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Immune responsiveness in renal transplant recipients: Mycophenolic acid severely depresses humoral immunity in vivo

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Immune responsiveness in renal transplant recipients: Mycophenolic acid severely depresses humoral immunity in vivo.

Background. Current immunosuppressive drug treatments for renal transplant recipients result in high one-year graft survival rates. Despite adequate suppression of the immune response directed to the allograft, the immune system remains able to cope with many infectious agents.

Methods. To define the influence of distinct immunosuppressive treatment protocols, primary and secondary cellular and humoral immune responses in groups of renal transplant recipients were studied: patient treated with prednisolone and cyclosporine A (P/CsA); with IgA CD3 monoclonal antibody as a rejection treatment superimposed on prednisolone and cyclosporine A (IgA CD3 mAb+P/CsA); and with prednisolone, cyclosporine A and mycophenolate mofetil (P/CsA/MMF).

Results. Primary in vitro proliferative responses to the protein antigen keyhole limpet hemocyanin (KLH) were not significantly disturbed in P/CsA treated patients, or in IgA CD3 mAb+P/CsA and P/CsA/MMF treated patients. In vitro proliferative responses to the recall antigen tetanus toxoid (TT) were similarly unaffected. Antigen-specific antibody responses to immunization with KLH and TT were not affected by treatment with P/CsA, or by IgA CD3 mAb+P/CsA, but were severely disturbed in patients treated with P/CsA/MMF. All patients displayed a profound inhibition of the delayed-type hypersensitivity skin reactivity to KLH and recall antigens. Nevertheless, in most patients with P/CsA treatment, T cell infiltrates were observed in skin biopsies from the site of KLH challenge, while expression of intercellular cell adhesion molecule-1 (ICAM-1) expression in challenged skin was significantly decreased in these patients. The balance between T helper 1 and T helper 2 cells was unaffected by immunosuppressive treatments during one year of follow-up.

Conclusions. Immunosuppressive drug treatment with P/CsA inhibits delayed-type hypersensitivity skin reactions to both primary and frequently encountered antigens. Histological studies indicate an effect on ICAM-1 expression, leaving the influx of $CD3^{pos}$ T cells unaffected. Administration of a 10-day course of IgA CD3 mAb does not add profound immunosuppressive effects on the measured parameters. In contrast, addition of treatment with MMF profoundly decreases both primary and secondary humoral immune responsiveness in vivo. Finally, no effect of the studied immunosuppressive drugs on Th1/Th2 balance in vivo was measured.

Key words: immunosuppression, kidney transplantation, graft survival, prednisolone, cyclosporine A, mycophenolate mofetil.

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We recently demonstrated that a substantial proportion of renal transplant recipients who received double immunosuppressive therapy consisting of prednisolone and cyclosporine A mounted readily detectable T helper cell responses to newly acquired human cytomegalovirus (hCMV) [1]. Moreover, both the anti-hCMV antibody response and the hCMV-specific CD8 responses rapidly followed the T helper cell response (Gamadia et al, unpublished observations). Recently, we found that protection against hCMV infections in immunosuppressed renal transplant recipients is achieved mainly through increased frequencies of activated, cytotoxic effector cells [2]. Thus, immunosuppressive drug treatment that is often sufficient to protect renal transplant recipients from rejection of the allograft, also allows for effective cellular and humoral immune responses against infections.

In a previous study, the influence of cyclosporine A monotherapy on primary and secondary cellular and humoral immune responses was investigated in renal transplant recipients [3, 4]. Nowadays, the immunosuppressive drug regimen administered to renal transplant recipients consists of two and more frequently three drugs, leading to better graft survival at the expense of more profound immunosuppression. In vitro, glucocorticoids affect the Th1 polarizing properties of dendritic

cells [5, 6]. The effects of glucocorticoids on human T cells are complex and depend, among other things, on the differentiation state of the T cells [7]. Mycophenolic acid (MMF) has been shown to reduce acute rejection episodes in renal transplant recipients [8–10]. Moreover, MMF was shown to reduce the risk of developing chronic allograft nephropathy, independent of the reduced incidence of acute cellular rejection [11].

We evaluated the cellular and humoral immune responses in three groups of renal transplant recipients who were under different immunosuppressive protocols. In renal transplant recipients and healthy control individuals, immunization protocols were performed and the effects of treatment consisting of prednisolone and cyclosporine A (P/CsA), IgA CD3 mAb as a rejection treatment in addition to prednisolone and cyclosporine A (IgA CD3 mAb+P/CsA) as well as prednisolone, cyclosporine A and mycophenolate mofetil (P/CsA/MMF) on primary and secondary, cellular and humoral immune responses were assessed in vivo as well as in vitro. In addition, after short-term ex vivo stimulation, the frequencies of Th1 and Th2 cells in peripheral blood from these patients were evaluated.

METHODS

Subjects

Twenty-one renal transplant recipients on basic immunosuppressive therapy consisting of prednisolone 10 mg per day orally and cyclosporine A microemulsions (Neoral®) orally in a dosage scheme adjusted to blood trough levels of about 150 ng/mL (P/CsA-group) were included into this study (median age at entry, 43 years old; range 21 to 71 years old; female/male ratio, 10/11). In addition, six renal transplant recipients receiving a 10 day course of CD3 directed monoclonal antibody (mAb) of mouse IgA subclass as a rejection treatment for biopsy-proven acute rejection episodes were investigated (IgA CD3 mAb+P/CsA-group; median age at entry, 47 years old; range 18 to 53 years old; female/male ratio, 2/4) [12]. IgA CD3 mAb was administered as 5 mg/day IV (bolus injection) preceded by promethazine 50 mg orally. On the first day of IgA CD3 mAb treatment, the patients received 500 mg of methyl prednisolone IV prior to IgA CD3 mAb administration. Moreover, seven renal transplant recipients on immunosuppressive drug therapy consisting of prednisolone, cyclosporine A, and mycophenolate mofetil entered this study (P/CsA/MMF-group; median age at entry, 50 years old; range 30 to 59 years old; female/male ratio, 1/6). In these patients, MMF was given in a dose of 1 gram b.i.d. orally. Time of entry into the study was the day of transplantation for the P/CsA group, the first day of rejection treatment for the IgA CD3 mAb+P/CsA group, and at least three months after initiation of MMF administration for the P/CsA/MMF group. Renal transplant recipients from the P/CsA group were immunized at three months after transplantation; patients from the IgA CD3 mAb+P/CsA group were immunized at three months after the initiation of rejection treatment; patients from the P/CsA/MMF group were immunized at least three months after initiation of the triple immunosuppressive drug regimen. At that time, no significant difference was found in renal transplant function between the different patient groups.

