MAJOR ARTICLE

Assessment of Cytomegalovirus-Specific Cell-Mediated Immunity for the Prediction of Cytomegalovirus Disease in High-Risk Solid-Organ Transplant Recipients: A Multicenter Cohort Study

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Background. Cytomegalovirus (CMV) disease remains an important problem in solid-organ transplant recipients, with the greatest risk among donor CMV-seropositive, recipient-seronegative (D^+/R^-) patients. CMV-specific cell-mediated immunity may be able to predict which patients will develop CMV disease.

Methods. We prospectively included D^+/R^- patients who received antiviral prophylaxis. We used the Quantiferon-CMV assay to measure interferon-γ levels following in vitro stimulation with CMV antigens. The test was performed at the end of prophylaxis and 1 and 2 months later. The primary outcome was the incidence of CMV disease at 12 months after transplant. We calculated positive and negative predictive values of the assay for protection from CMV disease.

Results. Overall, 28 of 127 (22%) patients developed CMV disease. Of 124 evaluable patients, 31 (25%) had a positive result, 81 (65.3%) had a negative result, and 12 (9.7%) had an indeterminate result (negative mitogen and CMV antigen) with the Quantiferon-CMV assay. At 12 months, patients with a positive result had a subsequent lower incidence of CMV disease than patients with a negative and an indeterminate result (6.4% vs 22.2% vs 58.3%, respectively; $P < .001$). Positive and negative predictive values of the assay for protection from CMV disease were 0.90 (95% confidence interval [CI], .74–.98) and 0.27 (95% CI, .18–.37), respectively.

Conclusions. This assay may be useful to predict if patients are at low, intermediate, or high risk for the development of subsequent CMV disease after prophylaxis.

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Keywords. Quantiferon-CMV; late-onset CMV disease; protection; antiviral prophylaxis.

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Cytomegalovirus (CMV) is the most common viral infection after solid-organ transplantation (SOT) and is associated with significant morbidity [1, 2]. CMV disease commonly presents as a viral syndrome with fever, malaise, and leukopenia. If left untreated, it can progress to severe tissue invasive disease. The SOT recipients at greatest risk for CMV disease are those who are seronegative recipients of organs from seropositive donors (D^+/R^-), representing 15%–25% of the transplant population [1]. Antiviral agents have proven to be useful in the prevention of CMV disease in SOT recipients, including the high-risk D^+/R^- patients [3]. Upon completion of prophylaxis, however, CMV disease occurs in 25%–35% of D^+/R^- SOT recipients within the first year after transplant [4, 5]. This is termed "late-onset" CMV disease and continues to be a significant problem in high-risk transplant recipients [6].

Accurate methods to predict the development of CMV disease following the completion of prophylaxis would have significant clinical benefit. Routinely available diagnostic tests are not reliable for predicting the risk of CMV disease in this setting. CMV RNA testing after prophylaxis in D^+/R^- patients has been shown to have poor predictive value for subsequent CMV disease [7, 8]. CMV serology testing posttransplant was also shown to be only of marginal use in predicting the risk of late-onset disease [9]. Cell-mediated immunity is known to be more important than humoral immunity in controlling CMV infection [10]. CMV infection elicits a strong virus-specific $CD4^+$ and $CD8^+$ T-cell response. Therefore, measuring an individual's cell-mediated immunity response to CMV may be a useful predictor of the risk of CMV infection or disease after prophylaxis [11–13]. However, many assays for cell-mediated immunity require laboratory expertise and specialized technology, which may not be widely available and often lack standardization [2].

The Quantiferon-CMV assay measures the interferon (IFN)–γ responses to a range of T-cell epitopes of CMV proteins including pp65, pp50, the glycoprotein gB, and the immediate early IE-1 antigen that cover a wide range of human leukocyte antigen (HLA) class I specificities [14]. The aim of the present study was to determine the utility of monitoring CMV-specific cell-mediated immunity to predict CMV disease after discontinuation of prophylaxis in an international cohort of D^+/R^- SOT recipients.

MATERIALS AND METHODS

Study Design and Participants

Participating transplant centers were identified through the American Society of Transplantation Infectious Diseases Community of Practice. Adult SOT recipients were eligible if they had a pretransplant D^+/R^- CMV serostatus and they were scheduled to receive antiviral prophylaxis with either ganciclovir or valganciclovir. Duration of prophylaxis was allowed to be between 3 and 6 months. Immunosuppression protocols were as per the center specific standard. We obtained approval from institutional review boards before initiation of enrollment at every site and all participants provided written informed consent.

