Vascular pathophysiology of cytomegalovirus infection after kidney transplantation
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A SENSITIVE METHOD FOR QUANTIFYING CYTOMEGALIC ENDOThelial CELls IN PERIPHERAL BLOOD FROM CYTOMEGALOVIRUS-INFECTED PATIENTS

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Quantification of cytomegalic endothelial cells in PB

Abstract
A sensitive method has been developed for the quantification of cytomegalic endothelial cells in peripheral blood (CEC in PB) of patients with an active cytomegalovirus (CMV) infection. The three subsequent key steps of this method are density centrifugation to enrich endothelial cells in the mononuclear cell (MNC) fraction, endothelial cell specific staining, and fluorescence activated cell sorter (FACS) sorting of endothelial cells (EC) onto adhesion slides. The method of FACS sorting was compared with the conventional method of cytocentrifugation of the MNC fraction onto slides, followed by EC specific staining. The main advantage of the additional steps for the isolation and quantification of CEC in PB by FACS sorting is a ten times greater sensitivity than by cytocentrifugation of the MNC fraction alone. The recovery percentages of EC from whole blood were comparable for both methods. Recoveries of EC obtained after FACS sorting were 53 ± 16.5 % and recoveries of EC obtained after cytocentrifugation of the MNC fraction were 43 ± 4.3%. In patients with an active CMV infection 5 to 72 CEC were detected using FACS sorting, equivalent to 0.8 to 9.0 CEC/ml blood. With this method for isolation and quantification, the characterization of CEC in PB of patients with CMV associated clinical symptoms can be performed as well as the quantification of EC in PB of patients with pathophysiological manifestations involving endothelial damage different from CMV infections.

Introduction
In immunocompromised patients e.g. organ transplant recipients and HIV infected patients cytomegalovirus (CMV) infection may cause symptomatic infection involving several organs [30]. Patients with an active CMV infection may show subtle disturbances in organ function, even without clinically manifest CMV disease symptoms. An indicator of subtle disturbances in the lungs is a decrease in pulmonary diffusion for CO [31]. Effects of CMV infection on the intestines was shown by an increased intestinal permeability to lactulose [3]. Although mechanisms of CMV-induced pathophysiology in patients are not clear, we think endothelial cells are involved. Results from histochemical studies of CMV-infected lung and gastrointestinal tissues show that endothelial cells represent important targets for virus, together with epithelial cells, fibroblasts, and smooth muscle cells [23]. Another important finding is the occurrence of cytomegalic endothelial cells in peripheral blood (CEC in PB) described by Grefte et al [9,10], which appear during or shortly after the peak in CMV pp65 antigenemia in patients with an active CMV infection. These CEC in PB might be correlated with the severity of CMV disease and organ involvement [17,8], although we could not confirm a relationship between clinical symptoms and the mere presence of CEC in blood [9]. Therefore, the development of a quantitative method to detect CEC in PB should give more insight in a relationship between CEC counts in PB and organ involvement. In addition, with this method further studies towards characterization of CEC should be possible.

In addition to CMV infections, endothelial cells or endothelial cell carcasses circulating in blood have been noticed in several other pathophysiological conditions, including damage due to heart catheterization, infections, or intravascular coagulation [7,14,20,27]. At present,
different strategies to identify endothelial cells in blood have been described. One procedure makes use of Ficoll Hypaque density centrifugation followed by cytocentrifuge preparation of cells onto slides and the subsequent immunocytochemical staining of endothelial cells. This strategy was described for endothelial cells in the mononuclear cell fraction (MNC) of peripheral blood from patients after heart catheterization [20]. Also the CMV-infected endothelial cells were detected in the MNC fractions [9].

Another method was originally designed for the isolation of rare cell populations from blood, for instance epithelial cells in blood from cancer patients or isolation of stem cells from human cord blood [12,19], and involves fluorescence activated cell sorting (FACS) sorting.