All blood samples were drawn just before intake of immunosuppressive drug medication. Finally, 10 healthy control volunteers were included into this study (C.I., median age at entry 47.5; range 38 to 57; female/male ratio 6/4). All patients and healthy control individuals consented to the study, and the study was approved by the local medical ethical committee.

Reagents

Keyhole limpet hemocyanin was prepared as described [13]. Tetanus toxoid for intramuscular injection (containing at least 40 International Units; WHO) was obtained from Pasteur Merieux MSD (Brussels, Belgium). For culture experiments, tetanus toxoid from the RIVM (Bilthoven, The Netherlands) was used in a final concentration of 19 limits of flocculation (Lf)/mL.

Immunization, blood sampling, skin tests and skin biopsies

One milligram of KLH was administered subcutaneously in the right arm. In the left arm, 1 mL of tetanus toxoid was administered in the deltoid muscle. Before as well as 14 days after immunization, blood was drawn to analyze proliferation, frequencies of Th1 and Th2 cells and antibody production. Fourteen days after immunization, intracutaneous KLH (100 μg) administration was performed in the gluteal region of the right leg. In addition, the CMI-multitest (intracutaneous administration of tetanus toxoid, *Streptococcus*, diphtheria, tuberculin, *Candida*, *Trichophyton* and *Proteus*; Pasteur Mérieux, Lyon, France) was applied at the frontal side of the antebrachium, according to the manufacturer's instructions. Forty-eight hours later, local induration at the skin test area was palpated, the borders of the induration were marked off and its diameters were measured in two perpendicular directions. Results are expressed as either mean diameters of the indurations (KLH) or number of indurations equal to or larger than 5 mm (CMI multitest). In addition, a 4 mm diameter skin biopsy of the KLH-challenge site was performed under local anesthesia. Tissue was snap-frozen in liquid nitrogen and stored at −80°C.

Proliferative responses of PBMC to KLH and tetanus toxoid

Peripheral blood mononuclear cells (PBMC) were cultured in six replicates as 50,000 PBMC per well in

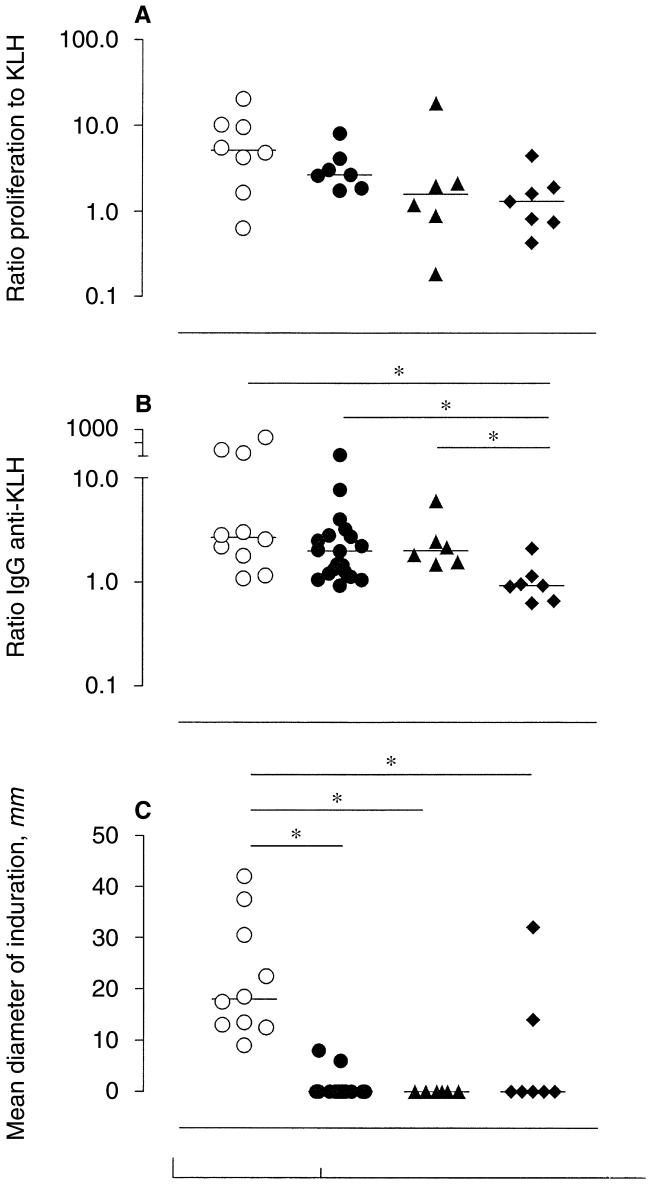

Fig. 1. Cellular and humoral immune responses and delayed-type hypersensitivity skin reactions to keyhole limpet hemocyanin (KLH). (*A*) Ratio of the proliferative response to KLH of values from 14 days after immunization to values from before immunization (Y-axis, ratio, log scale) in either healthy control individuals (C.I.; ○; median before 538.8, range 172 to 1312 cpm; median after 3707, range 384 to 6802 cpm), renal transplant recipients receiving prednisolone and cyclosporine A (P/CsA; ●; median before 291, range 25 to 909 cpm; median after 1096, range 101 to 1991 cpm), renal transplant recipients who had received a rejection treatment with IgA CD3 monoclonal antibody (mAb) 3 months before, plus basic immunosuppressive treatment consisting of prednisolone and cyclosporine A (IgA CD3 mAb+P/CsA; ▲; median before 165, range 32 to 4103 cpm; median after 598, range 38 to 2597 cpm); or renal transplant recipients receiving the triple immunosuppressive regimen consisting of prednisolone, cyclosporine A and mycophenolate mofetil (P/CsA/MMF; ◆; median before 382, range 49 to 10786 cpm; median after 500, range 93 to 4633 cpm). (*B*) Ratio of IgG anti-KLH production of values from 14 days after immunization to values from before immunization (Y-axis, ratio, log scale) in individuals as in panel A: C.I. (○; median before 399, range 136 to 6035; median after 1163, range278 to 1,630,000), P/CsA (●; median before 464, range203 to 2289; median after 763, range 201 to 5082), IgA CD3 mAb+P/CsA (▲; median before 1163, range 288 to 3558; median after 2083, range 1093 to 5534), and P/CsA/MMF (◆; median before 129, range 45 to 378; median after 191, range 36 to 353). (*C*) Mean diameter of induration of the skin area, 48 hours after intracutaneous skin testing with 100 μg KLH (Y-axis, millimeters) of the same individuals as in panel A. There were no statistically significant differences, $P = 0.055$ by the Kruskal-Wallis test, except for $*P < 0.05$ by the Mann-Whitney test.

