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Diagnostic Usefulness of Cytomegalovirus (CMV)-Specific T Cell Immunity in Predicting CMV Infection after Kidney Transplantation: A Pilot Proof-of-Concept Study

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Background: Cytomegalovirus (CMV) is one of the most important opportunistic infections in transplant recipients. Currently sero-positivity for CMV IgG before solid organ transplantation is the laboratory test of choice for stratifying the risk of CMV reactivation after solid organ transplantation. Theoretically, CMV-specific cell-mediated immune responses before solid organ transplantation should further categorize patients as high or low risk of CMV development. We therefore evaluated the usefulness of the CMV-specific enzyme-linked immunospot (ELISPOT) assay in kidney transplant (KT) candidates for predicting the development of CMV infections after transplantation.

Materials and Methods: All adult CMV IgG (+) recipients admitted to the KT institute between March 2014 and June 2014 were enrolled, and CMV infections after KT were observed between March 2014 and December 2014. All patients underwent CMV pp65 and IE1-specific ELISPOT assays before transplantation. CMV infection was defined in the presence of CMV antigenemia, CMV syndrome, or tissue-invasive CMV disease. We used the data to select optimal cut-off values for pp65 and IE1, respectively, on ROC curves.

Results: A total of 69 transplant recipients involving 54 (78%) living-donor KT, 9 (13%) deceased-donor KT, 3 (4%) kidney-pancreas transplants, and 3 (4%) pancreas transplants were enrolled. Of the 69 patients, 27 (39%) developed CMV infections. There was no association between the IE1-specific ELISPOT assay and CMV infection. However, only 15 (31%) of the 48 patients with positive pp65-specific ELISPOT results (>10 spots/2.0 × 105 cells) developed CMV infections, whereas 12 (57%) of the 21 patients with negative pp65-specific ELISPOT results developed CMV infection ($P = 0.04$).

Conclusion: Negative pp65-specific ELISPOT assay results before transplantation appear to predict the subsequent development of CMV infections after transplantation in CMV IgG (+) KT recipients. Therefore, risk stratification of CMV IgG (+) recipients using the CMV-specific ELISPOT, together with preventive strategies, may further reduce CMV development.

Key Words: Cytomegalovirus; Enzyme-linked immunospot assay; Kidney transplantation

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Introduction

Cytomegalovirus (CMV) is one of the most important opportunistic infections in transplant recipients [1]. Currently sero-positivity for CMV IgG before solid organ transplantation is the laboratory test of choice for stratifying the risk of CMV reactivation after solid organ transplantation (SOT) [2]. It is known that D+/R (donor seropositive and recipient seronegative) has the highest risk of CMV infection after SOT, followed by D+/R+ and D-/R+. D-/R- has the least risk [1]. Experts recommend universal ganciclovir prophylaxis for the highest risk group [1]. Either universal prophylaxis or preemptive ganciclovir therapy based on monitoring for early CMV replication is recommended for the moderate risk groups [1], into which more than 95% of Korean SOT candidates fall [3]. Theoretically, CMV-specific cell-mediated immune responses before SOT would further categorize the moderate risk groups (D+/R+; D-/R+) into patients with higher or lower risk of CMV development after SOT. If that were the case, risk stratification of recipients using the CMV-specific enzyme-linked immunospot (ELISPOT) together with preventive strategies might further reduce CMV development. We therefore evaluated in kidney transplantation (KT) candidates the usefulness of the CMV-specific ELISPOT assay for predicting the development of CMV infections after KT. We report the results of a preliminary proof-of-concept study.

Materials and Methods

1. Study population

All patients admitted for transplantation to a renal transplant unit between March 2014 and June 2014 in a 2,700-bed, tertiary-care hospital in Seoul, South Korea, were prospectively screened. Tests for CMV IgG were performed in the KT recipients and donorss. Exclusion criteria were refusal of informed consent and pediatric renal transplant candidates (<16 years old). The universal oral valgaciclovir for 3 months was given only to the highest CMV risk group (D+/R-). CMV antigenemia assays were performed weekly during the first month, bi-weekly during the 1-3 months after KT, and then monthly to 6 months after KT. CMV antigenemia of >50 cells per 200,000 cells were indications for preemptive therapy. Conventional-dose ganciclovir (5 mg/kg twice daily) as preemptive therapy was given daily for at least 2 weeks and until patients were negative for CMV antigenemia. All individuals were informed of the nature of the study, and all participants provided written informed consent. This investigation was approved by the Institutional Review Board of our hospital.

2. The CMV antigenemia assay and CMV ELISPOT assay

The CMV antigenemia assay was performed as previously described [4]. EDTA-treated whole blood samples were fractionated by dextran sedimentation and lysis of erythrocytes. The granulocytes were then centrifuged to prepare a cytospin slide. The cells were then fixed with formaldehyde, sequentially immunostained by monoclonal antibodies C10/C11 (Clonab CMV; Biotest, Dreieich, Germany). Counts are expressed as positive cells per 200,000 leukocytes.

