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A multiparametric flow cytometry method for detection of modifications of antigen expression in polymorphonuclear cells infected by human cytomegalovirus

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Abstract

Human cytomegalovirus (CMV) has been shown to alter adhesion molecule expression on permissive cells such as endothelial cells. The aim of the present study was to investigate expression of receptors for these molecules on CMV infected polymorphonuclear leukocytes (PMNLs). CMV-induced variations on cellular integrin expression were examined using an in vitro system to obtain infected PMNLs. A triparametric flow cytometry approach was developed, which allows combined detection, in a single experiment, of both viral intranuclear antigen in the selected PMNLs and cellular CD11/CD18 expression. Comparison of infected PMNLs with uninfected cells showed a decrease of up to 50% in the expression of CD11b, CD11c, and CD18. This study thus demonstrates that the presence of CMV in PMNLs, which characterizes active infection, modifies the expression of integrins and may thus affect cell-to-cell interactions and immune functions.
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Keywords: Cytomegalovirus; Polymorphonuclear cells; Flow cytometry; Integrins

1. Introduction

Cytomegalovirus (CMV) is one of the most common opportunistic microbes associated with infectious complications in immunosuppressed patients, leading to life-threatening organ involvement (Fishman and Rubin, 1998; Paya, 1994). Polymorphonuclear leukocytes (PMNLs) have been recognized as a major blood cell target during active infection (Gerna et al., 1998; Poirier-Toulemonde et al., 2000; Revello et al., 1998), and are notably responsible for the propagation of the virus within the host. Although previous studies have demonstrated the presence of infectious virions in these cells (Revello et al., 1998), the relationship between this virus and PMNLs remains unclear. Virus uptake by these cells is mediated by microfusion events with infected permissive cells, particularly endothelial cells (Grundy et al., 1998; Kas-Deelen et al., 2001; Waldman et al., 1995). PMNLs are also able to support the immediate early phase of the viral replication cycle (Gerna et al., 2000, 1992; von Laer et al., 1995), although the functional consequences of CMV infection have not yet been assessed. As infection may impair cell-to-cell interactions, this specific point ought to be investigated further.

Conducting such a study is, however, particularly difficult due to the fact that PMNLs have a very short half-life and that they cannot be maintained in culture. These limitations may be overcome by using an in vitro infection system-based on co-culture of fresh PMNLs with monolayers of infected fibroblasts—to generate infected PMNLs. However, as the efficiency of infection is low (less than 10% of infected leukocytes), accurate selection of infected PMNLs is necessary. A multi-parameter flow cytometric approach was developed, allowing the presence of cell markers, expressed in infected and uninfected PMNLs, to be assessed in a single experiment. This procedure allows selection of PMNLs from contaminating fibroblasts, discrimination between infected and uninfected cells by detection of an intracellular viral antigen (pp65), and simultaneous study

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of a number of adhesion molecules. This study focused on integrins, notably LFA-1 (CD11a/18), Mac-1 (CD11b/18), and p150,95 (CD11c/18) (Harris et al., 2000), which are receptors for molecules whose expression is modified by CMV infection in fibroblasts and endothelial cells.

2. Methods

2.1. Propagation of virus strains in fibroblasts

Five clinical human cytomegalovirus strains were recovered from clinical samples of patients with active CMV infection treated at the Nantes Hospital. Patient specimens were urine (He, Si, and Me strains) or respiratory secretions (Po, Bo). Viral strains isolated from these clinical samples were propagated for two to six passages in human embryonic fibroblasts (MRC-5, Biomérieux, Lyon, France), cultured in minimal essential culture medium (MEM, GibcoBRL div. of Invitrogen, Gaithersburg, USA) supplemented with HEPES, antibiotics (penicillin, colistin, and streptomycin). The growth medium was supplemented with 8% fetal calf serum (FCS), whereas medium used for maintenance contained only 2%. Cultures were performed in 25 cm^2 culture flasks. Cells were trypsinized and split when 80% characteristic cytopathic effect (CPE) was reached. The same protocol was used for propagation of the reference viral strains Toledo and Davis.