Table 1. KLH-specific immune responses after immunization with KLH

	Proliferative response to KLH in vitro[b]			IgG response to KLH in vivo[c]		
Ratio[a]	≤1	>1		≤1	>1	
CI	1	7	8	0	10	10
P/CsA	0	7	7	1	18	19
IgA CD3 mAb + P/CsA	2	4	6	0	6	6
P/CsA/MMF	3	4	7	5	2	7
Total	6	22	28	6	36	42

Abbreviations are: CI, healthy control individuals; P/CsA, renal transplant recipients receiving immunosuppressive drug therapy consisting of prednisolone and cyclosporine A; IgA CD3 mAb+P/CsA, renal transplant recipients receiving a rejection treatment of IgA CD3 monoclonal antibody plus basic immunosuppressive drug therapy consisting of prednisolone and cyclosporine A; P/CsA/MMF, renal transplant recipients receiving immunosuppressive drug treatment consisting of prednisolone, cyclosporine A and mycophenolate mofetil.

[a] Ratio of values from 14 days after immunization to values from before immunization. The figures represent numbers of subjects within each treatment group, either with a ratio ≤1 or a ratio >1

[b] Chi-square test, not statistically significant

[c] Chi-square test, $P < 0.05$

Iscove's modified Dulbecco's medium (IMDM), supplemented with 5% human pooled serum, penicillin and streptomycin and β-mercapto-ethanol in a final volume of 170 μL per well. KLH was added as 38 μg/mL (final concentration), and tetanus toxoid was added as 19 Lf/mL (final concentration). Thirty-seven MBq/mL [methyl-^{3}H]thymidine (20 μL/well; Nycomed Amersham PLC, Buckinghamshire, UK) was added at day 6 to the KLH- or tetanus toxoid-stimulated cultures. The plates were incubated for eight hours at 37°C and 5% CO_2 in a humidified chamber. DNA was harvested on filter plates (UniFilter® GF/C™, Groningen, The Netherlands) using the Packard Harvester Filtermate 196 (Packard Instrument Company, Downers Grove, IL, USA) and dried. Subsequently, scintillation fluid (Packard Bioscience, Groningen, The Netherlands) was added and filter plates were sealed (Packard Plate Sealer Micromate 496). Incorporated ^{3}H-thymidine was measured with a liquid scintillation counter (Packard Topcount microplate scintillation counter). Counts per minute were used as the readout for proliferation.

Antibody response against KLH and against tetanus toxoid

IgG anti-KLH and IgG anti-tetanus toxoid were measured by enzyme-linked immunosorbent assay (ELISA) as described [3, 13].

