Modification of the HCMV-specific IFN-γ release test (QuantiFERON-CMV) and a novel proposal for its application

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Abstract
Human cytomegalovirus (HCMV) is universally distributed among humans without any adverse effects; however, it induces severe diseases in immunocompromised patients such as organ transplant recipients and AIDS patients. To manage these immunocompromised patients, an easy clinical examination for the monitoring of disease risk is required. In this study, we modified the interferon-γ (IFN-γ) release test (QuantiFERON®-CMV) using HCMV immediate early-1 (IE-1) or pp65 whole proteins, or UV-inactivated HCMV particles as an antigen. The response of heparinized peripheral blood from healthy volunteers to the pp65 protein showed an obvious dose-dependent sigmoid curve, although no correlation was observed between results of this assay and an ELISPOT assay. The addition of pp65 to the blood samples at a final concentration of 1×10^3 to 1×10^5 pg/ml was found to be optimum. Using this assay, we observed a significant enhancement in cellular immunity in volunteers after the daily ingestion of yogurt for 8 weeks, which suggested a novel application of the assay in addition to monitoring HCMV infection risk. IFN-γ secretion from peripheral blood cells on HCMV-antigen stimulation differed significantly between individuals; therefore, the assay could not be normalized. Nevertheless, it was found to be particularly useful for observing fluctuations in cellular immune activity on an individual level.

Key words: Human cytomegalovirus, Interferon-γ, Cellular immunity

Introduction
Human cytomegalovirus (HCMV) belongs to the subfamily β-herpesvirus. The virus possesses a 235 Kbp double-stranded DNA, which is the largest among human herpesviruses and codes about 250 open reading frames1). Most human beings (50-85%) are infected with HCMV without apparent disease2). However, in immunocompromised patients, such as organ transplantation recipients and AIDS patients, HCMV-infection can cause fatal diseases such as opportunistic infections3-5). In addition, congenital infection of fetuses with immature immunity results in microcephaly, hearing loss and mental retardation in about 20% of cases6,7).

It has been reported that HCMV establishes latent infection in CD34-positive myeloid progenitor cells8), and it is clinically recognized that reactivation of the virus from latently infected cells occurs through the reduced immunity associated with immunosuppression, inflammation and stress9,10); however, a number of points regarding the underlying mechanisms remain to be clarified. For example, while HCMV pneumonia frequently occurs in organ transplant patients11), HCMV retinitis occurs in AIDS patients12). In addition, HCMV enteritis readily occurs as a complication of ulcerative colitis but not of Crohn’s disease13,14). To understand the pathogenesis of HCMV diseases, it is important to monitor cellular immunity against HCMV. To this
end, two laboratory techniques, the human leukocyte antigen (HLA) type-matched tetramer method\textsuperscript{15} and the ELISPOT method\textsuperscript{16,17}, have been established. However, these methods require expensive materials, precision instruments and technical expertise. For example, the tetramer method has high sensitivity and excellent quantification, but the tetramer is expensive and HLA identification is necessary. The ELISPOT method can evaluate the number of antigen-specific T cells with high sensitivity, but the purification of monocytes is required and the dynamic range of this assay is much lower than that of the tetramer method. Furthermore, as both methods require relatively large numbers of lymphocytes, a large volume of blood must be collected from immunocompromised patients with leucopenia.

Recently, Walker et al. developed QuantiFERON\textsuperscript{®}-CMV as a simple and sensitive diagnostic method for monitoring HCMV-specific CD8\textsuperscript{+} T cell responses as follows. Heparinized whole blood is incubated with 21 synthetic oligopeptides that are T cell epitopes in HCMV pp65, pp50, immediate early-1 (IE-1), IE-2, and glycoprotein B. After culturing overnight, the supernatants are harvested and analyzed by ELISA for interferon-\textgamma (IFN-\textgamma) secreted from the T cells\textsuperscript{18}. This kit has been approved as CE Marked for commercial use in Europe and has been applied to several clinical studies\textsuperscript{18,19,20,21,22}, although it is not yet US FDA-approved. This method appears to afford advantages as a clinical test in terms of cost, sensitivity and ease of use. However, it is possible that the sensitivity of the assay might vary with subject HLA type as different T cell and macrophage HLA types are stimulated by different numbers of epitopes among the 21 epitopes used. Moreover, this assay does not rely on antigen presentation cell functions as the oligopeptides can bind and stimulate the T cell receptors directly. Therefore, we sought to modify the assay using virus particles or a whole protein as the antigen and herein propose the use of this assay for the evaluation of cellular immunity in healthy individuals as a novel application.

