In Vitro Transmission of HCMV between Fibroblasts and Peripheral Blood Leukocytes in the Presence of IL-4

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Summary: We demonstrated transmission of human cytomegalovirus (HCMV) from the human lung fibroblast MRC-5 to peripheral blood leukocytes (PBLs). mRNA of the HCMV immediately-early (IE) antigen was detected in PBLs cultured with IL-2 or IL-2 + IL-4 that made direct contact with HCMV-infected MRC-5, whereas it was not detected in PBLs prevented from making cell-to-cell contact. However, mRNA of HCMV IE was not detected in PBLs cultured with IL-2 and IFN-gamma that made direct contact with HCMV-infected MRC-5. Transmission of the pp65 antigen was increased in culture medium containing IL-4. At a higher viral infection titer, cell-free HCMV infected adherent PBLs cells. The subset, which did not adhere, did not infect cell-free viruses even at a very high multiplicity of infection. Moreover, the adhered subset of PBLs infected with HCMV was able to transmit HCMV to non-infected fibroblasts. Our results suggest that cell-to-cell contact (when PBLs make direct contact with HCMV-infected cells) is important in the mechanism of HCMV transmission and that the adherent cells of PBLs are one of the most important vehicles for HCMV infection. Moreover, we suggest that type 2 cytokines such as IL-4 enhance the transmission of HCMV to PBLs.

Key words human cytomegalovirus, fibroblasts, leukocytes, interleukin 4, in vitro

INTRODUCTION

Human cytomegalovirus (HCMV) is a pathogen of opportunistic infection. The presence of HCMV in blood is a significant indicator of the development of HCMV disease [1]. For immunocompromised host-like recipients of bone marrow transplantation or AIDS patients, HCMV frequently causes severe pneumonitis. Moreover, amplification of HCMV DNA from peripheral blood is significant in determining the prognosis of diseases [2-4]. We previously reported that detection of the HCMV antigen in monocytes is related to HCMV activation, and that detection in neutrophils indicates that the patient has an HCMV-associated disease [4,5]. Kondo et al. [6] suggest that granulocyte-macrophage progenitor (GM-P) in bone marrow is a potential reservoir of latent HCMV. They reported that HCMV infection of GM-P is non-cytocidal and that viral gene expression is restricted. Numerous studies report that peripheral blood leukocytes (PBLs) are able to harbor the virus in an infectious state [7-9]. However, the origin of the virus carried by PBLs and the mechanism of the transmission is not known.

Increased production of type 2 cytokines (e.g., IL-4 and IL-10) has been highlighted in the suppression of cellular immunity. IL-4 is secreted not only from CD4 positive T cells but also from several CD8 positive T cells in a compromised host [10]. It is a most important factor for the development of humoral responses. In contrast, IL-4 inhibits cytokine production by activated monocytes. The transcription of IFN-alpha and IL-12 are inhibited. IL-4 suppresses the development of type 1 cells through down-
regulation of IL-12 production [11,12]. Since infections with intracellular pathogens like HCMV are greatly regulated by cellular responses, a predominance of cellular responses to type 2 cytokines may contribute to the decreased resistance to infection. Matsuo et al. [13] reported that IL-4 and IL-10 production was increased by burn-associated CD8 positive cells in the thermal-injured mouse spleen. They showed that predominance of type 2 cytokine suppresses resistance to HSV infection. In the present experiment, we observed the influence of type 2 cytokine on HCMV infection of peripheral blood cells in vitro.

**MATERIALS AND METHODS**

**Virus and cells**

Human cytomegalovirus laboratory strain AD169 was obtained from the American Type Culture Collection (Manassas, VA). Virus was propagated in low-passage MRC-5 human diploid fetal lung fibroblasts. Human embryonic lung fibroblast MRC-5 was obtained from Riken Cell Bank (Saitama, Japan). The culture of MRC-5 was maintained in Dulbecco's medium (GIBCO/BRL., Rockville, MD) supplemented with 10% fetal calf serum (FCS), 1% MEM non-essential amino acid solution (GIBCO/BRL., Rockville, MD), penicillin G (100 units/ml) and streptomycin (100 μg/ml). Infected cells were lysed by three freeze-thaw cycles, and the cell lysates clarified by centrifuging at 500 g for 20 min. Infectious virus in the supernatant was quantified by plaque forming assay.