For the development of a quantitative method, we isolated endothelial cells from whole blood by density centrifugation, followed by an endothelial cell specific staining and subsequently FACS sorting of the MNC fraction onto adhesion slides. The method of FACS sorting was compared to centrifugation of the MNC fraction onto slides, followed by endothelial cell specific staining. Experiments were performed with uninfected EC or HCMV infected EC and using these cell populations no differences in recovery were observed for both methods.

We report FACS sorting as a method with an improved sensitivity to study the kinetics in the occurrence of CEC in PB during CMV infection and for the further characterization of the isolated CEC in peripheral blood of CMV patients.

**Material and Methods**

**Antibodies**

Endothelial cell specific antibodies were E1/1 2.3, a mouse monoclonal antibody directed to a 90 kD cell surface antigen [18] and a polyclonal rabbit antibody against vWf (Dakopatts A/S, Glostrup, Denmark). Antibodies directed against exon 2 of major IE were Moab E13 [15] (FITC) (Biosoft, Paris, France) and C10/C11, a mixture of two mouse monoclonal antibodies directed to CMV pp65 [29].

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins [13,16]. Briefly, endothelial cells (EC) were harvested from umbilical cords using chymotrypsin and grown on 1% gelatine in endothelial growth medium (RPMI1640, 20% pooled human serum, or 20% foetal calf serum, endothelial cell growth factor 50 μg/ml, heparin 5 U/ml, glutamine 2 mM, penicillin 100 U/ml and 0.1 mg/ml streptomycin). EC were used at passage two or three.

**CMV-infected EC**

The endotheliotropic CMV clinical isolate TB42 [24] was used to infect endothelial cell cultures. Viral infection of EC was achieved by seeding trypsinized CMV-infected EC together with uninfected EC at a ratio 1:10 in culture flasks. After 6 days more than 95% of the EC were infected as determined by immunostaining of cytocentrifuged cells (Cytospin II,
Shandon, UK). Cells were analysed by immunofluorescence staining for CMV major IE viral protein.

**Patients**

Blood samples were obtained from two patients, three blood samples from the same patient at different timepoints and one blood sample from another patient. The samples were drawn from a cubital vein via venapuncture after 10 minutes of venous stasis (by tourniquet) and gently rubbing of the forearm. The samples were from two kidney transplant recipients with an active CMV infection diagnosed by positive CMV antigenemia [29].

**CMV antigenemia**

The CMV antigenemia assay was performed according the method described by Van der Bij et al [29]. Briefly, peripheral blood leukocytes were dextran-sedimented followed by lysis of remaining erythrocytes with NH₄Cl. After two washes the leukocytes were counted and cytospots were prepared. Spots were indirectly stained with C10/C11, a mixture of two mouse monoclonal antibodies directed against CMV pp65 [11]. Cells positive for pp65 cells were counted, the number of negative cells were counted by automated image analysis (Quantimet, Leica) and positive cells were expressed per 50,000 leukocytes screened. Two spots were analyzed for each patient sample.

**Enrichment of EC by density gradient centrifugation**

Using an *in vitro* model to study EC in blood, we added EC to whole blood or to MNC fractions. Diluted EC were counted twice in a Nageotte (Omnilabo, The Netherlands) haemocytometer before the cells were added to 1 ml whole blood or 1 x 10⁶ MNC. Blood obtained by venapuncture from healthy donors was collected in siliconized tubes (Vacutainer, Becton Dickinson, France) containing EDTA or heparin. Cell differentiation of whole blood samples was performed on a Coulter STKS (Coulter Electronics, Hialeah, Florida). The MNC fraction with or without added EC was isolated by LymfoPrep™ (Nycomed Pharma AS, Oslo, Norway, d = 1.077 g/cm²) density-gradient centrifugation and washed twice with RPMI1640. Cells were counted in a Coultercounter (Cell-Dyn 610, Abbott Diagnostics, Irving, Texas).

