Choice of Antibody Immunotherapy Influences Cytomegalovirus Viremia in Simultaneous Pancreas-Kidney Transplant Recipients

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OBJECTIVE — Simultaneous pancreas-kidney (SPK) transplantation in type 1 diabetic patients requires immunotherapy against allo- and autoreactive T-cells. Cytomegalovirus (CMV) infection is a major cause for morbidity after transplantation and is possibly related to recurrent autoimmunity. In this study, we assessed the pattern of CMV viremia in SPK transplant recipients receiving either antithymocyte globulin (ATG) or anti-CD25 (daclizumab) immunosuppressive induction therapy.

RESEARCH DESIGN AND METHODS — We evaluated 36 SPK transplant recipients from a randomized cohort that received either ATG or daclizumab as induction therapy. Patients at risk for CMV infection received oral prophylactic ganciclovir therapy. The CMV DNA level in plasma was measured for at least 180 days using a quantitative real-time PCR. Recipient peripheral blood mononuclear cells were cross-sectionally HLA tetramer-stained for CMV-specific $CD8^+$ T-cells.

RESULTS — Positive CMV serostatus in donors was correlated with a higher incidence of CMV viremia than negative serostatus. In patients at risk, daclizumab induction therapy significantly prolonged CMV-free survival. CMV viremia occurred earlier and was more severe in patients with rejection episodes than in patients without rejection episodes. CMV-specific $CD8^+$ T-cell counts were significantly lower in patients developing CMV viremia than in those who did not.

CONCLUSIONS — Despite their comparable immunosuppressive potential, daclizumab is safer than ATG regarding CMV infection risk in SPK transplantation. ATG-treated rejection episodes are associated with earlier and more severe infection. Furthermore, high CMV-specific tetramer counts reflect antiviral immunity rather than concurrent viremia because they imply low viremic activity. These findings may prove valuable in the discussion on both safety of induction therapy and recurrent autoimmunity in SPK and islet transplantation.

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T ype 1 diabetes is an autoimmune disease characterized by T-cell–mediated destruction of insulin-producing β-cells (1). Simultaneous pancreas-kidney (SPK) transplantation is a well-established treatment option for type 1 diabetic patients with (or approaching) end-stage renal failure (2–5). The foremost challenge in SPK transplantation is to prevent alloreactivity as well as recurrence of autoimmunity against β-cells.

Recurrent autoimmunity and alloreactivity can be effectively reduced by immunosuppressive induction therapy (6,7), in combination with maintenance immune suppression (8). Polyclonal rabbit antithymocyte globulin (ATG) has been widely accepted as an effective form of induction therapy in pancreatic and islet transplantation (9). It depletes different subsets of the T-cell repertoire (10) and is also commonly used as rejection therapy for steroid-resistant rejection episodes (11). Unfortunately, it can cause a number of unwanted side effects, the most important being prolonged immunodeficiency and a subsequent increased risk of infections (12). In our institute, ATG Fresenius (ATG_F) (derived from a rabbit anti-Jurkat cell line) (13) is used for induction therapy, whereas ATG Merieux (ATG_M) (derived from a rabbit anti-human thymocyte line) (10) is used as rejection therapy in SPK transplantation.

More recently, monoclonal antibodies directed against specific T-cell surface molecules have been developed for clinical use for immunosuppression. One of these is anti-CD25 (daclizumab), a humanized IgG1 monoclonal antibody directed against the low-affinity interleukin-2 receptor α-chain (14). This antibody is supposed to solely affect activated T-cells (15). Its use in a clinical setting has increased in recent years (16–19). Similar immunosuppressive properties for both ATG and daclizumab in terms of preventing alloreactivity have been reported (14).

The most common opportunistic pathogen complicating the care of immunosuppressed solid organ transplant recipients is cytomegalovirus (CMV). It causes both direct effects, including tissue injury and clinical disease, and a variety of indirect effects, such as allograft rejection (20). Because protection from CMV infection is mainly dependent on cellular-mediated immunity (21), CMV-related problems are typically encountered primarily between 1 and 6 months after transplantation as a consequence of the

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Table 1—*Characteristics of the study population according to type of induction therapy*