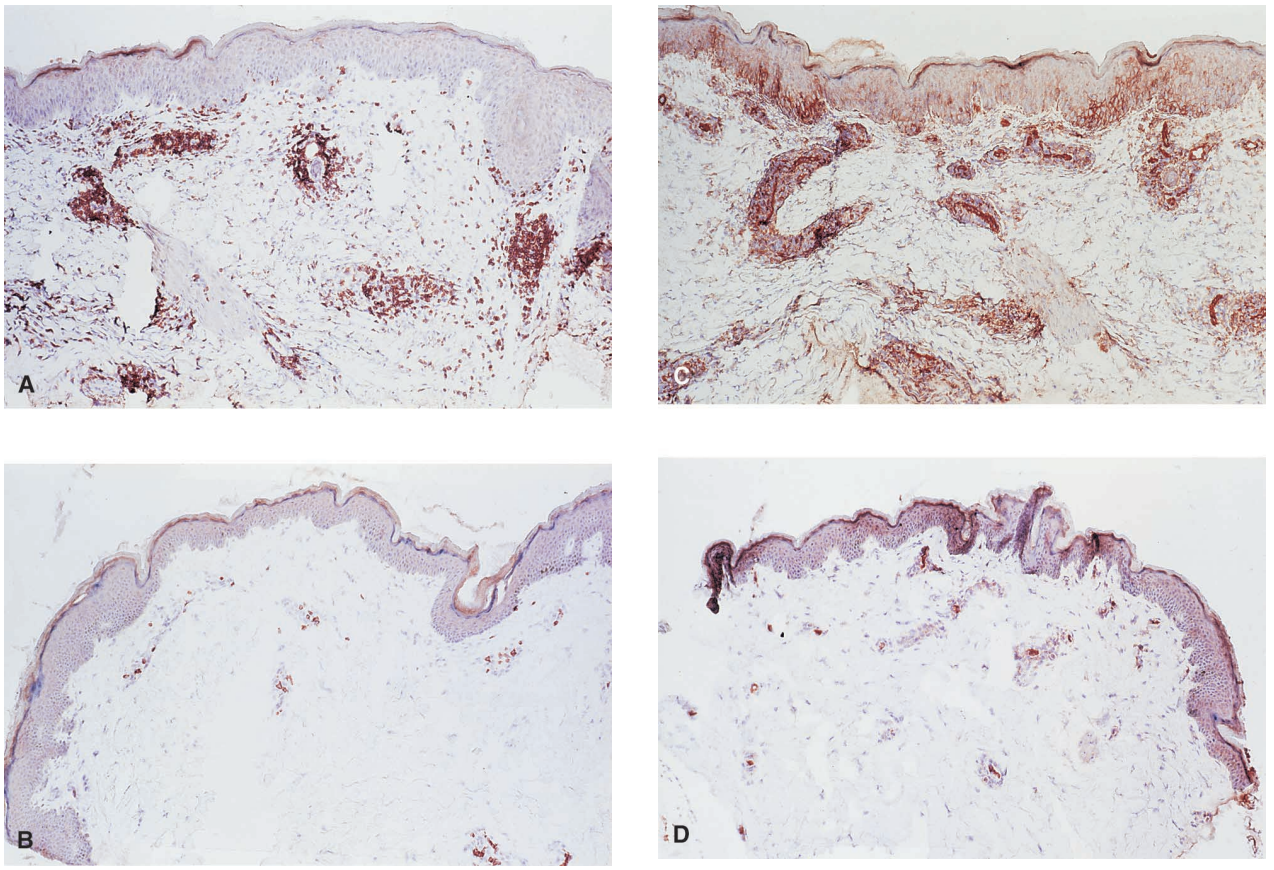

Fig. 2. Reduced intercellular adhesion molecule-1 (ICAM-1) expression in delayed-type hypersensitivity skin of patients treated with prednisolone/cyclosporine A. CD3 expression in challenged skin, 48 hours after intracutaneous challenge with 100 μg KLH from either a representative healthy control individual (*A*) or a representative renal transplant recipient receiving prednisolone and cyclosporine A (*B*). (*C* and *D*) ICAM-1 expression in challenged skin, 48 hours after intracutaneous challenge with 100 μg KLH from the same subjects as in panels A and B, respectively.

Flow cytometric determination of Th1 and Th2 subsets in peripheral blood

Frequencies of interferon-gamma (IFN-γ)- or interleukin 4 (IL-4)-producing T helper cells were determined according to the method described by Picker et al [14]. Briefly, PBMC were incubated for four hours in the presence of phorbol 12-myristate 13-acetate (PMA; 1 ng/mL final concentration; Sigma Aldrich Chemie GmbH, Deisenhofen, Germany), ionomycine (1 μg/mL final concentration; Sigma) and brefeldin A (10 μg/mL final concentration; Sigma) in IMDM, containing 10% human pooled serum, β mercapto-ethanol and antibiotics, at 37°C, in a humidified chamber.

Cells were transferred to FACS tubes, surface stained with CD4-APC (BD) and CD8-PerCP, fixed in 2 mL per tube FACS lysing solution (BD), permeabilized in 0.5 mL per tube FACS permeabilizing solution (BD) followed by intracellular staining with anti IFN-γ-FITC (BD) or anti IL-4-PE (BD). To control for cellular stimulation, intracellular CD69 expression was analyzed. Flow cytometric analyses were performed on a FACS Calibur flow cytometer. Lymphocytes were gated based on forward scatter and sideward scatter. These events were further gated based on either positive CD4 or brightly positive CD8 expression. Within these gated events, the percentages of cells expressing either IFN-γ or IL-4 were evaluated.

Immunohistochemical staining of skin biopsies

Frozen 6-μm tissue sections were harvested onto slides, fixed in cold acetone for 10 minutes, air dried and stored at −20°C until use. Sections were stained with either CD3 (OKT-3 ascites final dilution 1/1000), anti-vascular cell adhesion molecule-1 [VCAM-1; clone BBIG-V1 (4B2); 0.4 μg/mL final concentration; R&D Systems, Wiesbaden, Germany], anti-E-selectin [clone BBIG-E4 (5D11); 4 μg/mL final concentration; R&D Systems], anti-CLA (HECA-452), and anti-intercellular cell adhesion molecule-1 [ICAM-1; clone BBIG-I1(11C81) 0.4 μg/mL final concentration; R&D Systems] according to a previously described protocol [15]. Infiltration of $CD3^{pos}$ cells and the expression of E-selectin, ICAM-1 and VCAM-1 were scored semiquantitatively as described [16, 17]. CLA positive cells (that is, cells binding the