Enrollment was carried out between April 2008 and March 2011. Patients were followed longitudinally to assess the development of CMV-specific cell-mediated immunity. Cell-mediated immunity was measured at 3 time points in each patient: at the time of prophylaxis discontinuation (ie, 3–6 months posttransplant), at 1 month postprophylaxis discontinuation, and at 2 months postprophylaxis discontinuation. Patients were followed for the development of CMV disease for 12 months.

Procedures

Cell-mediated immunity was determined using the Quantiferon-CMV assay (Cellestis Ltd, a QIAGEN company) [15]. One-milliliter aliquots of whole blood were collected into 3 heparinized tubes. One tube contained a mix of 22 CMV $CD8^+$ T-cell synthetic epitopes (CMV tube); one tube contained phytohemagglutinin (mitogen or positive control); and the third tube contained only heparin (no antigen or negative control). After collection, the 3 tubes were incubated overnight at 37°C. Following incubation, the tubes were centrifuged and plasma removed from each tube and placed in a plasma storage container. These containers were then frozen at −70°C (consistent with the manufacturer's recommendations regarding storage and processing), and IFN-γ measurement using an enzyme-linked immunosorbent assay (ELISA) was subsequently performed in batch testing in 3 centers (Cleveland, Ohio; Edmonton, Canada; and Leiden, the Netherlands). The assay was performed in a blinded manner by one technician in each center with experience in the use of the Quantiferon platform.

According to the manufacturer, a cutoff of 0.2 IU/mL of IFN-γ is used for defining positivity of the assay [15]. However, based on previous data in D^+/R^- patients, the a priori cut-point for defining positivity was set at 0.1 IU/mL of IFN-γ [16]. If the level was <0.1 IU/mL and the mitogen control was positive (≥0.5 IU/mL), the test was considered to be negative. Technically, if the level of IFN-γ in the CMV antigen tube is <0.1 IU/mL and in the mitogen tube is <0.5 IU/mL, the result is indeterminate. For the purposes of the analysis, negative and indeterminate results were also classified together as being nonreactive.

The primary study endpoint was the incidence of CMV disease within the 12 months after transplantation. We assessed the value of the assay for prediction of protection against CMV disease at the time of discontinuation of

prophylaxis and at subsequent time points. The definition of CMV disease was based on the criteria recommended by the American Society of Transplantation for use in clinical trials [17]. In brief, CMV viremia was defined by the detection of replicating CMV in blood by either quantitative nucleic acid testing or by the pp65 antigenemia assay. CMV disease was defined as evidence of CMV infection with compatible symptoms [17]. CMV disease was classified as tissue-invasive disease if there was evidence of localized CMV infection in a biopsy or another appropriate specimen, or as CMV syndrome if there was no such evidence.

Statistical Analysis

We calculated the sample size on the basis of the primary endpoint of a lower incidence of CMV disease in patients with a positive cell-mediated immune response. We assumed that the percent of patients with a positive cell-mediated immune response after prophylaxis was 30%. The overall CMV disease rate was estimated to be approximately 25%: 5% in patients with a positive cell-mediated immune response and 30% in patients with a negative cell-mediated immune response. With these assumptions, we estimated that 125 patients would be a sufficient sample size ($\alpha = .05$, power = 0.80, 2-tailed). Baseline characteristics of the patients were compared using the χ^2 test for categorical variables and the *t* test and the Mann-Whitney test for continuous variables, when appropriate. The performance of the assay for detecting protection from CMV disease was assessed by calculation of the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). We calculated these parameters at the first time point of testing and at multiple time points. Any samples collected after the occurrence of CMV disease were excluded for the analysis of predictive value. The incidence of CMV disease according to the result of the Quantiferon-CMV assay was calculated using Kaplan-Meier curves. The cutoffs of IFN-γ levels associated with the best sensitivity and specificity were analyzed by means of receiver operating characteristic (ROC) curve analysis. We calculated ROC curves with IFN-γ levels obtained in both CMV and mitogen tubes. Variables associated with an indeterminate result of the Quantiferon assay were investigated by univariate analysis using the χ^2 test. All analyses were performed using PASW Statistics 20 software (IBM Corporation), and a *P* value < .05 was considered to be statistically significant.