**Materials and Methods**

**Cells**

Monocytes (PMBCs) from the peripheral blood of healthy donors were isolated by Ficoll-Hypaque density-gradient separation (Lympholyte-H; Cedarlane Labs, Ontario, Canada) and resuspended in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal calf serum. The PBMCs were stained with 0.4% Trypan Blue Dye (Bio-Rad, CA, USA), counted using a TC20TM fully automatic cell counter (Bio-Rad), and then stored in small aliquots at $-80^\circ$C. A human foreskin fibroblast cell line, hTERT-BJ1, was derived from an immortalized BJ-1 cell by the expression of the human telomerase reverse transcriptase subunit (Clontec, CA, USA). The cells were cultured with Dulbecco’s modified Eagle’s minimum essential medium (DMEM; Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal calf serum.

**Anti-HCMV antibody titer**

Anti-HCMV IgG titers were measured by avidity test using a Mini VIDAS\textsuperscript{®} system (Sysmex bioMerieux, Co., Ltd., Lyon, France). The Mini VIDAS\textsuperscript{®} system is able to measure the HCMV avidity index based on the following criteria; low value (L) <0.4, decision pending (G) 0.4≤~<0.65, and high value (H) ≥0.65. A low value suggests the person was primarily infected with HCMV less than 4 months prior to testing and a high value indicates latent infection with a lapse of over 4 months from primary infection.

**Purified HCMV antigens**

As HCMV antigens, purified IE-1 and pp65 polypeptides were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

**Production of the UV-inactivated HCMV**

HCMV strain AD-169 was grown in hTERT-BJ1 cells and purified by ultra-centrifugation on a 10-50% sucrose gradient according to the method of Zucker\textsuperscript{23}. The virus was titrated by plaque-forming assay using hTERT-BJ1 cells and stored in small aliquots at $-80^\circ$C. Purified HCMV was irradiated with 5×10\textsuperscript{3} µJ/m\textsuperscript{2} using a UV crosslinker (Agilent Technologies, CA, USA). No infectious virions were detected under these conditions.

**HCMV antigen-specific IFN-\textgamma release assay**

Ten µl of IE-1 polypeptide, pp65 polypeptide or UV-inactivated HCMV particles were added to 100 µl samples of peripheral venous blood at final concentrations of $6.7\times10^4$~$1.6\times10^5$, $2.6\times10^4$~$6.7\times10^4$ pg/ml or $7.2\times10^3$~$3.7\times10^4$ pfu/ml, respectively, in a 96-well plate. After culturing for 24 h at 37°C in a CO\textsubscript{2} incubator, the entire culture solutions were transferred into micro-tubes and then centrifuged at $800\times$g at 4°C for 10 min with a micro-ce-
trifuge. After centrifugation, supernatants were collected and stored at −80°C until IFN-γ measurement. The IFN-γ level in the supernatant was quantified by an ELISA assay using Human IFN-γ ELISA Ready-SET-GO!® (eBioscience, CA, USA) in accordance with the manufacturer’s protocol.

ELISPOT assay

PBMCs stocked at −80°C were dissolved and seeded into an anti-IFN-γ antibody-coated Multi-Screen 96-well plate (Merck Millipore, Darmstadt, Germany) at 2×10⁵ cells/well with or without 1.0×10⁸ pg/ml of IE-1 or pp65. After culturing for 24 h at 37°C under 5% CO₂, IFN-γ-producing cells were stained and counted using a Human IFN-γ ELISPOT Ready-SET-GO! Kit (eBioscience) in accordance with the manufacturer’s protocol. Both 12-O-Tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) and ionomycin (Sigma-Aldrich) were added at 4.2 μg/ml each, instead of the HCMV antigen, to induce the expression of IFN-γ as a positive control.