A peripheral venous blood sample (~8 mL) was collected from each patient for the CMV ELISPOT assay for T cells producing IFN-γ (*i.e.* T-track CMV, Regensburg, Germany). Briefly, peripheral blood mononuclear cells (PBMC) were immediately (within 30 minutes) separated and collected. The collected cells were resuspended at 2.0×10^6 cells/mL, placed (2.0×10^5 cells/well) in wells pre-coated with anti-human IFN-γ antibody. The samples were stimulated with phytohemagglutinin (positive control), pp65, IE1, and media only (negative control) and incubated for 18 hours. The resulting spots were counted with an automated microscope (ELiSpot 04 HR; Autoimmune Diagnostika GmbH, Strassberg, Germany). Background counts, obtained in negative control wells, were subtracted.

3. Assessment of outcomes

The primary outcome was the development of CMV infection. In this study, patients with CMV antigenemia or CMV disease were considered to have a CMV infection [2, 5, 6]. CMV antienemia was defined as CMV antigenemia identified by pp65 antigenemia, and CMV disease was defined as CMV syndrome or tissue-invasive CMV disease. CMV syndrome was defined as CMV antigenemia and at least one of the following: fever >38°C; new onset severe malaise; leucopenia in two successive measurements (WBC count of <3,500 cells/mm^3); atypical lymphocytes of >5%; thrombocytopenia of < 100,000/mm^3. Tissue-invasive CMV was defined as evidence of localized CMV infection (cells with CMV inclusions, in situ detection of CMV antigen by immnunohistochemistry, or DNA) in a biopsy or other appropriate specimen (*e.g.*, bronchoalveolar lavage, cerebral spinal fluid) and symptoms of organ dysfunction.

4. Statistical analysis

Since this study was a preliminary proof-of-concept study,

the sample size was not calculated. We planned to enroll over a 4-month period and monitor the development of CMV infection for an additional 6 months. For each of the tests used here to predict CMV infection namely the pp65- and IE1-specific ELISPOT assays, we examined receiving operator characteristic (ROC) curves that plotted sensitivity against the rate of false-positive results over a range of cut-off values [7]. We chose the optimal cut-off value as the point on each ROC curve farthest from the diagonal line that maximized the sum of sensitivity and specificity. Diagnostic performance was expressed in terms of sensitivity, specificity, positive predictive value, and negative predictive value. Categorical variables were compared using Pearson's Chi-square tests. All tests of significance were two-tailed and a *P*-value of less than 0.05 was considered to indicate statistical significance. Calculations were performed using the SPSS for Windows software package, version 14.0K (SPSS Inc., Chicago, IL, USA) and MedCalc software (MedCalc Software bvba, Mariakerke, Belgium).

Results

1. Patients characteristics

Figure 1 is a flow diagram covering all the patients admitted to our hospital for KT between March 2014 and June 2014, and presents the results of observations on the development

Table 1. Characteristics of transplant recipients

Patient characteristic	N = 69 (%)
Mean age, years (± SD)	46 ± 12
Male gender	46 (67)
Primary reason for transplant	
Glomerulonephritis	17 (25)
Hypertension	10 (15)
Diabetes mellitus	12 (17)
Unknown	19 (28)
Polycystic kidney disease	2 (3)
Others	9 (13)
Transplant type	
Living donor kidney	54 (79)
Deceased donor kidney	9 (13)
Pancreas and kidney	3 (4)
Pancreas alone	3 (4)
ABO-mismatch transplantation	18 (26)
Primary transplant induction therapy at transplantation	
Anti-IL2 receptor antibodies	57 (83)
Antithymocyte antibodies	6 (9)
Rituximab	22 (32)
CMV serostatus	
D+/R+	63 (91)
R+ (donor serology unknown)	5 (7)
D-/R+	1 (2)
CMV infection	
CMV antigenemia	27 (39)
CMV antigenemia > 50 CMV-positive cell/2,000,000 leukocytes	7 (10)
CMV syndrome	0
Tissue-invasive CMV	4 (6)

IL, interleukin; CMV, cytomegalovirus; D, donor; R, recipient.

of CMV infections after KT between March 2014 and December 2014. A total of 111 patients were initially enrolled in the study. Of these, 42 (38%) were ultimately excluded; 40 for refusal of informed consent and 2 pediatric patients. The remaining 69 patients were enrolled in the final analysis. Demographic data for the study patients are shown in Table 1.