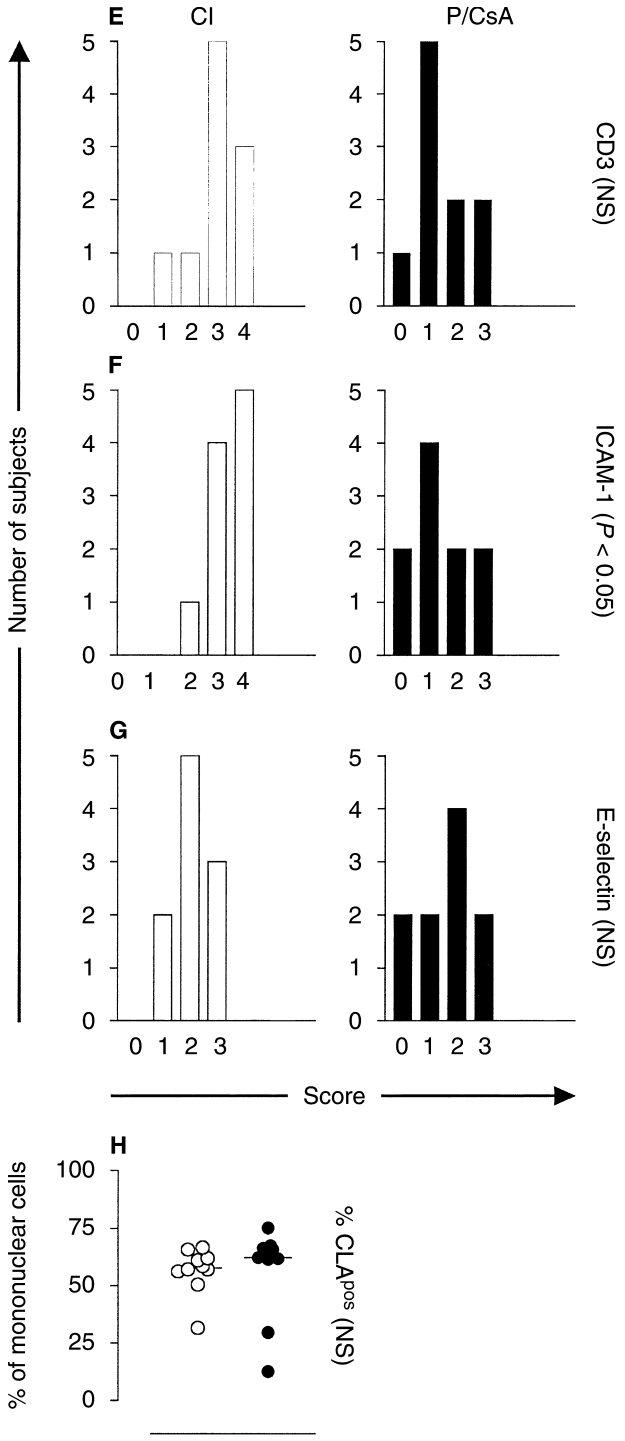

Fig. 2. (Continued). Frequency distributions of the semiquantitative score after immunohistochemical staining of KLH challenged skin biopsies with CD3 (*E*), anti-ICAM-1 (*F*) or anti-E-selectin (*G*) in skin biopsies at 48 hours after KLH challenge from either healthy control individuals (C.I., □) or renal transplant recipients receiving prednisolone and cyclosporine A (P/CsA, ■). (*H*) Frequencies of mononuclear cells expressing the cutaneous lymphocyte-associated antigen (CLA) as detected by the HECA-452 antibody (Y-axis) in either healthy control individuals (○) or renal transplant recipients receiving P/CsA (●). There were no statistically significant differences in panels E to G by the χ^2 test, and in panel H by the Mann-Whitney test; $P < 0.05$ in panel F.

HECA-452 antibody) were scored as a percentage of mononuclear cells.

Statistical analysis

Between-group differences of continuous variables were analyzed using Kruskal-Wallis test and Mann-Whitney test. Within group analyses of continuous variables were analyzed using the Wilcoxon's signed rank test. Chi-square testing of frequencies was performed. Tests were performed using SPSS 8.0.2. software program (SPSS, Inc., Chicago, IL, USA).

RESULTS

In vitro proliferative responses to KLH after in vivo immunization are relatively intact in immunosuppressed renal transplant recipients

Since most subjects are considered immunologically "naive" for KLH, this was used as a model antigen for the study of primary immune responses toward protein antigens. Proliferative responses to KLH were evaluated before and 14 days after subcutaneous immunization with 1 mg of KLH. In healthy individuals, the median ratio of these proliferative responses (that is, counts/min 14 days after immunization divided by counts/min before immunization) was 5.1 (range 0.6 to 20.3; Fig. 1A). In renal transplant recipients, the ratio of the in vitro proliferative response to KLH was not statistically different from the healthy control individuals (P/CsA group, median 2.7, range 1.7 to 8.1; IgA CD3 mAb+P/CsA group, median 1.6, range 0.2 to 18.1; P/CsA/MMF group, median 1.3, range 0.4 to 4.4). The number of subjects who responded to KLH immunization with a ratio of their proliferative response higher than 1 was calculated (Table 1). Although not statistically significant, the number of subjects with a ratio higher than 1, as well as the magnitude of the T helper cell response tended to be hampered by addition to the P/CsA treatment of IgA CD3 mAb or of MMF. We conclude that, on average, T helper cell responses to KLH can be induced by immunization to KLH in renal transplant recipients.

MMF addition to P/CsA drug therapy abrogates IgG anti-KLH production in most subjects

IgG anti-KLH responses were analyzed before and 14 days after immunization with 1 mg KLH. Healthy individuals demonstrated a median 2.4-fold rise in IgG anti-KLH antibody concentration (range 1.1 to 270.0; Fig. 1B). In renal transplant recipients receiving prednisolone and cyclosporine A, the ratio of IgG anti-KLH production was not affected (median ratio 2.0; range 0.9 to 12.4). In addition, rejection treatment with the CD3 directed mAb did not affect IgG anti-KLH production (median ratio 2.0, range 1.5 to 6.0). In contrast, in patients receiving triple immunosuppressive therapy (P/CsA/

MMF), no B cell response was observed in five out of seven patients (median ratio 0.9; range 0.6 to 2.1; Table 1). From these data, it may be inferred that the addition of MMF abrogates B cell responses toward a new protein antigen upon in vivo immunization, despite the presence of residual KLH-specific T helper cell responses.

Delayed-type hypersensitivity reaction to KLH is markedly inhibited under systemic treatment with P/CsA and P/CsA/MMF

Fourteen days after primary immunization with KLH, skin reactions to intracutaneous re-challenge with 100 μg of KLH were performed. Forty-eight hours thereafter, erythema and induration at the injection site were scored. Figure 1C demonstrates that vigorous skin reactions were observed in all tested healthy control individuals. In contrast, none of the renal transplant recipients receiving IgA CD3 mAb rejection treatment in addition to P/CsA, and only two of the patients from the P/CsA and P/CsA/MMF groups had indurations to intracutaneous KLH re-challenge (Fig. 1C).

Systemic immunosuppression with P/CsA reduces ICAM-1 expression in skin during delayed-type hypersensitivity reactions to KLH, while E-selectin expression is not affected

The observation of profoundly reduced skin reactions in the presence of residual proliferative T helper cell responses to the same antigens prompted us to investigate the influx of T cells into the skin test site. In most immunosuppressed individuals, perivascular infiltrates were observed in the dermis in response to the vaccination (Fig. 2B). Compared to healthy individuals, CD3 expression in the challenged skin area from renal transplant recipients was not significantly depressed (Fig. 2 A, B, and E). Expression of E-selectin on endothelium of challenged skin was similar in healthy individuals and renal transplant recipients (Fig. 2G), as well as the frequency of mononuclear cells expressing the E-selectin ligand CLA. In contrast, in comparison with healthy individuals, renal transplant recipients expressed lower levels of ICAM-1 in challenged skin (Fig. 2 C, D and F). No effect of any of the immunosuppressive drug treatments on VCAM-1 expression was observed (not shown).