Clinical test of yogurt ingestion

In order to estimate the ability of the IFN-γ release test to assess the cellular immune status in individuals, the effect of yogurt ingestion for 8 weeks on immunity was analyzed using the assay. After obtaining approval for this clinical test from the Institutional Ethics Committee of Fukushima Medical University and written informed consent from all volunteers, peripheral blood was collected in heparinized test tubes from 39 healthy volunteers (15 men and 24 women, average 43.8±11.7 years old). The daily ingestion of 80 g of yogurt fermented with Streptococcus thermophiles, Lactobacillus delbrueckii subspecies bulgaricus and Lactobacillus acidophilus (Tohoku Kyodo Nyugyo Corporation, Fukushima, Japan) was then begun. At the last day of the 8-week ingestion period, peripheral blood was again sampled. These samples were then applied to anti-HCMV antibody titer measurement and HCMV antigen-specific IFN-γ release assay as described above.

Table 1. Sero-status against HCMV of the volunteers

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Anti-HCMV IgM and IgG antibody titers, and the IgG Avidity index of plasma obtained from 17 healthy volunteers are shown. The criteria for CMV IgG Avidity index are as follow: Low value (L) < 0.40, Decision pending (G) 0.4 ≤ - < 0.65, and High (H) ≥ 0.65
NT: not tested
Statistical analysis

Results are expressed as mean±S.D. Significant differences between two mean values were determined by unpaired t-test. The correlation between assays was analyzed by the linear approximation of least squares method and calculations were carried out using Microsoft Excel software version 2013.

Results

Sero-status against CMV of the healthy blood donors

In order to establish a HCMV antigen (Ag)-specific IFN-γ release test, 17 healthy blood donors were enrolled and their sero-status evaluated on the basis of anti-HCMV IgM and IgG titers, and IgG avidity index (Table 1). Only one volunteer, ID #1, was not infected with HCMV, whereas the other 16 volunteers were estimated to be infected with HCMV more than a few months prior to this study based on their IgM negativity and/or high avidity index.

Establishment of a HCMV antigen-specific IFN-γ release assay

The amount and type of antigens; i.e., UV-inactivated HCMV particles, IE-1 polypeptide or pp65 polypeptide, were optimized to establish the HCMV-Ag specific IFN-γ release test. One of these antigens was added to 100 μl of peripheral blood collected in heparinized test tubes from the healthy donors and the supernatant was collected after 24 hours of incubation at 37°C. The amount of IFN-γ secreted to the supernatant from the PBMCs in the blood sample was then measured with an ELISA assay.

On UV-inactivated HCMV particle stimulation, IFN-γ was secreted in a dose-dependent manner and the samples were divided into two groups based on the amount of Ag required for IFN-γ secretion (Fig. 1A). In one group, IFN-γ production was detected at 1×10^4 pfu/ml of UV-inactivated HCMV, whereas the other group required ten times more Ag. IFN-γ production was observed even in seronegative samples (#1-2) at 4×10^4 pfu/ml of CMV particles. These results indicated that a CMV particle concentration of between 1×10^4 and 2×10^4 pfu/ml was optimum for the detection of HCMV-specific IFN-γ production without the induction of any non-specific immune reaction.

IFN-γ production was also observed in a dose-dependent manner on stimulation with the purified polypeptide Ag, IE-1 or pp65 (Fig. 1B and 1C). Among these Ags, pp65 was observed to be superior in certain regards. A higher dose of IE-1 induced inversely lower IFN-γ production, as observed in #9, #13, #16 and #17; therefore, quantitative analysis of the immune response by IE-1 stimulation appears to be impossible. On the other hand, the pp65-induced IFN-γ expression showed a more obvious sigmoid curve. Moreover, stimulation by pp65 was slightly more effective than that by IE-1 at low concentrations. The correlation between IE-1 stimulation at 1.6×10^4 pg/mL and pp65 stimulation at 6.7×10^4 pg/mL was higher than the other correlations at different Ag concentrations, but was nevertheless low at R^2=0.0469 (Fig. 2).