Characteristic	Induction therapy		*P* value
	ATG	Daclizumab	
n	19	20	
Recipient age (years)	44.1 ± 8.3	40.3 ± 7.4	0.14
Recipient sex (male/female)	10/9	14/6	0.33
Diabetes duration (years)	29.2 ± 8.3	26.9 ± 6.5	0.35
Diabetic retinopathy (%)	100	100	1.00
Diabetic neuropathy (%)	88.9	70.0	0.24
Maintenance dialysis (%)	68.4	75.0	0.73
Time on dialysis (years)	2.2 ± 1.3	1.3 ± 0.7	0.03
HLA-A mismatch	1.4 ± 0.6	1.4 ± 0.6	0.88
HLA-B mismatch	1.4 ± 0.6	1.7 ± 0.5	0.12
HLA-DR mismatch	1.4 ± 0.6	1.2 ± 0.8	0.33
Donor age (years)	39.3 ± 8.4	32.2 ± 12.6	0.04
Donor sex (male/female)	10/9	11/9	1.00
Cold ischemic time pancreas (h)	12.0 ± 3.4	13.3 ± 3.4	0.23
Cold ischemic time kidney (h)	12.2 ± 4.1	13.6 ± 3.4	0.28
CMV IgG serostatus (%)			
D+/R+	16	20	1.00
D+/R−	26	20	0.72
D−/R+	11	20	0.66
D−/R−	47	40	0.89
Ganciclovir prophylaxis (days)	92 ± 18.6	107 ± 19.4	0.55
Acute rejection at 6 months (%)	36.8	45.0	0.85
Patient survival at 6/12/36 months (%)	100/100/100	95/95/90	0.11
Kidney graft survival at 6/12/36 months (%)	100/94.7/94.7	100/100/94.7	0.98
Pancreas graft survival at 6/12/36 months (%)	89.5/84.2/84.2	100/100/94.7	0.27

Data are means ±SD unless otherwise indicated.

intensity of immunosuppressive therapy in that period (20,22). In pancreas and islet transplant recipients, the possible role of CMV in the pathogenesis of type 1 diabetes is of additional interest. This mechanism is proposed to be mediated by an autoimmune reaction provoked by molecular mimicry between CMV and autoantigen GAD65 (23) and/or by impaired insulin release (24). As a consequence, adequate prevention and treatment of CMV infection can have additional value for the prevention of recurrent autoimmunity in recipients of SPK transplants as well as islet allografts.

The severity of an episode of CMV viremia is determined not only by its level but also by its duration (25,26). Both quantities can be combined by calculation of the area under the curve of viral load over time (25), a universal means of assessing the interrelationship among peak viral load, initial viral load, and rate of increase of viral load, parameters that have been described as independent risk factors for CMV disease (26). In this retrospective study, (re)activation of CMV, as measured by DNA load in plasma, was used as a safety parameter to evaluate the

efficacy of ATG versus daclizumab in SPK transplant recipients. Additionally, CMV-specific tetramer staining was used as a marker for antiviral immunity to further assess its role in CMV (re)activation in this patient group.

RESEARCH DESIGN AND
METHODS — Thirty-nine consecutive patients received SPK transplants at the Leiden University Medical Center between October 1999 and May 2002. In all patients duodenocystostomy was used for exocrine drainage of the pancreatic graft. Patients were randomly assigned to receive either a single dose of ATG_F (9 mg/kg) intraoperatively or five consecutive doses of daclizumab (1 mg/kg) administered in 2-week intervals, starting before transplantation. Relevant patient characteristics were comparable between groups. No differences in clinical outcome were observed between either induction protocols or occurrence of CMV viremia with regard to transplant survival, insulin independence, and cumulative numbers of rejection episodes (Table 1). From 36 patients, sufficient plasma samples could be collected for the CMV DNA

quantification used in this study. Two patients lost their pancreas graft at an early stage (3 and 4 days after transplantation, respectively) due to technical complications (venous graft thrombosis), and one patient died with functioning grafts 70 days after transplantation.

CMV serostatus of both donor and recipient was determined before transplantation. Patients at risk for CMV infection (based on donor [D]/receptor [R] serostatus: D+/R−, D+/R+, or D−/R+) received antiviral prophylaxis (1,000 mg ganciclovir orally three times daily for 3–4 months) starting within 14 days after transplantation. Maintenance immunosuppression in all patients consisted of cyclosporin A microemulsion (Neoral) with dose adjustments based on trough level monitoring, mycophenolate mofetil 1,000 mg twice per day, and prednisolone, which was gradually tapered to 10 mg/day by 3 months. Clinical rejection episodes were treated with high-dose intravenous steroids (Solu-Medrol 1,000 mg/day for 3 consecutive days). Recurrent or steroid-resistant rejection episodes were treated with a 10-day course of ATG_M (starting at 4 mg/kg), with sub-

sequent dosing guided by absolute lymphocyte counts in peripheral blood.

