these results. Alternatively, the very low level of MPA that

TABLE 2. The immunocompetence effect of MMF is measurable regardless of whether the patients are on cyclosporine or tacrolimus[a]

Calcineurin treatment	Comparison treatment					
	MMF		AZA		Neither	
	n	IC±SD	n	IC±SD	n	IC±SD
CsA	53	9.62±18	63	37.8±53	91	25.7±42[b]
Tacrolimus	96	13.4±25	23	54.7±76	99	24.4±46[c]

[a] Patients were liver or kidney recipients, on corticosteroids and other treatment as indicated.
[b] MMF/CsA vs. neither, $P=0.002$; MM/CsA vs. AZA, $P=0.00017$.
[c] MMF/FK vs. neither, $P=0.038$; MM/FK vs. AZA, $P=0.016$.

TABLE 3. Trough levels of calcineurin inhibitors amongst the comparison groups (mean levels, ng/ml ± SD)

Patient Group	MMF	AZA	Neither
Kidney recipients			
CsA	201 ± 95	219 ± 136	197 ± 68
FK	9.4 ± 4.0	9.0 ± 3.5	8.9 ± 4.0
Liver Recipients			
CsA	204 ± 27	256 ± 84	184 ± 69[a]
FK	8.7 ± 3.1	(no patients)	8.4 ± 4.0

[a] CsA, AZA vs. neither, $P=0.01$.