From these results, we determined that 1×10^3 to 1×10^5 pg/ml of pp65 was the best Ag for the HCMV Ag-specific IFN-γ release test.

Correlation between the IFN-γ release assay and ELISPOT assay results

To evaluate whether the HCMV Ag-specific IFN-γ release assay could adequately determine cellular immunity, the same blood samples were analyzed using both the IFN-γ release assay and ELISPOT assay. In the ELISPOT assay, from 4 to 96 and from 4 to 26 spots were observed in the samples from HCMV-negative donor #1 on IE-1 and pp65 stimulation, respectively. This indicated that our ELISPOT assay involved non-specific reactions. We could count the number of spots in all but one sample (#11) in the assay after IE-1 stimulation, but could not count the spots in 11 samples as they exceeded the limit (>200 spots) for the assay after pp65 stimulation (Fig. 3).

The correlation between the IFN-γ release assay and ELISPOT assay was then analyzed (Fig. 4). The correlation coefficients between the IFN-γ release assay and ELISPOT were low at 0.3313 and 0.0978 for IE-1 and pp65, respectively (Fig. 4).

Application of the IFN-γ release assay to the study of the immune-stimulatory effect of yogurt

About 80% of Japanese adults are infected with HCMV. Therefore, it is possible that the HCMV specific IFN-γ release assay could be applied clinically for the evaluation of cellular immune status. To ascertain this possibility, we analyzed peripheral blood samples collected from 39 healthy volunteers (15 men and 24 women 43.8±11.7 average age) before and after ingesting 80 g of yogurt every day for 8 weeks. Among these 39 volunteers, 10 were excluded from this analysis because their IFN-γ con-
concentration was too high (> 600 pg/ml) before yogurt ingestion ($n=8$) or they were anti-HCMV antibody negative ($n=2$). The results for the remaining 29 volunteers before and after yogurt ingestion are summarized in Fig. 5.

IFN-γ secretion from PBMCs was enhanced by some factor(s) associated with the in vitro culture, with the IFN-γ levels in supernatant being 10.6±22.3 and 20.1±20.0 pg/ml before and after yogurt ingestion without pp65 stimulation, respectively (Fig. 5A). These results indicated that yogurt ingestion slightly activated the basal level of monocyte activity, although the difference was not statistically significant ($p=0.0926$). Further, pp65-specific PBMCs were significantly enhanced by yogurt ingestion ($p=0.0037$), with 123.8±183.2 and 438.4±540.4 pg/ml of IFN-γ released by PBMCs from volunteers before and after yogurt ingestion, respec-

Fig. 1. HCMV antigen-specific IFN-γ release assay.
Peripheral blood from healthy donors was mixed with UV-inactivated HCMV particles (A), IE-1(B) or pp65(C).
#1: CMV antibody-negative donor
#2-17: CMV antibody-positive donors
**Discussion**

HCMV is one of the most common and problematic pathogens in immunocompromised patients. In particular, HCMV infection is a major problem in terms of morbidity and mortality in transplant recipients. In Europe and United States, long-term valganciclovir is administered prophylactically to solid organ transplant patients. However, preemptive treatment with an antiviral drug is recommended in Japan in consideration of the cost and drug side effects. In the case of hematopoietic stem cell transplantation, antiviral prophylaxis is not generally applied or, rather, is contraindicated in the immediate post-transplantation period due to the delay in engraftment caused by antiviral drugs. Therefore, clinical examinations to indicate HCMV infection or risk of infection are important for the management of opportunistic HCMV infection. Quantiferon®-CMV was developed as an assay for the evaluation of disease risk. In this study, we sought to modify the assay to enable the monitoring of not only T cell function but also Ag processing and presentation using whole polypeptides or UV-inactivated virus particles.