**Viral infection to monolayer cells**

MRC-5 was seeded into 6-well plates at 2×10⁴ cell/well and maintained with medium until it showed confluent growth. The cells were then inoculated with 1×10⁵ pfu of virus for 1 hr at 37°C. After the inoculation, these cells were washed twice with medium and maintained with D-MEM for 5 days. Virus concentration was determined from a preliminary experimental result that almost 80% of cells show a cytopathic effect 5 days after inoculation.

**Co-culture PBL with HCMV infected fibroblasts**

Human peripheral blood leukocytes (PBLs) were collected from buffycoats by ficoll density centrifugation and washed twice in ice-cold PBS. Erythrocytes were removed with ACK lysing buffer (0.15 M NH₄Cl, 1.0 M KHCO₃, 0.1 mM Na₂EDTA, pH 7.4) and resuspended in RPMI 1640 with 10% FCS. Cell culture insert membrane (3.0 μm pore size membrane) was purchased from Beckon Dickinson Co. (CA). Just before adding PBLs to MRC-5, the supernatant in the 6-well plate was removed and washed with fresh D-MEM twice. PBLs were resuspended in RPMI 1640 with 10% FCS containing (a) 100 units/ml of IL-2, (b) 100 units/ml of IL-2 and 15 units/ml of IFN-gamma, or (c) 100 units/ml of IL-2 and 0.2 ng/ml of IL-4. Concentrations (ED₅₀) of each cytokine were determined according to the manufacturer's instructions (R & D systems, Inc., Minneapolis, MN). Membranes were placed in each well over the monolayer of HCMV-inoculated MRC-5 cells to allow the virus to move up through. PBLs were spread on this membrane (indirect contact) or on HCMV-inoculated MRC-5 cells under the membrane (direct contact), at 2×10⁶ cell/well each. PBLs were collected 1×10⁵ cell each from each well after 48 hrs of incubation.

**PBLs selected by anti CD45 positive selection**

PBLs cultured with HCMV infected MRC-5 were spun-down briefly and adjusted to 1×10⁶ cell/50 μl. Purified mouse anti-human CD45 monoclonal antibody was purchased from Beckon Dickinson Co. (CA). Two microliters (50 μg) of monoclonal antibody was added to the 1×10⁶ cells of PBLs and incubated for 30 min at 4°C. To capture antibody-coated PBL, excess antibodies were removed by washing twice with washing buffer (ice-cold PBS containing 20% FCS) and the cells then re-suspended in 50 μl of PBS. Dynabeads M-450 goat anti-mouse IgG (Dynal A. S., Oslo, Norway) was employed. Dynabeads was used according to the manufacturer's protocol and 1:10 of cell/beads ratio was used. During incubation, selection and washing procedures, cells and reagents were kept at 4°C to prevent non-specific attachment of cells to Dynabeads. PBLs positively selected by Dynabeads were then transferred to a 6-well tissue culture plate to determine under light microscopy that no MRC-5 cells were contaminated.