RESULTS

Study Population

Overall, 127 patients were included in the study (Table 1). Most patients were kidney (53.5%) or liver (21.2%) transplant recipients. Immunosuppressive and prophylactic regimens are summarized in Table 1. As per protocol, all patients received antiviral prophylaxis either with valganciclovir or oral ganciclovir; 13.4% of the patients received intravenous ganciclovir immediately posttransplant before switching to an oral agent. Median duration of antiviral therapy was 98 days (interquartile range [IQR], 92–178 days).

During the first year after transplant, 50 of 127 (39.3%) patients developed CMV viremia, and 28 of 127 (22.0%) patients developed CMV disease, at a median of 181 and 209 days posttransplant, respectively. Twenty-two patients had CMV viral syndrome and 6 patients had CMV tissue-invasive disease. No episode of CMV disease was observed while patients were on prophylaxis. No differences in terms of organ transplanted, immunosuppression, or antiviral prophylaxis were observed between patients with and without CMV disease.

Cell-Mediated Immunity

The Quantiferon-CMV assay was performed at all 3 time points in 90 patients (70.8%), at 2 time points in 23 patients (18.1%), and at 1 time point in 12 patients (9.4%). In 2 patients, the Quantiferon-CMV assay was not performed, and therefore these 2 patients were excluded from the analysis (Figure 1). As CMV disease induces a subsequent cell-mediated response, the result of the assays performed after development of CMV disease (n = 24) were not considered for the purpose of our analysis (ie, prediction of subsequent CMV disease). Of note, 1 patient developed CMV disease before the first assay was performed and was excluded from the analysis. At the first time-point testing, the Quantiferon-CMV assay was positive in 15 of 124 patients (12.1%), and nonreactive in 109 patients (87.9%; negative in 80 patients [64.5%] and indeterminate [negative for both mitogen and CMV antigen tube] in 29 patients [23.3%]) (Table 2). Subsequently, using any of the 3 time points of testing, the Quantiferon-CMV assay was positive in 31 of 124 patients (25%), and nonreactive in 93 (75%) patients (negative in 81 patients [65.3%] and indeterminate in 12 patients [9.7%]).

Predictive Value of Cell-Mediated Immunity

When analyzing the incidence of CMV disease according to the results of the Quantiferon-CMV assay at multiple time points, patients with a positive result of the assay had a lower subsequent incidence of CMV disease (6.4%) than patients with a negative (22.2%) and an indeterminate (58.3%) result ($P < .001$; Figure 2*A*). When classifying both negative and indeterminate results as nonreactive, the incidence of subsequent CMV disease was 6.4% vs 26.8% ($P = .02$; Figure 2*B*). The performance of the assay for predicting protection against CMV disease is shown in Table 3. Of note, sensitivity of the assay was low (0.14; 95% confidence interval [CI], .08–.23)

Table 1. Baseline Characteristics of the Patients Included in the Study

	Total (N = 127)	CMV Disease (n = 28)	No CMV Disease (n = 99)	*P* Value
Age, y, mean (SD)	50.0 (12.9)	51.4 (13.9)	49.6 (12.8)	.66
Sex, M/F, No.	87/40	18/10	69/30	.58
Type of transplant				.09
Kidney	68 (53.5%)	11 (39.3%)	57 (57.6%)	
Kidney-pancreas	10 (7.8%)	2 (7.1%)	8 (8.1%)	
Liver	27 (21.2%)	7 (25.0%)	20 (20.2%)	
Lung	14 (11.0%)	5 (17.8%)	9 (9.1%)	
Heart	4 (3.1%)	1 (3.6%)	3 (3.0%)	
Other[a]	4 (3.1%)	2 (7.1%)	2 (2.0%)	
Antiviral prophylaxis[b]				
Intravenous ganciclovir	17 (13.4%)	3 (10.7%)	14 (14.1%)	.57
Valganciclovir	122 (96.1%)	28 (100%)	94 (94.9%)	.22
Oral ganciclovir	9 (7.1%)	2 (7.1%)	7 (7.1%)	.99
Duration of prophylaxis, d, median (IQR)	98 (92–178)	124 (98–180)	98 (91–178)	.2
Induction therapy				.31
None	17 (13.4%)	4 (14.3%)	13 (13.1%)	
Basiliximab	60 (47.2%)	10 (35.7%)	50 (50.5%)	
Thymoglobulin	47 (37.0%)	14 (50.0%)	33 (33.3%)	
Alemtuzumab	3 (2.4%)	0 (0%)	3 (3.0%)	
Maintenance				
Steroids	101 (79.5%)	23 (82.1%)	78 (78.8%)	.7
Tacrolimus	113 (89.0%)	24 (85.7%)	89 (90.0%)	.53
Cyclosporin	9 (7.1%)	4 (14.3%)	5 (5.0%)	.1
MMF/MPA	103 (81.1%)	22 (78.6%)	81 (82.0%)	.7
Azathioprine	7 (5.5%)	1 (3.6%)	6 (6.1%)	.57
mTOR inhibitors	8 (6.3%)	3 (10.7%)	5 (5.0%)	.3
Other	3 (2.4%)	1 (3.6%)	2 (2.0%)	.63