**FACS sorting**

EC were added to whole blood, to MNC, or first stained with E1/1 2.3, quantified and added immediately prior to FACS sorting. MNC with or without EC were stained with E1/1 2.3 on ice for 30 min, washed twice with ice-cold Hanks’ balanced salt solution, 5% human poolserum and subsequently labelled with FITC conjugated rabbit anti mouse IgG on ice for 30 min. Cells were washed twice and collected in Hanks’ balanced salt solution, 5% human pool serum. The FACS sorting was performed on a coulter Epics Elite equipped with a Gated Amplifier (Coulter Electronics, Hialeah, Florida, USA) and upgraded with enhanced system performance (ESP). A sortgate was set by measuring MNC and E1/1 2.3 labelled EC,
whereby the gate was selected for log forward scatter (FSC)/log side scatter (SSC) and FITC positive cells. Cells were triggered for sorting by a positive FITC signal. The FITC positive cells were sorted onto adhesion slides (Biorad, München, Germany) and fixed with 1% paraformaldehyde in phosphate buffered saline. Afterwards, the adhered cells were stained with 4’,6-diamidine-2’-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim) which binds selectively to DNA. Recovery was determined by counting FITC-positive cells with a pale oval nucleus. Three samples were processed and tested for each measurement, unless mentioned otherwise.

**Cytocentrifugation**

MNC fractions with or without EC were cytocentrifuged onto slides at 550 rpm for 5 min (Cytospin II). Cytospots were fixed with 1% paraformaldehyde in phosphate buffered saline and stained for EC markers. DAPI (Boehringer Mannheim) was used for counterstaining. The recovery was determined by counting the FITC-positive stained cells. Three samples were processed and tested for each measurement, unless mentioned otherwise.

**Statistical analysis**

The Unpaired t-test was used to compare differences in recovery.

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**Figure 1**

Expression of E1/1 2.3 on CMV-infected (white histograms) and non-infected EC (black histograms). For each group an E1/1 2.3-stained sample and an unstained sample are shown. moab, monoclonal antibody

**Results**

*EC recovery after FACS sorting*

To develop a quantitative method we determined EC recoveries for the different steps during the isolation, and we correlated EC losses to losses of MNC. The FACS sorting method was composed of three key steps: density centrifugation, EC cell specific staining, and FACS
sorting of EC out of the MNC fraction onto adhesion slides. Cell losses after density centrifugation step were 17.6 ± 15% of MNC and also of the EC. EC were obtained solely from the MNC fraction after density centrifugation, as no EC were detected in the granulocyte fraction. The largest loss of MNC including the EC was 30 ± 25% due to the washings of the cells during the staining procedure. Losses due to FACS sorting onto adhesion slides and adhesion of the sorted EC were negligible. Loss percentages of MNC and EC isolated from whole blood were similar, respectively 30 ± 25% and 38% (EC recovery 62 ± 17.2%). During the FACS purification itself virtually no EC were lost (EC recovery 98 ± 3.6 %), whereas most of the blood MNC (>99 %) were removed. Thus, recovery of EC added to whole blood is 53 ± 16.5%, caused by losses due to isolation of the MNC fraction and EC specific staining (Table 1).

**EC recovery after cytocentrifugation of MNC fractions**

Similarly, the effect of every step on specific loss of added EC was examined for the cytocentrifugation procedure. Density centrifugation resulted in a loss of 25.8 ± 13.8% of the cells, and cytocentrifugation of the MNC fraction caused an additional loss of MNC of approximately 33%. The recovery of EC from whole blood after density centrifugation and cytocentrifugation was 43 ± 4.3%. For each sample of the MNC fraction four or more cytospots were analyzed for quantification of EC among the MNC on spot. We also determined the variance between samples and the different spots per sample. The spread in recovery of EC between spots appeared to be larger (intra-test variance 23.5%) than between different samples (inter-test variance 6.0%). Thus EC isolated from whole blood using FACS sorting or cytocentrifugation of MNC resulted in similar recovery percentages of pre-added EC from whole blood: 43 ± 4.3 % and 53 ± 16.5 % respectively.