Sample collection, quantification of CMV DNA load in plasma, and determination of area under the viremia curve

EDTA plasma samples were collected at a frequency of about once a week for at least 180 days after transplantation and stored at −80°C until further processing. Nucleic acids were extracted from 0.2-ml plasma samples with the automated purification procedure of the MagNA Pure LC system (Roche Molecular Systems, Almere, the Netherlands) using the total nucleic acid isolation kit. Subsequently, CMV DNA quantification was performed using an internally controlled real-time quantitative CMV PCR. Sensitivity, specificity, and reproducibility of this assay were described in more detail previously (27). The course of CMV DNA load in plasma was documented longitudinally for each patient within 180 days of follow-up. Individual areas under the CMV viremia curves between 0 and 180 days after transplantation were calculated using the trapezoidal rule as described previously (25,28).

CMV tetramer staining

HLA-A2–restricted, CMV-specific phytoerythrin-labeled tetramers have been shown to be a valuable tool both for the detection of cytotoxic lymphocytes directed against CMV and potentially for diagnostic use (29). Blood from 16 HLA-A2–positive SPK transplant recipients was drawn and heparinized cross-sectionally 1–2 years after transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and washed in 0.9% phosphate-buffered saline. One million cells were incubated in PBS containing 0.1% FCS at room temperature for 30 min with a CMV-specific tetramer developed in our lab. Cells were washed and stained with fluorescein isothiocyanate–labeled anti-CD3 monoclonal antibody (BD Biosciences, Oxford, U.K.) and allophycocyanin-labeled anti-CD8 monoclonal antibody (BD Biosciences) for 20 min at 4°C. After washing, fluorescence was measured immediately using a FACScan (BD Biosciences). Cells were analyzed using CellQuest software (BD Biosciences), measuring the percentage of CMV-specific cells in the $CD3^+/CD8^+$ living cell population.

Statistical analysis

Two-tailed Fisher's exact test was used to determine differences between serologic groups. Disease-free survival data were presented as Kaplan-Meier survival curves with log-rank analysis and Cox proportional hazard regression to determine differences in survival. Differences in total viral load and T-cell counts were measured using nonparametric Mann-Whitney U tests, assuming non-Gaussian distribution.

RESULTS

Donor serology is related to CMV viremia

With regard to the pretransplantation CMV serostatus of donor and recipient among the 36 SPK transplant recipients, 9 were D+/R−, 7 were D+/R+, and 6 were D−/R+. CMV viremia was detected in 13 of 16 patients (81%) receiving seropositive donor organs, compared with 2 of 20 patients (10%) receiving seronegative donor organs ($P < 0.0001$) (Table 2). In contrast, no significant difference was seen for the incidence of CMV viremia in seropositive recipients versus seronegative recipients (7 of 13 and 8 of 23, respectively). Regarding the serologic groups at risk for CMV, D+/R− patients tended to develop more CMV viremia, whereas D−/R+ patients showed a trend toward a reduced risk of CMV viremia compared with the other at-risk groups.

CMV viremia occurs earlier with ATG_F induction therapy

The two different antibody induction therapies were compared with regard to the moment CMV viremia occurred. CMV viremia was defined as detection of two consecutive CMV DNA loads of more than $_{10}$log 2.7 (= 500) copies/ml plasma. In the total population, a trend was noted toward shorter CMV-free survival in the ATG_F-treated than in the daclizumab-treated patients ($P = 0.10$). Considering the population at risk for CMV infection ($n = 22$, D−/R− excluded), CMV-free survival was significantly shorter in the ATG_F group ($P = 0.04$) (Fig. 1*A*). Both patient groups were comparable regarding age, sex, incidence of rejection, and CMV serostatus. The median area under the viremia curve tended to be higher in the ATG_F group (Fig. 1*B*), indicating more severe CMV viremia.