Data are No. (%) unless otherwise specified.

Abbreviations: CMV, cytomegalovirus; IQR, interquartile range; MMF, mycophenolate mofetil; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin.

[a] Two small bowel and 2 kidney and liver transplant recipients.

[b] Seventeen patients received initial intravenous ganciclovir prophylaxis; 4 patients received both valganciclovir and oral ganciclovir.

when using the result of the first assay, but increased using multiple time points of testing (0.30; 95% CI, .21–.40). The positive predictive value of the assay (ie, protection against CMV disease in patients with a positive result of the assay) remained high irrespective of the time point used. Incidence of CMV viremia (either asymptomatic or in patients with CMV disease) was not different in patients with a positive or negative result of the Quantiferon-CMV assay but was increased in patients with an indeterminate result (36%, 31.7%, and 72.7% respectively; $P = .013$).

We compared IFN-γ levels of the CMV tube and the mitogen tube in patients with and without CMV disease (Figure 3). There was a trend toward higher IFN-γ levels in patients without CMV disease in both the CMV and mitogen tubes. For the CMV tube, median IFN-γ levels were 0.02 IU/mL (IQR, 0–0.16) in patients without CMV disease and 0.005 IU/mL (IQR, 0–0.05) in patients with subsequent CMV disease ($P = .06$). For the mitogen tube, median IFN-γ levels were 9.65 IU/mL (IQR, 3.63–9.90) in patients without CMV disease and 1.17 IU/mL (IQR, 0.34–9.95) in patients with subsequent CMV disease ($P = .06$). Based on ROC curve analysis, levels of ≥0.08 IU/mL of IFN-γ in the CMV tube have a 32% sensitivity and 93% specificity for subsequent protection for CMV disease. In the mitogen tube, levels of ≥2.2 IU/mL of IFN-γ have an 83% sensitivity and a 59% specificity.

As patients with an indeterminate result of the Quantiferon-CMV assay had the highest incidence of CMV disease, we looked for variables associated with an indeterminate result. Patients who had received thymoglobulin for induction had a higher rate of indeterminate results (9/47 [19.1%] vs 3/80 [3.7%]; $P = .009$). Patients on prednisone had a lower rate of

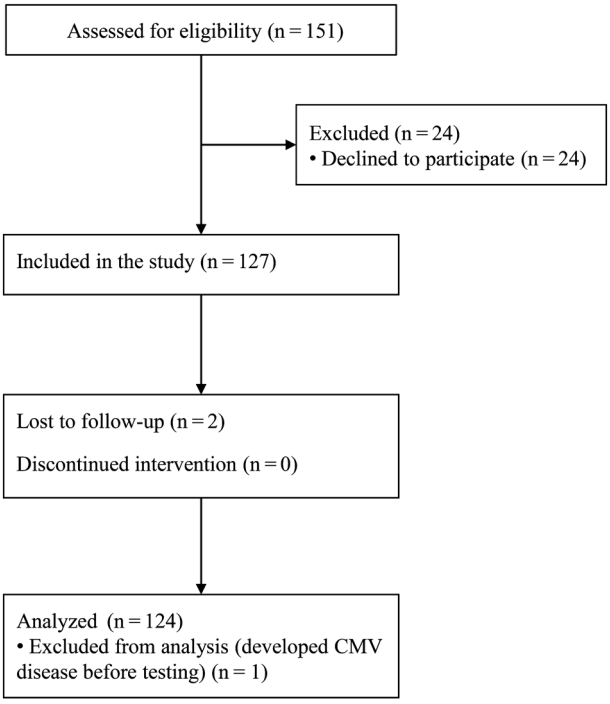

Figure 1. Flow diagram of the study. Abbreviation: CMV, cytomegalovirus.

indeterminate results (6/95 [6.3%] vs 6/20 [30%]; $P = .006$). No other immunosuppressive drug influenced the response of the Quantiferon-CMV assay.