The IE-1 protein, a HCMV UL 123 gene product, is a non-structural protein expressed in the earliest stage of the HCMV replication cycle in infected cells. The pp65 protein, a HCMV UL 83 gene product, is a structural protein located at the tegument in the viral particle. Both IE-1 and pp65 are known to be major targets for T cells. Previous studies indicated that CD4 T cells predominantly recognized structural proteins and CD8 T cells recognized non-structural proteins, as structural proteins present on MHC class II as exogenous Ags and non-structural proteins present on MHC class I as endogenous Ags. However, a recent study showed there were huge overlaps between the epitopes for CD4 and CD8 T cells. The internalization of exogenous Ag into dendritic cells was suggested as one explanation of this overlap; for example, dense bodies containing full HCMV proteins are secreted by infected cells and internalized by antigen-presenting cells resulting in the transport of exogenous Ag into the endogenous pathway. Non-infectious enveloped particles secreted by infected endothelial cells appear to have a similar function. In this study, we tested IE-1 and pp65 proteins as exogenous Ag and UV-inactivated HCMV particles as endogenous Ag. Therefore, the IE-1 and pp65 proteins seemed to induce IFN-γ from CD4 T cells by presentation on MHC class II antigen presentation cells, while the inactivated virus appeared to induce IFN-γ from both CD4 and CD8 T cells by presentation on MHC class I cells, respectively, although it is possible that exogenous Ag is internalized into the cytoplasm. Based on this hypothesis, our IFN-γ release test would, therefore, observe different cell functions from those of Quantiferon®-CMV using synthetic peptides that bind to T cell receptors directly.

The IE-1 and pp65 proteins were chosen as Ags as these proteins are well known as major Ags for T cells and the purified proteins are commercially available. A comparison of these proteins and UV-inactivated virus particles revealed that pp65 was the best Ag due to its dose dependency and
Fig. 3. HCMV antigen-specific IFN-γ ELISPOT assay.
A monolayer of sample monocytes was stimulated with 1.0×10^6 pg/ml of IE-1 or pp 65. Cells were treated without Ag as a negative control (NC) or with TPA and ionomycin as a positive control. The numbers of spots were counted and are shown under the photos.
easy availability. However, a recent study indicated that various virus proteins induce various immunological cells, not only CD4+ or CD8+ T cells but also CD28 null cells33). Moreover, some proteins, such as UL138, were shown to stimulate IL10 and transforming growth factor-β (TGF-β) expression30).

Therefore, it remains possible that pp65 and IE-1 stimulated different subsets of immunological cells and there is no correlation between the IFN-γ release assay using pp65 and IE-1. To understand the immunological response against HCMV infection in greater detail, not only viral proteins but also target immunological cells should be analyzed using various cytokine-release tests in the future.

Compared with Europe and North America, the prevalence of HCMV infection in adult Japanese is high24). We hypothesized that anti-HCMV cellular immunity could afford a general marker for cellular immune status in healthy HCMV-infected persons. To estimate the usefulness of the IFN-γ release test for this purpose, the cellular immunity of healthy volunteers was examined before and after the ingestion of yogurt as an immunological modifier. The IFN-γ secretion level of the 29 volunteers was significantly enhanced by the 8-week yogurt intake, demonstrating the usefulness of this test. However, the basal IFN-γ release level differed markedly

Fig. 4. Correlation between the IFN-γ release assay and ELISPOT assay.
IFN-γ production by IE-1 (A) and pp65 (B) stimulation, and the number of spots are plotted.
R²: coefficient of determination.
on an individual level, making it difficult to determine the normal value. In addition, we could not set a control group due to difficulties associated with the preparation of placebo yogurt, so we compared values before and after ingestion of the yogurt. Nevertheless, monitoring of the alterations in IFN-γ release level in an individual was useful for understanding cellular immunological status in general.

In conclusion, we established a HCMV-specific IFN-γ release test using whole proteins and UV-inactivated virus particles. This method could be applied to the monitoring of cellular immune function. Various viral proteins should be applied to this system in the future for further analysis of the cellular immune response against HCMV infection.

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Conflict of Interest Disclosure

All authors declare that they have no conflicts of interest.

References