Proliferative responses to tetanus toxoid are relatively intact in immunosuppressed renal transplant recipients

In addition to KLH vaccination, all subjects were simultaneously immunized with tetanus toxoid intramuscularly. Proliferative responses to tetanus toxoid were measured before and 14 days after immunization and are presented as a ratio of the proliferative response after immunization to the proliferative response before immunization. The tetanus toxoid-induced proliferative capacity of PBMC in response to immunization did not differ among the healthy individuals and the renal transplant recipients in three treatment groups. Median ratios of the proliferative responses were 2.9 (range 0.5 to 85.6) in the healthy control individuals, 3.9 (range 2.3 to 62.3) in the P/CsA group, 2.6 (range 0.2 to 13.3) in the IgA CD3 mAb+P/CsA group, and 1.9 (range 0.8 to 8.5) in the P/CsA/MMF groups (Fig. 3A). Importantly, after immunization, the majority of the patients from the IgA CD3 mAb+P/CsA and P/CsA/MMF groups demonstrated an enhanced proliferative response to tetanus toxoid (Table 2).

IgG anti-tetanus toxoid production cannot be boosted by immunization under systemic treatment with P/CsA/MMF

In most healthy control individuals, tetanus toxoid-specific IgG antibodies could be induced by the immunization protocol (median anti-TT ratio 8.7; range 1.0 to 67.7). In renal transplant recipients from both the P/CsA and the IgA CD3 mAb+P/CsA groups, IgG anti-tetanus toxoid antibodies also were induced after immunization with median ratios of 7.0 (range 1.1 to 56.2) and 2.8 (range 1.5 to 9.3), respectively (Fig. 3B). In contrast, in the renal transplant recipients treated with triple immunosuppressants, an antibody response to tetanus toxoid was completely absent in four out of six patients treated (Table 2). In the other two patients from this treatment group, the IgG anti-tetanus toxoid response was very small (complete P/CsA/MMF group, median ratio 0.94; range 0.7 to 1.3).

Systemic immunosuppression with P/CsA based medication reduced the skin reactions to recall antigens

To test whether the memory T cells that have been generated in the absence of immunosuppression are also defective in inducing delayed-type hypersensitivity reactions, intracutaneous administration were performed with tetanus toxoid and a panel of six additional antigens: *Proteus*, *Trichophyton*, *Candida*, *Streptococcus*, tuberculin and diphtheria. Figure 3C displays the frequency distributions of the number of significant skin reactions to any of these antigens in healthy control individuals and in the three groups of renal transplant recipients. The number of positive skin reactions was different among the groups. Since the different groups of immunosuppressed individuals had very similar low responses, the CMI multitest data from these groups of renal transplant recipients were pooled. Compared to healthy control individuals, the relative risk for these renal transplant recipients for scoring no positive skin reactions as opposed to 1 or more positive skin reactions was calculated as 3.0 (95% Confidence interval, 1.5 to 6.1).