**Detection of HCMV IE mRNA using the RT-PCR method**

PBLs, which were selected by anti CD45 positive selection, were harvested and transferred to a microcentrifuge tube, at 10³ cell/tube. Cells were spun-down briefly, the supernatant discarded, and then lysed with 50 μl of 2% Triton-X-100 buffer which also contained the reagents necessary for the RT reaction: 10 mM Tris-HCl, pH 9.3, 50 nM KCl, 3 mM MgCl₂, 0.1 mg/ml BSA, 10 units/μl of M-MLV reverse transcriptase, and 200 μM each dNTP, 50 units/μl of Taq DNA polymerase. The RT reaction mixture was incubated at 42°C for 1 h. The RT product was used as a template in the PCR reaction, which was performed using the following conditions: 10 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and a final extension at 72°C for 5 min. The PCR products were separated on a 3% agarose gel and visualized by staining with ethidium bromide.
reverse transcriptase (GIBCO/BRL), 0.5 mM dNTP (Promega), 1 unit/μl RNAsin (TOYOBO, Osaka, Japan) and 5 pM random hexamers (TAKARA, Japan) [1]. The RT reaction was performed for 45 min at 39 °C followed by 95 °C for 5 min. The following primers were used to detect IE-1 mRNA [14]: upstream, 5'-ACGAGAACCCCGAGAAAGAT-3'; downstream, 5'-CATCCACATCTCCCCGTAT-3'. The primers yield a 303 bp product from mRNA and a corresponding product of 454 bp from gDNA. HCMV cDNA generated by RT was amplified by PCR. Each reaction mix consisted of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X, 2.5 mM MgCl$_2$, 200 μM dNTP, 0.5 μM of each appropriated primer, 1.0 U of Taq DNA polymerase (Amersham, Ohio), and 0.056 μM of TaqStart Antibody (CLONTECH, CA) to a volume of 20 μl. Samples were amplified in a DNA thermal cycler (ASTEC PC-800). To amplify cDNA from IE-1 mRNA, 35 cycles of the program, denaturation at 94 °C for 30 sec, annealing at 60 °C for 10 sec, and extension at 72 °C for 20 sec were repeated. To verify that the RT-PCR procedure was working correctly, cellular beta-actin mRNA was amplified. The amplification process consisted of 30 cycles with denaturation at 94 °C for 10 sec, annealing at 58 °C for 10 sec, and extension at 72 °C for 20 sec. A 10 μl aliquot of each sample was analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide.

**Immunohistological staining to detect the CMV pp65 antigen**

PBLs, which were co-cultured with HCMV-inoculated MRC-5 in the presence of IL-2 or IL-2+IL-4, were removed by incubation with trypsin-EDTA (0.3%), washing twice in Ca$^{2+}$ and Mg$^{2+}$-free PBS and cytocentrifugation on heavy teflon coating slide (HTCS) glasses. The slides were dried and fixed with cold acetone for 5 min. The slides were stained with a commercially available kit (CMV antigen Mitsubishi, Dia-Iaton Co., Tokyo). Under light microscopy, CMV antigen-positive cells developed a distinct red-colored stain and were enumerated in triplicate.

**Inoculate HCMV to PBL in higher multiplicity of infection (MOI)**

To examine whether PBL, itself alone is permissive or not, PBLs were inoculated with various concentrations of HCMV. Virus pools were diluted with RPMI 1640 to 1×10$^4$, 1×10$^5$ and 1×10$^6$ pfu/ml. Each 6-well plate was coated with FCS at 4 °C for 4 hrs and washed with PBS twice. One milliliter of PBLs (1×10$^6$ cell) was transferred to each well, and then the cell suspension was gently swirled and incubated for 1 hr at 37 °C in a 5% CO$_2$ humidified incubator. Supernatant containing nonadherent cells was transferred to a 15 ml conical tube. Plates were washed with PBS twice to remove excess nonadherent cells and loosely adherent cells. Prepared virus solutions were added to a nonadherent cell (1×10$^6$ cells/ml) in the 15 ml conical tube or an adherent cell in plates, followed by incubation at 37 °C for 2 hrs in the CO$_2$ incubator. PBLs (adherent or nonadherent cells) were washed twice with RPMI 1640 with 10% FCS to remove excess virus. The cells were resuspended in medium and incubation was continued. Aliquots were harvested 7 days after inoculation. Half of PBLs were analyzed to determine the expression of IE-1 mRNA. The other half of the PBLs was transferred to MRC-5 monolayer inoculated cultures and the appearance of the cytopathic plaque effect (cpe) was observed for 7 days.