In both groups, a number of patients received a 10-day course of ATG_M rejec-

Table 2—Impact of donor serology on incidence of CMV viremia

Patient group	CMV viremia	No CMV viremia	P value
D+	13	3	<0.0001
D−	2	18	
R+	7	6	0.31
R−	8	15	
D+/R−	8	1	0.16*
D+/R+	5	2	1.0*
D−/R+	2	4	0.054*
D−/R−	0	14	

*Compared with other groups at risk for CMV.

tion treatment, influencing CMV load (see rejection treatment results below). Excluding these patients from the induction group analysis did not influence patient group characteristics, and shorter CMV-free survival ($P = 0.01$) and more severe infection ($P = 0.05$) were seen in the ATG_F compared with the daclizumab group (Fig. 1*C* and *D*, respectively).

Rejection episodes treated with ATG_M are related to earlier and more severe CMV viremia episodes

Next, the correlation between rejection episodes treated with ATG_M and CMV viremia in the patient group at risk for CMV was assessed. One patient was excluded from this analysis because he received only Solu-Medrol as rejection treatment. Figure 1*E* shows the disease-free survival curves for patients receiving ATG_M rejection therapy versus patients without rejection episodes. A significantly shorter disease-free survival was seen in the ATG_M rejection therapy group ($P = 0.02$). In these patients, CMV viremia occurred after administration of rejection treatment, except for one patient in whom detection of CMV coincided with rejection treatment. Total viral load as measured by the area under the curve from 0 to 180 days was higher ($P = 0.01$) than in patients without rejection episodes (Fig. 1*F*).

Cox proportional hazard regression identified both ATG_M rejection therapy and ATG_F induction therapy as independent risk factors for shorter CMV-free survival (ATG_M hazard ratio 6.191 [95% CI 1.792–21.393], $P = 0.004$; ATG_F 5.447 [1.598–18.564], $P = 0.007$).
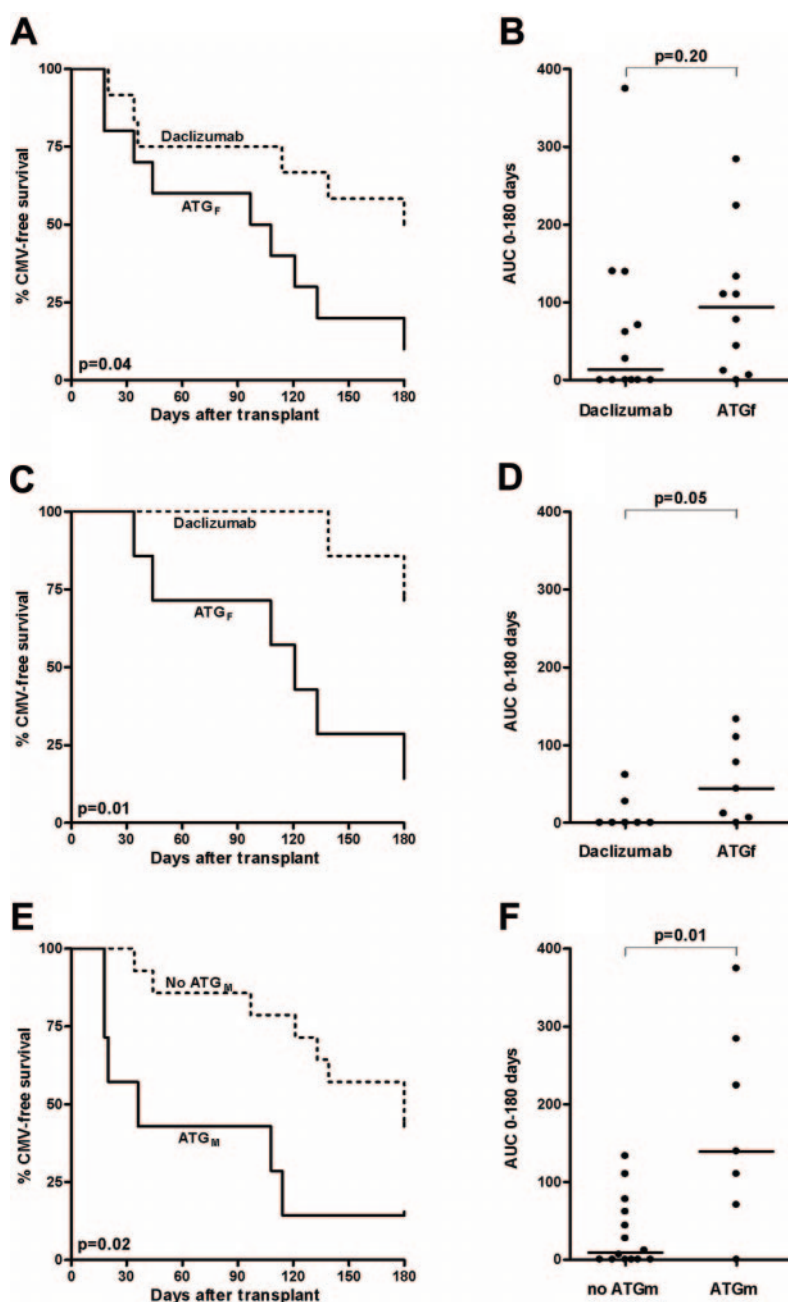
Figure 1—*Pattern of CMV viremia of SPK transplant recipients at risk for CMV. Shown are CMV-free survival (Kaplan-Meier) and total viral load over 180 days. A and B: Differences between ATG_F (n = 10) and daclizumab (n = 12) induction therapy for all patients at risk. First detection of CMV viremia (median + range in days): ATG_F 97 (18–180) and daclizumab 75 (20–180). C and D: Differences between ATG_F (n = 7) and daclizumab (n = 7) induction therapy for patients at risk who did not receive ATG_M rejection therapy. First detection of CMV viremia (median + range in days): ATG_F 114.5 (34–180) and daclizumab 159.5 (139–180). E and F: Differences between patients at risk who received ATG_M rejection therapy (n = 7) and patients who did not (n = 14), regardless of induction therapy. First detection of CMV viremia (median + range in days): ATG_M 28 (18–114) and no ATG_M 127 (20–180) (P = 0.03, Mann-Whitney U test). Median time between ATG_M rejection treatment and occurrence of CMV viremia was 9 (0–75) days.*