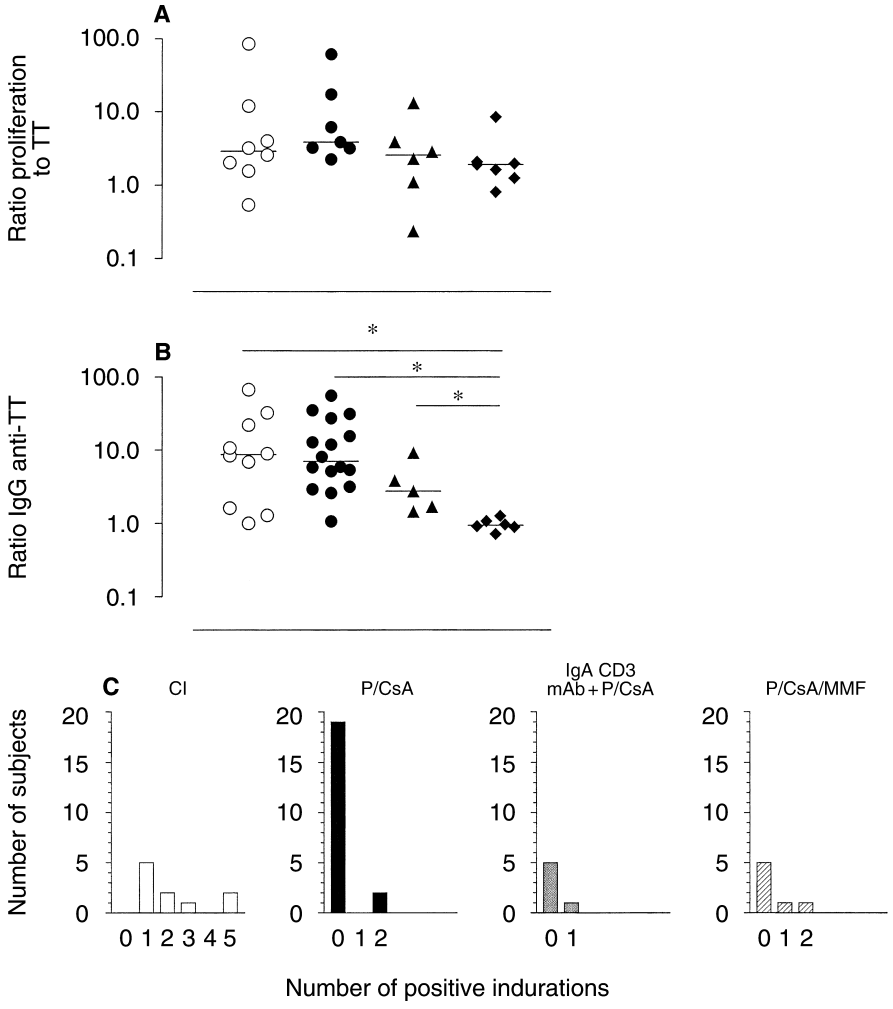

Fig. 3. Cellular and humoral immune responses to tetanus toxoid and delayed type hypersensitivity skin reactions to recall antigens. (*A*) Ratio of the proliferative response to tetanus toxoid (TT) of values from 14 days after immunization to values from before immunization (Y-axis, ratio, log scale) in healthy control individuals (C.I., ○; median before 1145, range 193 to 6398 cpm; median after 7901, range 152 to 16,510 cpm), renal transplant recipients receiving P/CsA (●; median before 856, range 21 to 5288 cpm; median after 3481, range 611 to 24,612 cpm), renal transplant recipients who had received a rejection treatment of IgA CD3 monoclonal antibody 3 months before, plus basic immunosuppressive treatment consisting of P/CsA (IgA CD3 mAb+P/CsA; ▲; median before 1044, range 103 to 121,891 cpm; median after 2549, range 235 to 14,236 cpm) and renal transplant recipients receiving "triple" immunosuppressive regimen consisting of P/CsA/MMF (◆; median before 880, range 35 to 31,456 cpm; median after 1867, range 73 to 39,350 cpm). (*B*) Ratio of IgG anti-tetanus toxoid (TT) production of values from 14 days after immunization to values from before immunization. (Y-axis, ratio, log scale) in the same subjects as in panel A: C.I. (○; median before 962, range 64 to 8828; median after 9048, range 2090 to 16,779), P/CsA (●; median before 295, range 14 to 13,827; median after 5851, range 83 to 17,735), IgA CD3 mAb+ P/CsA (▲; median before 215, range 39 to 5341, median after 1991, range 73 to 9069), and P/CsA/MMF (◆; median before 1356, range 1158 to 4313; median after 1404, range 837 to 4653). (*C*) Skin test to the recall antigens showing the frequency distributions of the number of positive indurations 48 hours after intracutaneous challenge with 7 different protein antigens (>5 mm mean diameter of induration, X-axis) versus the number of subjects (Y-axis) in healthy control individuals and three groups of immunosuppressed individuals as in panel A.

Table 2. Tetanus toxoid-specific immune responses after immunization with tetanus toxoid

	Proliferative response to tetanus toxoid in vitro[b]			IgG response to tetanus toxoid in vivo[c]		
Ratio[a]	≤1	>1		≤1	>1	
CI	1	7	8	1	9	10
P/CsA	0	7	7	0	16	16
IgA CD3 mAb + P/CsA	1	5	6	0	5	5
P/CsA/MMF	1	6	7	4	2	6
Total	3	25	28	5	32	37

Abbreviations are in Table 1.
[a] Ratio of values from 14 days after immunization to values from before immunization. The figures represent numbers of subjects within each treatment group, either with a ratio ≤1 or a ratio >1
[b] Chi-square test, not statistically significant
[c] Chi-square test, $P < 0.05$

Long-term systemic immunosuppression with P/CsA does not affect the ex vivo IFN-γ or IL-4 production profiles of T cells from peripheral blood

Delayed-type hypersensitivity reactions are generally considered to be T helper 1 type cell-mediated immune reactions. Moreover, T helper 1 cells are more likely to enter inflamed skin [18]. Therefore, we examined whether immunosuppressive regimens influence the Th1/Th2 balance in humans. In both healthy control individuals and renal transplant recipients, some variation in the frequencies of IFN-γ– and IL-4–producing T helper cells was found. However, these changes were not statistically significant during the one year follow-up (Fig. 4 A, B). Moreover, no effect on the ratio of IFN-γ–producing to IL-4–producing cells was observed in these patients (not shown.)

DISCUSSION

Although much is known on the effects of immunosuppressive drugs on human immune reactivity in vivo and