DISCUSSION

In this multicenter study, we evaluated the predictive value of CMV cell-mediated immunity testing using the Quantiferon-CMV assay in a common clinical setting posttransplant: predicting the development of CMV disease in high-risk D^+/R^- patients after discontinuation of antiviral prophylaxis. Patients with a positive Quantiferon-CMV result had a significantly lower incidence of subsequent CMV disease; patients with a negative assay had an intermediate risk, and those with an indeterminate result had a high risk. Therefore this test would likely be useful in the clinical setting to stratify the risk of CMV disease after discontinuation of prophylaxis. Given the costs and potential toxicity of antiviral drugs, the assay may help guide the duration of prophylaxis; once cell-mediated immunity is detected, the antiviral drug could likely be safely discontinued. Patients with a negative assay and especially those with an indeterminate result would likely benefit from either more prolonged prophylaxis or closer monitoring.

Table 2. Results of the Quantiferon-Cytomegalovirus Assay at Each Time Point

	First Sample (n = 124)	Second Sample (n = 107)	Third Sample (n = 73)
Positive	15 (12.1%)	21 (19.6%)	19 (26.0%)
Negative	80 (64.5%)	64 (59.8%)	43 (58.9%)
Indeterminate	29 (23.3%)	22 (20.6%)	11 (15.1%)

Samples collected after the development of CMV disease were excluded.

The clinical experience with the use of the Quantiferon-CMV assay to predict the development of CMV disease after transplant is limited (reviewed in [14]). This is the first study to evaluate the predictive clinical utility of this assay in a large cohort of exclusively D^+/R^- SOT recipients, who are at the highest risk for the development of late-onset CMV disease. We observed that 24% of patients developed a cell-mediated immunity against CMV in the weeks following the discontinuation of prophylaxis. In a previous single-center study that included various serogroups of transplant recipients (including 35 D^+/R^- patients), patients with a detectable IFN-γ response subsequently had a lower incidence of CMV disease as compared to patients with a negative response (5.3% vs 22.9%; $P = .038$) [16]. In D^+/R^- patients, 26.8% had a detectable cell-mediated response at the time of discontinuation of prophylaxis; these patients had a trend toward lower incidence of CMV disease (10% vs 40% in patients with a nonreactive result). Likewise, in a recent study involving 67 lung transplant recipients [18], patients with a positive Quantiferon-CMV assay had lower incidence of subsequent CMV viremia (25% vs 72% in patients with a negative result), and all 4 patients who eventually developed CMV disease had a previous undetectable cell-mediated immune response [18]. One of the limitations of the Quantiferon assay in transplant patients appears to be a low sensitivity, and so even lower cutoffs for defining a positive response may be appropriate [19, 20]. For example, with a cutoff of 0.08 UI/mL of IFN-γ, we showed a slightly higher sensitivity without losing specificity. However, the results of our study are only applicable to D^+/R^- patients. Test performance including sensitivity and predictive value may be different in seropositive patients.

In our study, patients at the highest risk for the development of late-onset CMV disease were those with an indeterminate Quantiferon-CMV assay result in which both mitogen and CMV antigen responses are absent. This likely reflects a high net state of immunosuppression. Patients who had received thymoglobulin, a polyclonal depleting antibody against T cells, had higher rates of indeterminate results. The incidence of indeterminate results decreased with subsequent testing, and it was similar to that reported in previous studies (ranging from approximately 20% to 30%) [18, 19, 21–23]. Therefore, patients with indeterminate results of the