**RESULTS**

**Detection of HCMV IE mRNA by RT-PCR**

As shown in Fig. 1, mRNA of HCMV IE was

![Fig. 1. Detection of CMV IE mRNA in PBLs. a: PBLs made direct contact with HCMV-infected MRC-5 cells and were cultured for 48 hrs. b: PBLs and HCMV-infected MRC-5, separated by cell membrane insert to prevent PBLs contact with MRC-5, were cultured. Then, PBLs were positively selected using anti-CD45 monoclonal antibody and prepared for RT-PCR. Lane 1 is 1 Kb DNA ladder (GIBCO/BRL., Rockville, MD). Lane 2 is negative control. Lane 3 is positive control (CMV-infected MRC-5 cell). Lanes 4-6 are IL-2, IL-2+IFN-gamma, IL-2+IL-4, respectively. Arrows show the bands of CMV cDNA (303 bp) and gDNA (454 bp).**
detected in the sample of PBLs which made direct contact with HCMV-infected MRC-5 (Fig. 1a), but was not detected in that which was separated by the cell membrane insert (Fig. 1b). However, mRNA of HCMV IE was not detected in the sample of PBL cultured with IL-2 and IFN-gamma, which made direct contact with HCMV-infected MRC-5. The positive PCR product amplified from cDNA was 303 bp. Lane 2 shows negative control. Lane 3 is the positive control and lanes 4-6 were supplemented with IL-2, IL-2+IFN-gamma, and IL-2+IL-4, respectively.

Detection of pp65 antigen by immunohistological staining

PBLs were stained immunohistochemically and examined. Figure 2 shows positive staining from a co-culture sample supplemented with IL-2 and IL-4. All positively stained leukocytes showed a polymorphonuclear shape and some had granules in the cytoplasm. Positively stained cells were observed in PBLs attached to MRC-5 but not in PBLs which had no direct contact with MRC-5. CMV antigen-positive cells were counted under light microscopy. The degree of antigen was expressed as the number of CMV antigen-positive cells per 1×10⁶ cells. The CMV antigen detected in PBLs cultured with IL-2 or IL-2+IL-4 was 5.5±2.3, 17±5.5, respectively (mean ±SD, n=3). CMV antigen-positive cells were significantly increased in PBLs cultured with IL-2+IL-4 (two sample t test: P<0.05).

High MOI inoculation

To examine whether a higher infectious titer causes infection of the non-attached leukocyte, PBLs were incubated with virus solution at a higher multiplicity of infection (MOI). MOI is defined as the ratio between the number of infectious units to the number of target cells [15]. Incubating 10⁴ pfu virus with 1×10⁶ cells will yield an MOI of 10⁴/10⁶ =0.01. In the present experiment, inoculations were performed with MOI values ranging between 0.01 and 1.0. PBLs observed to adhere to the bottom of the well were infected with HCMV. Figure 3a shows the PCR amplification result of adherent PBLs. Amplification was observed in non-adherent cells (data not shown). Amplification was observed only at a higher MOI 1.0 in adherent cells. Figure 3b shows the cytopathic effect on the MRC-5 mono-

Fig. 2. Detection of pp65 antigen by immunohistological staining. Positively stained cells are shown (magnification; ×400), which were co-cultured with HCMV-inoculated MRC-5, with 100 units/ml of IL-2+0.2 ng/ml of IL-4.

Fig. 3. a: Detection of CMV IE mRNA in adherent cells inoculated at higher MOI. Adherent cells were inoculated with virus stock at varying concentrations. Lane 1 is Kbp DNA ladder. lane 2; MOI 0.01. Lane 3; MOI 0.1, Lane 4; MOI 0.1, Lane 5; MOI 1.0. Arrow shows the band of CMV cDNA. b: Adherent cells inoculated at higher MOI were transferred to MRC-5 monolayer to examine whether it can transmit HCMV. Cytopathic effects in MRC-5 were observed (magnification; ×100).
layer. Adhered PBLs inoculated with HCMV (MOI 1.0) were harvested, and transferred to the MRC-5 monolayer. After 7 days of incubation, the cpe was observed as shown. Non-adhered PBLs were treated using the same method as adherent PBLs. Cpe was not observed (data not shown).