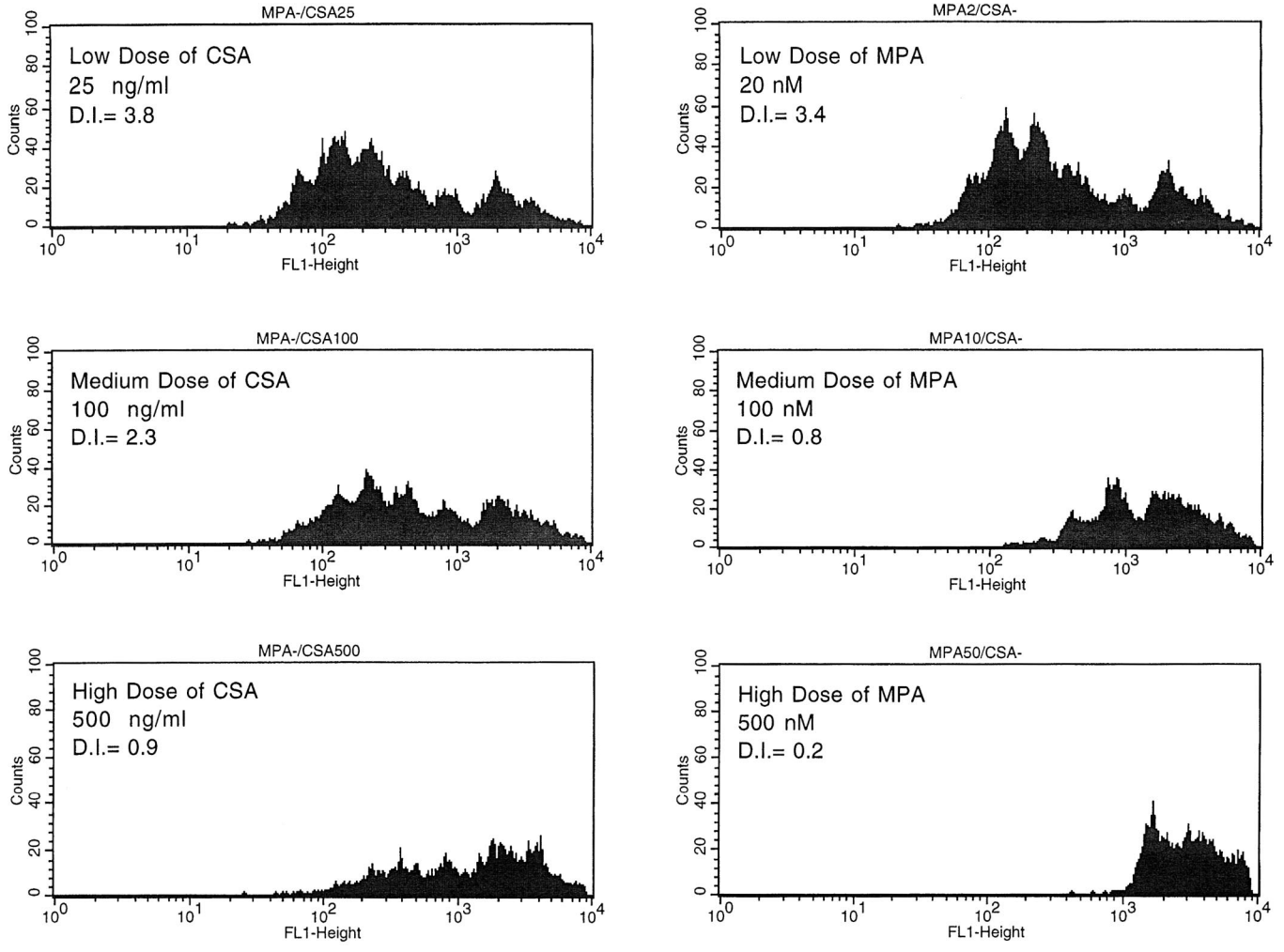

FIGURE 1. Effect of CsA or MPA in increasing doses on proliferative patterns measured by the CFSE enumeration of generations. Flow cytometry histograms represent numbers of cells versus fluorescence intensity. Peaks correspond to serially divided subsets with half the CFSE of the previous generation; subsequent generations move to the left (lower fluorescence). Note the tendency towards decreased number of generations with MPA versus decreased numbers of cells in each of several sequential generations found with CsA.

occurs soon after dosing could be sufficient to have an effect on the PBL that is sufficiently subtle that only the more discriminatory MLC result can detect it (as opposed to a polyclonal stimulator such as PHA). The effect might also be caused by some other long-term downstream effect (*7*) on patients during chronic administration. It is unlikely that the result caused by a confounding variable that affects immunocompetence, because we compared the groups for differences that would artifactually produce an effect of MMF. In particular, calcineurin inhibitor trough levels were measured (Table 3) and were not found to be an alternate explanation.

The IC results showing that MMF is more immunosuppressive than AZA (or neither AZA nor MMF) are consistent with findings from the randomized trials (*1*). Using this same immunocompetence approach, we previously showed that African Americans are less immunosuppressed (have a higher IC level) than others in the second and third years after transplantation, despite nominally identical immunosuppressive regimens (*14*), similar to a previous report of less

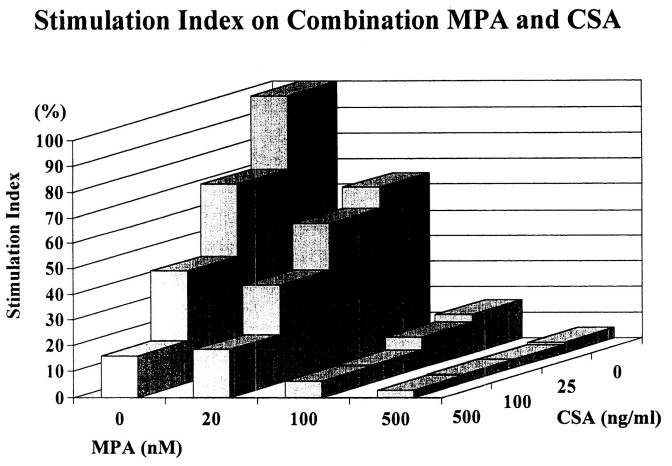

FIGURE 2. Strong additive effect of combining CsA and MPA on SEB-stimulated normal PBL. The CFSE-based division index was used to calculate a stimulation index as a percentage of normal response with no added immunosuppressant.

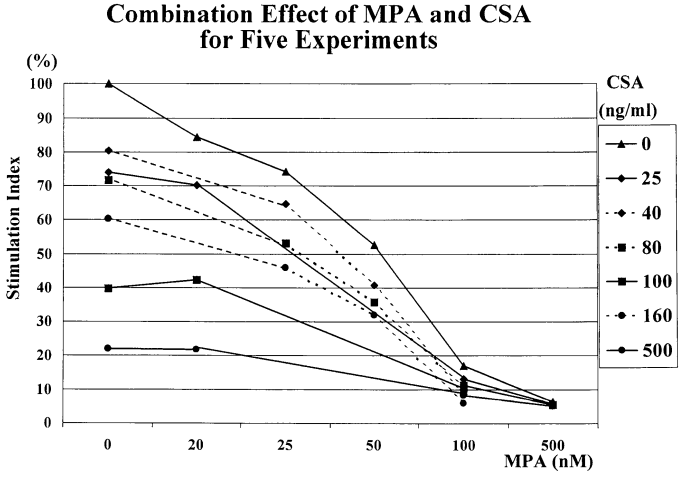

FIGURE 3. Summary of five separate experiments (one of which is shown in Figure 2) on CsA and MPA inhibition of stimulation by SEB of normal PBL. The stimulation is only suppressed below 20% with added MPA.

donor-specific hyporesponsiveness (*15*). These and the present results are consistent with the finding in the MMF (*16*) and sirolimus (*2*) trials that a higher dose was required for optimal immunosuppression in African-Americans. When MPA is present in moderate to high doses, as in Figure 1, there is an increasing trend to decreasing numbers of cell divisions allowed, as opposed to the effect of CsA, which allows proliferation into subsequent generations, but suppresses the numbers in each generation. This seems to relate to the capacity of PBL to divide in response to residual IL-2 produced in the presence of these levels of CsA, whereas the cell cycle blocking effect of MPA under these in vitro conditions is more complete. Moreover, we previously found that FasL expression for activation-induced cell death is prevented by CsA but not MPA (*17*). Apoptosis of dividing cells could partially explain the greater decrease in multiply dividing cells with MPA, compared with CsA, in Figure 1.