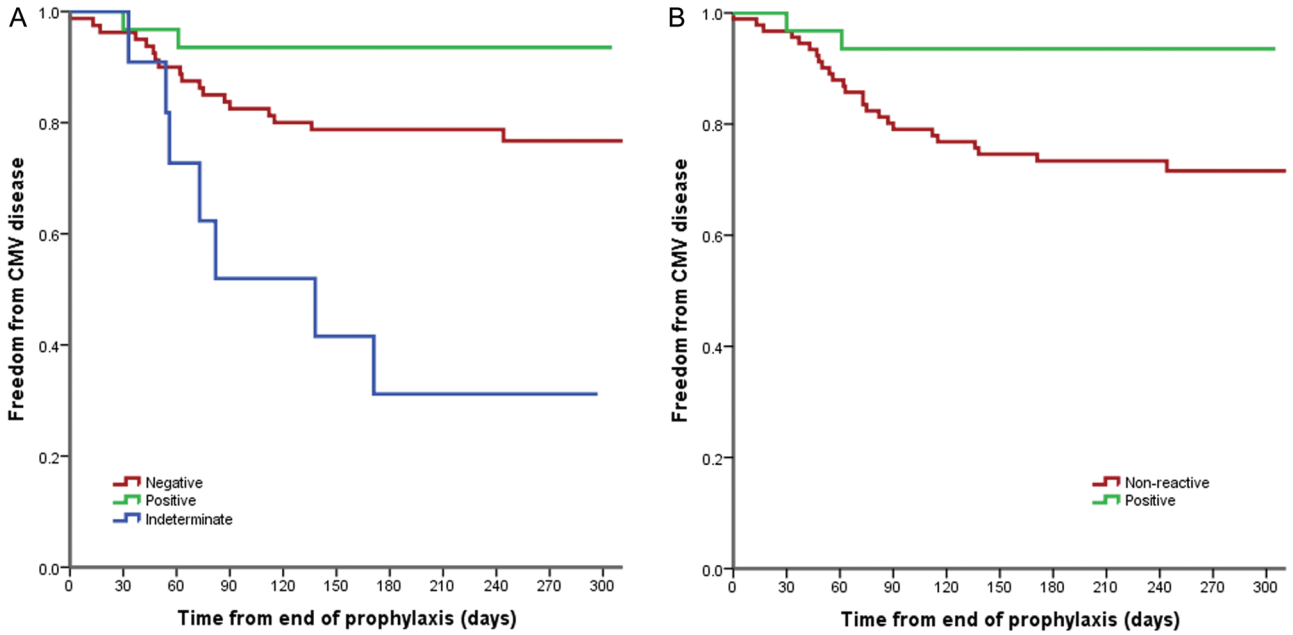

Figure 2. Kaplan-Meier curves of the incidence of cytomegalovirus (CMV) disease according to the result of the Quantiferon-CMV assay. *A*, Positive vs negative vs indeterminate result of the assay (log-rank test, $P < .001$). *B*, Positive vs nonreactive result of the assay (log-rank test, $P = .024$). Abbreviation: CMV, cytomegalovirus.

Quantiferon-CMV assay may benefit from a reduction of immunosuppression or an extension of antiviral prophylaxis until immunosuppression reduction can safely occur.

In addition to the Quantiferon-CMV assay, several other assays are available to assess CMV-specific cell-mediated immune responses. These include the enzyme-linked immunosorbent spot (ELISPOT) assay, major histocompatibility complex peptide multimers, and intracellular cytokine staining (ICS) by flow cytometry [10]. Although none of these assays can be considered the "gold standard" for the detection of CMV-specific cell-mediated immunity, they are widely used in research laboratories. Comparison between assays has been assessed in a limited number of studies. The sensitivity of the Quantiferon-CMV and of the ELISPOT to detect a cell-mediated immune response was equivalent in a study involving 10 healthy seropositive volunteers [15]. In hematopoietic stem-cell transplant recipients, the Quantiferon-CMV assay was compared to an ICS assay [19]. With a cutoff for positivity of 0.2 UI/mL of IFN-γ for the Quantiferon-CMV test, concordance between assays was 69% (κ value = 0.69). However, using the ICS method as reference, the Quantiferon-CMV assay detected a cell-mediated response in only 76% of cases. The Quantiferon-CMV assay is HLA restricted, so some rare HLA haplotypes may not be represented in the assay (<2% of the population) [14]. Also, the assay primarily detects a $CD8^+$ T-cell response, but not a $CD4^+$ T-cell response. Of note, in the study by Clari and colleagues, the Quantiferon-CMV assay appropriately correlated with polyfunctional (both antiviral and cytotoxic) CMV-specific $CD8^+$ T-cell responses [19].