**DISCUSSION**

We demonstrated herein that HCMV IE mRNA and pp65 antigens were detected in PBLs that made direct contact with HCMV-infected MRC-5. PBLs were infected when they were placed under the cell membrane insert, enabling direct cell-to-cell contact to occur. In contrast, infection did not occur in the parallel experiment under same conditions when the cell membrane insert prevented direct contact. Grundy et al. [16] demonstrated that CMV-infected endothelial cells can recruit neutrophils by the secretion of C-X-C chemokines and can transmit the virus to them by direct cell-to-cell contact. Our results supported this (Fig. 1). The direct contact of PBL with HCMV-infected cells appears to be an importance in the transmission of HCMV.

In our present study, pp65 antigens and IE mRNA expression were observed in PBLs which were co-cultured with CMV-infected fibroblasts in the presence of type 2 cytokines such as IL-4 (Figs 1 and 2). However, transmission was not observed in the presence of the type 1 cytokine IFN-gamma. We suggested that the transmission of HCMV was affected by the presence of IL-4. This finding shows the possibility of transmission to PBLs is increased by the presence of type 2 cytokines. In addition, HCMV infection to MRC-5 cell induces the production of proinflammatory cytokine (IL-6) from the MRC-5 cell [17]. IL-6 is a cytokine that promotes inflammation by stimulating the proliferation of B and T lymphocytes to type 2 predominant. IL-6 may enhance the effect of IL-4.

We demonstrated the presence of HCMV IE mRNA in PBLs following incubation with cell-free virus (Fig. 3a). PBLs were divided into two groups: adherent cells and nonadherent cells. Adherent cells consisted of neutrophils and monocytes. Nonadherent cells included lymphocytes and natural killer cells. Positive PCR results were obtained from the sample of adherent cells. Small aliquots transferred to the non-infected MRC-5 monolayer caused a cytopathic effect (Fig. 3b). These results suggest that although cell-to-cell contact is highly important for transmission, it is not essential.

Neutrophils are thought to be an important source of infectious HCMV in the peripheral blood of viremic patients. The presence of pp65 in the peripheral blood neutrophils of such patients forms the basis of the diagnostic antigenemia tests. It has been assumed that neutrophils acquire HCMV in vivo from the phagocytosis of virions or debris from infected cells. Grundy [16] suggested that in vivo neutrophils acquire the virus from direct contact with infected endothelial cells. The present in vitro results suggest the existence of both: phagocytosis of virions and direct contact. Cell-free contact infection is not realistic in vivo when we consider the higher MOI required for contact-free inoculation. Probably cell-to-cell contact is the main mechanism of transmission. We hypothesize that activation of the adhesion molecule located on the granulocyte and macrophage induces this mechanism. Neutrophils recruited and attached to infected cells produced IL-8 [18], which enhanced HCMV replication in fibroblasts [19]. This may contribute to the increase in inflammation that in turn enhances the viral replication and cytopathic effect. Phagocytosis eliminates debris and activates the production of IFN gamma and the presentation of antigens. Following these cascades, cytotoxic T cells may be activated and the inflammation may develop into pneumonia. We investigated only one strain, therefore, further study of other strains is required to determine whether type 2 cytokines are also capable of enhancing transmission from fibroblasts to PBLs.

In summary, we demonstrated HCMV transmission from fibroblasts to PBLs in direct cell-to-cell contact in vitro. However, adherent cells of PBLs were infected with cell-free HCMV only at an extremely high viral inoculation titer. All PBLs stained positive by the pp65 antigenemia assay showed a polymorphonuclear shape. Moreover, HCMV-infected PBLs transmitted HCMV to non-infected fibroblasts. The transmissions of HCMV were affected by the presence of IL-4. We suggest that the adhered subset of PBLs is one of the most important vehicles for HCMV infection and type 2 cytokines such as IL-4 enhance the transmission of HCMV.

**REFERENCES**