Tetramer staining shows fewer CMV-specific CD8+ T-cells in CMV-infected patients

To further investigate the mechanism underlying the pattern of CMV viremia in this patient group, HLA-A2–restricted CMV-specific tetramer fluorescence-activated cell sorter staining was performed on PBMCs of 16 HLA-A2+ patients. Several patients showed distinct populations of CMV-specific cells in the CD3+/CD8+ T-cell population. In the patients at risk, a trend was noted toward a higher percentage of CMV-specific CD8+ T-cells in the daclizumab-treated group compared with the ATG_F-treated group (Fig. 2A). When we stratified for CMV viremia, a significantly lower percentage of CMV-specific CD8+ T-cells was seen in patients who developed CMV viremia (P = 0.01) (Fig. 2B). To test the possibility of an ongoing infection at the time of blood withdrawal for isolation of PBMCs, the serum samples were analyzed for CMV viremia. No CMV DNA was detected in any of the samples (not shown). As a further control, PBMCs from HLA-A2+ patients not at risk for CMV infection (D−/R−) were stained, showing no CMV specificity at all (Fig. 2B).

CONCLUSIONS — In this study, it is shown that CMV viremia not only occurred earlier but was also more severe in SPK transplant recipients receiving single-shot ATG_F induction therapy compared with five-dose daclizumab and after rejection episodes treated with a 10-day course of ATG_M. Despite the limited number of patients included in the study, several potentially clinically relevant differences were found to be significant. In our study, we aimed to compare two different, but well-established, induction protocols. Although variations in timing and dosage conceivably affect the clinical outcome, this was not the subject of our studies because these variables are inherent to the protocols of choice.