Table 3. Sensitivity, Specificity, and Positive and Negative Predictive Values of the Quantiferon-Cytomegalovirus (CMV) Assay for Detecting Protection Against CMV Disease

	Patients With CMV Disease	Sensitivity (95% CI)	Specificity (95% CI)	Positive Predictive Value (95% CI)	Negative Predictive Value (95% CI)
First time-point	Positive QF assay: 1/15 (6.7%)	.14 (.08–.23)	.96 (.81–.99)	.93 (.68–.99)	.24 (.16–.33)
	Nonreactive QF assay: 26/109 (23.8%)				
Any time-point	Positive QF assay: 2/31 (6.4%)	.30 (.21–.40)	.93 (.76–.99)	.93 (.78–.99)	.27 (.18–.37)
	Nonreactive QF assay: 25/93 (26.8%)				

Abbreviations: CI, confidence interval; CMV, cytomegalovirus; QF, Quantiferon.

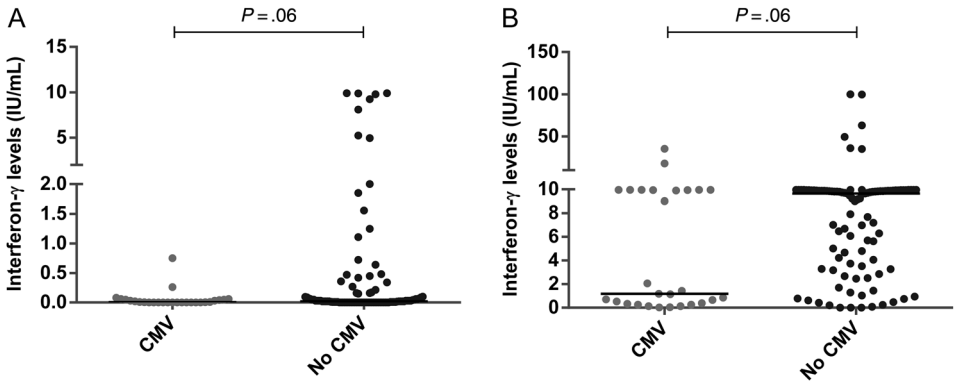

Figure 3. Interferon-γ levels in patients with and without cytomegalovirus (CMV) disease. Black lines are median levels. *P* values calculated using Mann-Whitney *U* test. *A*, Interferon-γ levels from the CMV tube. *B*, Interferon-γ levels from the mitogen tube. Abbreviation: CMV, cytomegalovirus.

Some limitations of our study need to be highlighted. First, several patients developed a detectable cell-mediated response only after subsequent testing in the 2 months following the discontinuation of prophylaxis. While multiple time point testing may improve the performance of the test, it may be logistically difficult to implement. Second, the method used to detect CMV viremia was dependent on the center-specific laboratory and included either quantitative nucleic acid testing or pp65 antigenemia testing. However, both these methods are recognized as appropriate for the diagnosis of CMV viremia according to current guidelines [1, 2]. In addition, we included only clinically symptomatic CMV disease, a stronger clinical endpoint, as the primary endpoint of the study. Finally, substantial heterogeneity existed in our study population (type of transplant, immunosuppression). However, all patients were D^+/R^-, which is the most important and consistent risk factor for CMV disease. This is one of the main strengths of our study in addition to the international recruitment reflecting current practices for CMV prevention around the world, and the use of an assay that can be easily implemented in the majority of transplant centers.

In conclusion, we showed that in high-risk D^+/R^- SOT recipients, assessment of cell-mediated immunity using the Quantiferon-CMV assay has clinical utility for the prediction of CMV disease. In those with a positive assay, the subsequent incidence of CMV disease was low and those with either negative or indeterminate results had a higher incidence of CMV disease. The Quantiferon-CMV assay may appropriately stratify D^+/R^- SOT recipients according to their individual risk for the development of CMV disease.

Notes

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Potential conflicts of interest. O. M. has received funding for research from Roche. D. K. has received funding for research from Roche and Sanofi-Pasteur, has participated in clinical trials sponsored by Astellas and Merck, and has received honoraria from Merck and Pfizer. M. G. I. has received funding for research from ViraCor. A. H. has received funding for research from Roche and has been a consultant for Astellas. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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