The data in Figures 2 and 3 showing the combined effects confirm the ability of MPA derived from MMF to add to the effect of CsA. We have not extended such experiments with the elegant median effect analysis used by Kahan and associates (*18*) to determine whether the effect is somewhat synergistic or inhibitory, but the effect seems to be essentially additive in the present data. CsA is bound to red blood cells and is considerably less active at a given concentration in blood than in TCM (*19*). MPA is water soluble and therefore it is mostly free in blood, not bound to cell lipids as is CsA. Although there is some protein binding (*20*), the effect on inosine monophosphate dehydrogenate, its principal target, was found to be the same in TCM as in blood (*5*). Although exact comparison of the steady-state exposure over several days in TCM with the clinical peak-to-trough situation for these drugs in vivo is inexact, the strong additive effect of MPA that is apparent with CsA levels of even 25 ng/ml in TCM (apparently corresponding to a CsA blood level of 250 ng or greater), is probably relevant to the effect in patients.

An IC level of approximately 12 in patients receiving MMF (Table 1) corresponds to an approximately 80% inhibition of the normal immune response (reduced from approximately 60; IC levels in PBL from normal volunteers of 40 to 80). Although the same effect could be achieved by increasing the dose of calcineurin inhibitor as opposed to adding MMF to the regimen, the need to add MMF to achieve these levels in vitro is confirmed in Figure 3, which shows that CsA without MMF does not achieve that degree of immunosuppression even at high doses. Although the mechanisms for the effect on immunocompetence in patients are not entirely defined, this quantitative comparison provides a rationale supporting the addition of MMF to a calcineurin inhibitor-based regimen, rather than increasing the dose of the calcineurin inhibitor, to optimize immunosuppression.

Patients immunosuppressed with AZA, in addition to prednisone and a calcineurin inhibitor, showed a trend toward higher IC levels than patients receiving neither AZA nor MMF. Although this was not statistically significant, it could represent clinical bias, in that patients discontinued from AZA or MMF may have had clinical evidence of more immunosuppression (e.g. infections) in response to equivalent chemical calcineurin inhibitor levels while on the additional agent. However, artifactual results caused by bias of patient selection are unlikely to explain the significantly different results with MMF, because the expected difference caused by this bias would be in the opposite direction, i.e., patients evincing over-immunosuppression clinically (and therefore low IC levels) would be expected to have had MMF discontinued, not instituted. We conclude that both groups that were not receiving MMF were in the relatively less-immunosuppressed IC range, and the addition of MMF to equivalent levels of calcineurin inhibitor produced a strong additional effect.

The approach leading to these experiments in posttransplantation immunocompetence analysis began with application of the observation that inhibition of T-cell responses by CsA required lower doses for indirect antigen presentation, than for direct antigen presentation (*21*). On this basis, organ recipients were characterized as responsive either to both direct and indirect antigen presentation (normal, more likely to have rejection), to neither (over-immunosuppressed, more likely to have infection), or, perhaps optimally, responsive to

direct, but not indirect, antigen presentation (*22–24*). We subsequently described the simplification used in the present study (*9*). These correlations show the potential for this type of assay to provide a surrogate for the "gold standard" clinical endpoints, of graft and patient survival. The alloimmune response seems to be more discriminatory than the response to PHA stimulation, although this was not tested formally. The results with MMF support the general principle that differences between patients are not solely caused by differences in calcineurin inhibitor levels, but also reflect other underlying endogenous or therapeutic influences on immunocompetence. Furthermore, this approach provides a rationale for comparing the immunosuppressive strength of drugs or drug regimens using groups that might not need to be as large and clinically homogeneous, or followed for as long, as required for trials using clinical endpoints. This type of approach could also be useful because transplantation results are sufficiently good that comparisons for equivalence of clinical endpoints are now accepted; a more informative comparison could include assessment of immunocompetence as a secondary endpoint. Unfortunately, the IC results described do not provide a sufficiently accurate and instantaneous measure to allow day-to-day individualization of patient regimens. Results from individual patients were often not stable over time (preliminary data, not shown). This is caused partly by the inherent variability of the isotopic uptake proliferation assay, and by changes in the patient's status or levels that may be misleading for an overall trend. As with drug levels (*4*), an isolated high or low value on a given day does not correlate tightly with infection or rejection at that point. Therefore, this approach is discriminatory only in distinguishing differences between groups.

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