2. Development of CMV infections and CMV ELISPOT

Of the 69 patients, 27 (39%) developed CMV infections after KT (Table 1). Of the 27 patients with CMV infections, 7 (10%) displayed significant levels of CMV antigenemia (>50 CMV-positive cells/200,000 cells, which was the threshold for ganciclovir preemptive therapy in our hospital). Of these 7 patients, 4 who had tissue-invasive CMV disease (all had CMV gastritis) and 1 without tissue-invasive CMV disease received ganciclovir therapy. The remaining 2 patients with >50 CMV antigenemia who did not received ganciclovir preemptive therapy recovered spontaneously without any complications.

On the basis of the ROC curves (Supplemental Fig. 1), we determined that the optimal cut-off values were >10 spots and

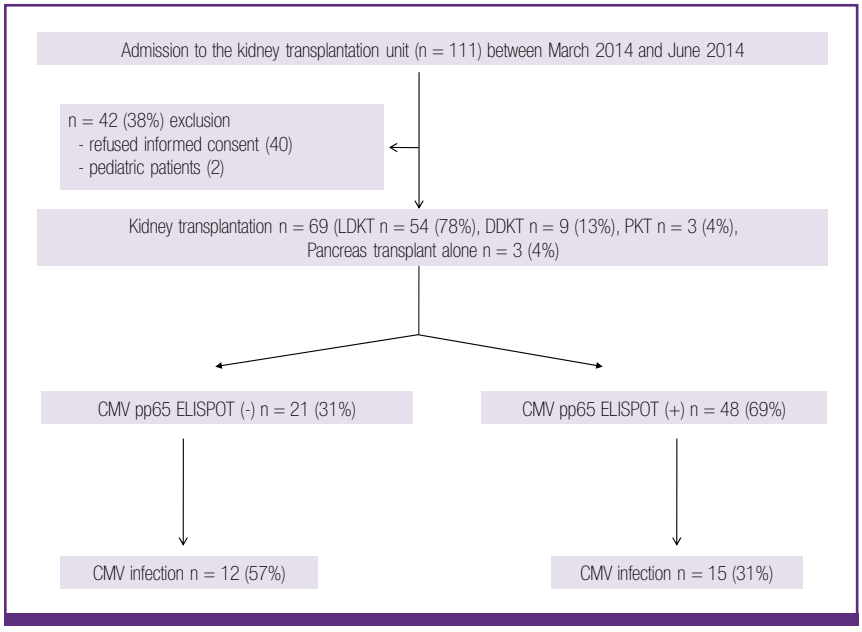

Figure 1. Flow chart of the study. LDKT, living-donor kidney transplantation; DDKT, deceased-donor kidney transplantation; PKT, pancreas-kidney transplantation. CMV, cytomegalovirus; ELISPOT, enzyme-linked immunospot.

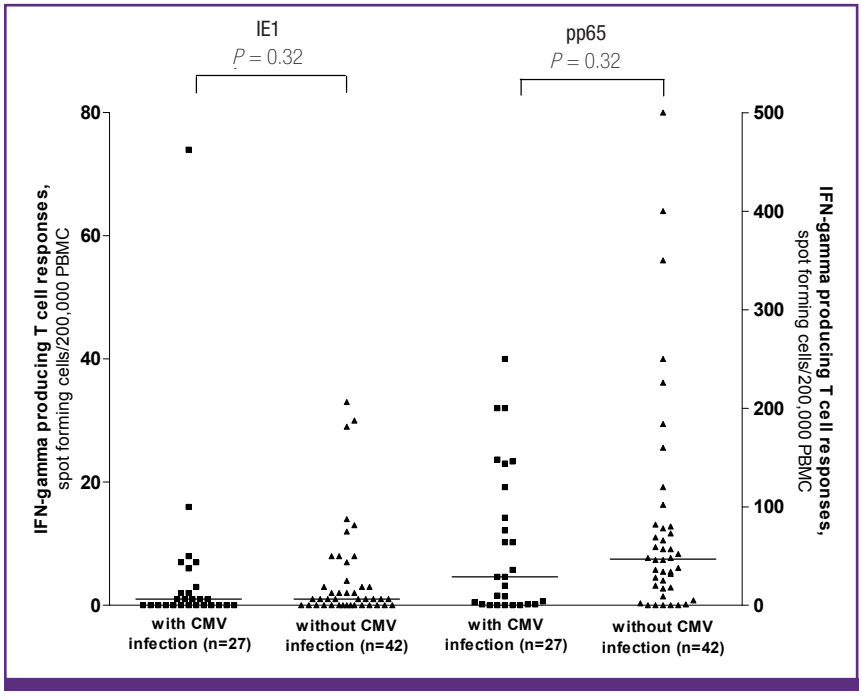

Figure 2. Response to IE1 and pp65 according to the presence of CMV infection after kidney transplantation. Bars indicate medians. The Mann-Whitney *U* test was used to compare the differences between groups. IFN, interferon; PBMC, peripheral blood mononuclear cells; CMV, cytomegalovirus.