The impact of donor pretransplant CMV serology clearly shows from these data. Patients receiving an organ from a seropositive donor had a much higher chance of developing CMV viremia than those receiving an organ from a seronegative donor. Remarkably, no direct influence of the patient's own pretransplant serology was noted. In the past, several studies have shown a higher risk for the development of CMV infection for patients who were de novo infected as a result of the transplantation (D+/R−) (17). In our patient group, only a trend in that direction was noted, conceivably due to the limited number of patients. Knowledge of pretransplant serology and subsequent adequate action could significantly decrease the risk of CMV infections. This is already being achieved by serological matching (positive organs to positive recipients and negative organs to negative recipients) (30). Unfortunately, donor
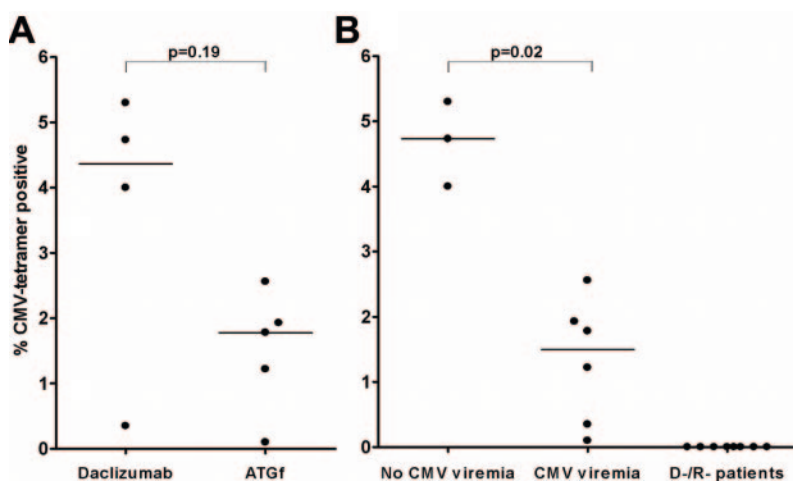
Figure 2— *Percentage of CMV-specific cells in the CD3+/CD8+ living cell population in HLA-A2+ SPK transplant recipients, stratified according to induction therapy (A) and the development of CMV viremia (B).*

shortage and limited ischemia times are restricting factors for the matching strategy. Another possible strategy would be to determine the immunosuppressive protocol individually for each patient based on CMV serology status.

Furthermore, this study stresses the need for careful monitoring of infections in patients treated with polyclonal ATG therapy. Antibody induction therapy for transplantation has become regular practice in recent years and in particular with SPK transplants (6). We conclude that antibody induction therapy with daclizumab (anti-CD25) is safer than antibody induction therapy with ATG_F regarding (re)activation of CMV in SPK transplant recipients because CMV viremia occurs later and the total viral load is lower. When patients receive ATG as rejection treatment, the effect on CMV viremia is even more pronounced. These findings are in accordance with findings in kidney transplant recipients (14) and can be explained by the proposed mechanisms through which both agents affect the immune system. Daclizumab treatment is said to affect activated T-cells only, thus leaving memory T-cell function relatively intact, whereas ATG profoundly depletes all T-cells, conceivably leading to a longer-lasting influence than with daclizumab (10,15). Nonetheless, in recent reports on nondepleting humanized anti-CD3 therapy in type 1 diabetes, it was suggested that modulation of T-cells can preserve β-cell function (31,32). The latter, however, was not the subject of our present studies.

Our findings are of importance because it is known that the consequences of CMV disease for morbidity and transplant survival are strongest in the first months after transplant. Furthermore, CMV dis-

ease indirectly affects transplant survival (33). In this study, however, none of the patients developed clinical CMV disease.

Tetramer staining for CMV-specific CD8+ T-cells gives additional insight into the mechanisms underlying the noted differences. The occasional high amounts of CMV-specific cells corresponded with absence of CMV viremia both in the first 6 months and at the time of staining rather than reflecting an ongoing infection. All three patients not developing CMV viremia (and with high CMV-specific T-cell counts) were treated with daclizumab, and, interestingly, the one patient developing CMV viremia in the daclizumab group had a low CMV-specific T-cell count. These findings suggest that having high CMV-specific tetramer counts is actually beneficial, rather than a surrogate for viremia, because they are correlated with low viremic activity after transplantation. In this respect, tetramer staining might become an important tool to prospectively identify patients at high risk for CMV infection in the future (29).

Although the number of patients limits definite conclusions, this study emphasizes the important role for cellular immunity in the prevention of CMV viremia after SPK transplantation and subsequently the impact antibody therapy has on the protective cytotoxic capacity of the immune system. With daclizumab induction therapy, this impact seems to be less vigorous than with ATG_F. Moreover, these results argue in favor of the use of daclizumab as induction therapy for pancreas and islet transplantation because of the reported potentiating effect of CMV on recurrent autoimmunity (23).

CMV disease in islet transplantation has not yet been studied extensively, but because recurrent autoimmunity may be

an important reason for the long-term loss of islet allografts (34), such studies are warranted. This recommendation also applies to trials in which immunosuppressive agents are used to try to halt type 1 diabetes early in the course of the disease. For pancreas-kidney transplantation, it can be concluded that the differences between daclizumab and ATG_F induction on CMV infection are relevant when choosing a certain induction or rejection therapy, considering that no difference in immunosuppressive potential has been noted.

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