2.2. Preparation of polymorphonuclear leukocytes

Human PMNLs were isolated from CMV seronegative EDTA-anticoagulated blood samples from three volunteers (Ce, Ma, St), using a sequential treatment with two separation media. Two milliliters of 6% Dextran T500 (Amersham Pharmacia Biosciences Europe, Sacaly, France) in Ca^{++}/Mg^{++}-free-phosphate-buffer-saline (PBS) were added to 10 ml of blood. Each sample was sedimented at room temperature for 30 min. Supernatant was layered onto an equal volume of Polymorphprep (Nycomed Pharma As, Oslo, Norway) and centrifuged at 300 × *g* for 30 min at 18 °C. PMNLs were harvested, washed with PBS, counted, and finally suspended in MEM with 2% FCS at a concentration of 5×10^5 PMNLs/ml. Purity was ≥90%, as determined by May–Grunwald–Giemsa staining.

2.3. In vitro generation of CMV-infected PMNLs

A total of 1×10^6 infected fibroblasts (with 80% CPE) were resuspended in 24 ml of MEM supplemented with 8% FCS. One milliliter of this suspension was distributed in each well of a 24-well plate containing confluent monolayers of uninfected fibroblasts. Co-cultures were incubated for 48 h at 37 °C in order to obtain a minimum of 80% CPE in each well. The supernatant was then removed and replaced with 1 ml of the PMNL suspension at a ratio of 5×10^5 PMNLs:1×10^5 fibroblasts. Co-cultures were incubated for 20 h at 37 °C in a 5% CO_2 humidified atmosphere. PMNLs were recovered from culture supernatant by gentle pipeting and aspiration, and thus separated from infected fibroblast monolayers. PMNLs were washed twice in PBS, manually counted in a Malassez hemocytometer, and suspended in PBS at a concentration of 5×10^5 cells/ml for subsequent flow cytometry analysis.

2.4. pp65 Antigenemia assay in PMNLs

Detection of the viral matrix phosphoprotein pp65 was achieved by flow cytometry, according to a procedure developed in the laboratory (Imbert-Marcille et al., 1997).

As pp65 is located in the nucleus, the first step was the fixation–permeabilization of PMNLs. This involved sequential incubation with 1% paraformaldehyde (PFA; vwr international Merck Eurolab S.A., Fontenay sous Bois, France) and then with cold 80% methanol or a one-step incubation with 4% PFA. Cells were kept at −20 °C for at least 24 h and washed in PBS.

In a final volume of 50 μl, 5×10^5 cells were incubated successively with an appropriate amount of MAb diluted in PBS, supplemented with 20% AB serum, in order to saturate Fc receptors and thus limit nonspecific binding. Cells were first incubated with 0.2 μg of a mixture of two mouse MAb directed against pp65 (1C3/AYM clones, CINApool, Argène Biosoft, Varihles, France) for 45 min at 37 °C. After washes, a second incubation was carried out for 30 min at +4 °C with 0.75 μg of a FITC-F(ab′)2 goat anti-mouse mAb (Beckman Coulter France S.A., Villepinte, France). Negative control was obtained by replacing primary antibody by PBS-AB alone.

For the multi-parameter analysis, an additional incubation was undertaken with 5 μl of mouse isotype control (Ms IgG control isotype, Coulter Clone, Beckman Coulter), in order to saturate the free Fc sites of the FITC-F(ab′)2 goat anti-mouse mAb.

2.5. Leukocyte antigen immunolabeling

Leukocyte immunolabeling was carried out as follows: 30 min incubation of 5×10^5 cells with the allophycocyanin (APC) or phycoerythrin (PE)-labeled antibodies, in a total volume of 50 μl of PBS with 20% AB serum. Mouse IgG1 antibodies were all purchased from Beckman Coulter (Villepinte, France) and the appropriate volumes of each of the following antibody solutions were used: pan leukocyte antigen CD45-APC (J33 clone), L-selectin CD62L-PE (DREG56 clone), αL chain of the LFA-1 complex CD11a-PE (25.3 clone), αM chain of the Mac-1 complex CD11b-PE (Bear1 clone), αX chain of gp 150 CD11c-PE (BU15 clone), and β_2 chain CD18 (7E4 clone). PMNLs' phenotype was also analyzed; the presence of myeloid (CD15-PE – 80H5 clone, CD13-PE – SJ1D1 clone, CD33-PE – D3HL60.251 clone), lymphoid (CD4-PE – 13B8.2 clone, CD8-PE – B9.11 clone, CD19-PE – J4.119 clone), and monocyte markers (CD14-PE – RMO52 clone) was assessed as previously described. Negative controls were obtained by replacing specific MAb by 3.5 μl of PE-labeled IgG1 isotype control (679.1Mc7 clone).

After incubation, cells were washed in PBS and fixed in 1% formaldehyde at 4 °C before flow cytometry analysis.