> 0 spots, for the pp65 and IE1 ELISPOT assays, respectively. When we used these cut-offs, only 15 (31%) of the 48 patients with positive pp65-specific ELISPOT results (>10 spots/2.0 × 10^5 cells) developed CMV infection, whereas 12 (57%) of the 21 patients with negative pp65-specific ELISPOT results developed CMV infection (P = 0.04). The sensitivity, specificity, positive predictive value, and negative predictive value of the pp65 ELISPOT for predicting CMV infection were 44% (95% CI, 25-65), 79% (95% CI, 63-89), 57% (95% CI, 34-78), and 69% (95% CI, 54-81), respectively. However, there was not any statistical significant association between the IE1-specific ELISPOT assay and CMV infection; 14 (33%) of the 42 patients with positive IE1-specific ELISPOT results (>0 spots/2.0 × 10^5 cells) developed CMV infection, and 13 (48%) of the 27 patients with negative IE1-specific ELISPOT results developed CMV infection (P = 0.22). The sensitivity, specificity, positive predictive value, and negative predictive value of the IE1 ELISPOT for predicting CMV infection were 48% (95% CI, 29-68), 67% (95% CI, 50-80), 48% (95% CI, 29-68), and 67% (95% CI, 50-80), respectively. Responses to IE1 and pp65 according to the presence of CMV infection after kidney transplantation are shown in Figure 2.

Discussion

Current clinical immune assessment of the CMV risk of infection before transplantation relies exclusively on donor and recipient CMV IgG serostatus [2]. However, given that most adults in Korea are CMV seropostive (>95%) [3], the serostatus of most SOT recipients is D+/R+. Thus, most Korean SOT recipients are homogenously classified as at moderate risk of CMV infection. In this clinical situation, a futher risk strification strategy is needed to reduce CMV development after SOT. In this study we showed that negative pp65-specific ELISPOT assay results before transplantation were associated with the subsequent development of CMV infections after transplantation in KT CMV IgG (+) recipients. So, our data indicate that risk stratification of CMV IgG (+) recipients using the CMV-specific ELISPOT, together with preventive strategies, may further reduce CMV development.

As for the interferon-gamma releasing assays (IGRAs) for tuberculosis, two commercial IGRAs for CMV are available; one is the ELISA-based Quantiferon-CMV (Cellestis, Valencia, CA, USA) and the other, the ELISPOT-based T-track CMV. Numerous studies using Quantiferon CMV have been reported in SOT recipients. Kumar, et al. [9] showed for 108 SOT recipients that those with detectable Quantiferon CMV responses after universal CMV prophylaxis were at lower risk of CMV disease [8]. Manuel, et al. also reported similar results in 127 D+/R- SOT recipients using Quantiferon CMV results after universal CMV prophylaxis [9]. Although there are a few studies of the utility of in-house ELISPOT assays for predicting CMV infection after SOT, there are no published data on the commercially available ELISPOT assay. To the best of our knowledge, this is the first effort to evaluate the clinical utility of the commercial CMV ELISPOT assay in transplant recipients.

It is noteworthy that the pp65-specific but not the IE1-specific T cell response was associated with the development of CMV after SOT. Both pp65 and IE-1 are considered dominant T cell targets. Bunde et al. [10] demonstrated that only IE1-specific CD8 T cells were associated with protection against CMV diseases [10]. In contrast, a recent study demonstrated that pp65-specific immunity was crucial for controlling CMV dissemination in an animal model [11]. There are reports that the IE1-specific T cell response is associated with CMV development after transplantation SOT [2, 12] whereas others have shown a positive correlation between pp65 T cell responses and CMV viremia [13, 14]. The reasons for these differences are not clear. Possible explanations could be differences between the various cellular immune assays, and different outcome measure in response to variable CMV stimuli. Further studies are needed in this area.

This study has some limitations. It is a preliminary proof-of-concept study that provides the information necessary to be able to calculate the sample size needed for a confirmatory study. Hence the present sample size was not sufficient to meaure the true difference in CMV infection rate between positive IE1-specific ELISPOTs and negative IE1-specific ELISPOTs. Indeed, statistical power was only 40% in this cohort study. Therefore, we are planning to enroll 191 patients to confirm the association of IE1-specific ELISPOT with CMV infection at a 5% alpha and a power of 80%.

In conclusion, negative pp65-specific ELISPOT assay results before transplantation appear to predict the development of CMV infections after KT in CMV IgG (+) recipients with CMV IgG (+) donors. We believe that risk stratification using the CMV-specific ELISPOT assay, combined with preventive strategies, may further reduce CMV development.

Conflicts of Interest

No conflicts of interest.

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Supplementary material

Supplementary data including one figure can be found with this article online http://www.icjournal.org/src/sm/ic-47-105-s001.pdf.

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