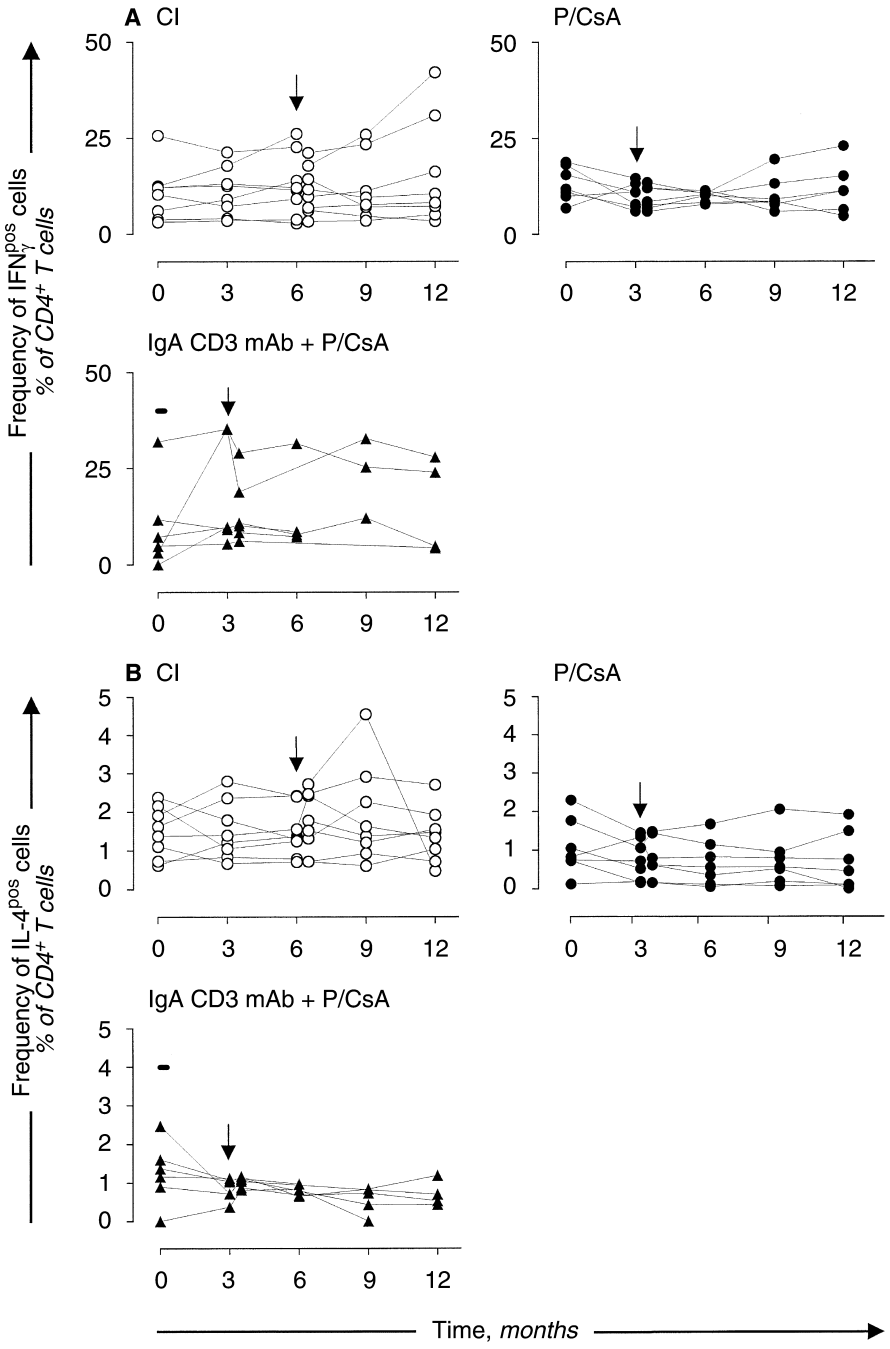

Fig. 4. Long-term systemic immunosuppression with prednisolone/cyclosporine A does not affect the ex vivo IFN-γ or IL-4 production profiles of T cells from peripheral blood. (*A*) Frequencies of IFN-γ producing cells within peripheral blood $CD4^{pos}$ T cells (Y-axis, % of $CD4^{pos}$ T cells) versus time after entry into the study (X-axis, months). Symbols are: (○) healthy control individuals (CI); (●) renal transplant recipients receiving P/CsA; (▲) renal transplant recipients receiving a rejection treatment of IgA CD3 mAb + immunosuppression treatment of P/CsA. The horizontal bar in the graph of IgA CD3 mAb+ P/CsA represents the time of the rejection treatment with IgA mouse anti-human CD3. Time of entry into the study is the day of transplantation for the P/CsA group and the first day of rejection treatment for the IgA CD3 mAb+P/CsA group. Small arrows indicate the time points of immunization with tetanus toxoid and KLH. (*B*) Frequencies of IL-4 producing cells within peripheral blood $CD4^{pos}$ T cells (Y-axis, % of $CD4^{pos}$ T cells) versus time after entry into the study (X-axis, months). The symbols are the same as in panel A.

in vitro, gaps remain in our understanding of their mechanism of action. Here, we studied both cellular and humoral immune responses in renal transplant recipients who were treated with different regimens of immunosuppressive drugs, after immunizations to elicit either a primary or a secondary immune response in vivo. This study shows that priming of T cells, specific for new protein antigens, still occurs in the presence of double immunosuppressive drug treatment consisting of prednisolone and cyclosporine A, and can even occur in the presence of triple immunosuppressive therapy with prednisolone, cyclosporine A and mycophenolate mofetil. In contrast, *effector* T cell responses are severely reduced: delayed-type hypersensitivity reactions were hampered, even if the antigen-specific T cells were educated in the absence of immunosuppression.

In a previous study, no effect of cyclosporine A monotherapy on the in vivo delayed-type hypersensitivity responses to recall antigens was found [3]. The discrepancy with our data may be explained by the difference in

pharmacological formulation of cyclosporine A. Neoral administration is known to result in higher drug level area under the curves as compared to Sandimmune [19]. In the above-mentioned study Sandimmune was administered, as opposed to Neoral, which was used in the present study. Moreover, the addition of 10 mg per day of prednisolone in our study may have exerted additional inhibitory effects on the delayed-type hypersensitivity reactions. A similar depressed skin reactivity was found in renal transplant recipients treated with prednisone in conjunction with azathioprine [20].

Histological studies on the delayed-type hypersensitivity reaction to KLH indicate an effect of P/CsA treatment on ICAM-1 expression, leaving the influx of T cells unaffected. Presumably, ICAM-1 expression is directly inhibited by the complex of the glucocorticoid receptor and prednisolone [21]. In addition, it may be speculated that tethering of lymphocytes on E-selectin in skin is unaffected by this drug regimen.

Immunosuppressive treatment with prednisolone and cyclosporine A did not significantly affect the frequencies of peripheral blood-derived Th1 or Th2 cells or the balance between these subsets. Differentiation of naive T cells into Th1 or Th2 cells is mainly driven by the cytokine environment in which naive to memory transition occurs, and is accompanied by switching on specific genetic programs while at the same time other genetic programs are switched off [22]. In vitro, treatment of dendritic cells with glucocorticoids impairs their Th1 skewing and enhances their Th2 skewing capacity [5, 6]. However, upon glucocorticoid treatment in vivo, many naive to memory transitions of T cells must occur before the peripheral blood Th2 deviation becomes apparent, thus requiring a more prolonged follow-up of patients. Alternatively, co-medication may have inhibited the Th2 skewing effects of prednisolone-treated dendritic cells.

Administration of a 10 day course of IgA CD3 monoclonal antibody does not add profound immunosuppressive effects on cellular or humoral immune responsiveness either in vivo or in vitro. In contrast, the addition of treatment with MMF to P/CsA profoundly decreases both primary and secondary humoral immune responsiveness in vivo. In response to KLH, three out of seven patients did not display a T helper cell response, which may explain in part the absence of the switch to IgG production. However, in response to immunization with tetanus toxoid, augmentation of the T helper cell responses were detectable in most subjects, whereas four out of six patients did not respond with increased antigen-specific IgG production. These results are in accordance with previously described impairments in antibody production under MMF treatment. In patients treated with P/CsA/MMF, significant impairment of antibody formation to the influenza A virus was observed [23]. In a comparison among patients with similar drug treatment protocols, antibody formation directed against equine-derived polyclonal anti-thymocyte globulin or against the murine CD3 monoclonal antibody OKT-3 was significantly impaired in MMF treated patients [24, 25]. This is in contrast to data obtained from previous studies, which showed no effect of the combination of prednisolone and azathioprine [20, 26], and a moderate effect on humoral immune responses in vivo with the addition of cyclosporine to that combination [27]. Taken together, our results show that addition of MMF to a drug regimen consisting of P/CsA acts directly on the humoral immune response while the T helper cell responses are relatively spared. These findings imply that vaccination of MMF-treated patients, aimed at eliciting an antibody response, is useless. Next, these findings—at least in part—may offer an explanation for the potential of MMF to reduce the occurrence of chronic allograft nephropathy, independent of the incidence of acute cellular rejection [11], Finally, the severely depressed antibody responses partially may explain the more severe course of CMV infection in MMF treated patients.

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