**Figure 2**

Recovery of CMV-infected or non-infected EC.

One hundred non-infected EC (open bars) or CMV infected EC (hatched bars) were added to 10⁶ MNC. No differences in recovery between CMV infected EC and non-infected EC were observed after FACS sorting (F) (P=0.239) or cytocentrifugation followed by subsequent immunofluorescent staining (C) (P=0.917) (unpaired t test). Experiments were performed in triplicate (FACS sorting of CMV-infected EC, n=2). For each sample, three or four cytospots were analyzed.
TABLE 1: Detection of CEC in blood of patients after FACS or after cytocentrifugation of MNC fractions

<table>
<thead>
<tr>
<th>Blood sample</th>
<th>Amt of blood analyzed (ml)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>No. CEC detected</th>
<th>No. of CEC / 500,000 MNC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FACS Cytospot</td>
<td>FACS Cytospot</td>
<td>FACS Cytospot</td>
</tr>
<tr>
<td>Sample 1</td>
<td>8.0</td>
<td>72</td>
<td>9.0</td>
</tr>
<tr>
<td>Sample 2</td>
<td>4.0</td>
<td>43</td>
<td>10.8</td>
</tr>
<tr>
<td>Sample 3</td>
<td>5.8</td>
<td>13</td>
<td>2.2</td>
</tr>
<tr>
<td>Sample 4</td>
<td>6.0</td>
<td>5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

1. Three blood samples were obtained from a single patient at different time points (samples 1 to 3) and one blood sample was from another patient (sample 4).
2. The amount of blood analyzed refers to the final number of MNC analyzed. MNC were originally obtained from the indicated blood volumes.
**CMV-infected EC**

The surface expression level of the antigen for MoAb E1/1 2.3 was more heterogeneous on CMV-infected EC and the mean fluorescence intensity was lower than of uninfected EC (Fig. 1). However, the expression level was sufficiently high to discriminate between FITC positive and negative cells (FITC-fluorescence is used as sortpulse). During the FACS sorting procedure or cytocentrifugation of MNC CMV-infected EC and uninfected EC behaved similarly (Fig. 2). FACS sorting of CMV-infected EC showed an extra decrease of 18% in recovery; uninfected EC did not show any decrease (Fig. 2). However, this difference was not statistically significant.

**Detection range**

Next, we studied the recovery of a dilution series of endothelial cells added to MNC varying from 5000 to 5 of EC to 1 x 10^6 MNC. A minimum of 5 EC was reproducibly detected using FACS sorting as well as by using cytocentrifugation and immunofluorescent staining (Fig. 3). The quantification of EC on cytospots with limited spread in recovery was only possible with a minimum of 50 EC added to 1 x 10^6 MNC. Below this level recovery decreased and the variation of the recovery increased (Fig. 3). Applying the FACS procedure, about 60% of the pre-added EC were recovered over the whole range of added EC tested. In addition, the recovery was also constant when 50 EC were isolated from either 1, 5 or 10 x 10^6 MNC (data not shown).

**Figure 3**

Lower limit of detection. A range of 5 to 5000 EC were added to 10^6 MNC. Recovery was determined by FACS (hashed bars) or by cytocentrifugation and immunofluorescent staining (open bars). Experiments are performed in triplicate (where no error bar is depicted, n=1). For each sample at least three or four cytospots were analyzed.
Quantification of CEC in PB from patients

To evaluate the quantification procedure for CEC in PB, we determined CEC counts in PB from four blood samples of patients with an active CMV infection and compared detected CEC numbers after FACS sorting or cytocentrifugation. After FACS sorting of a range of 4 to 8 ml of blood (Table 2), 5 to 72 CEC were detected, equivalent of 0.8 to 9.0 CEC/ml blood (sample 4, sample 1). Counting CEC on cytopsots of MNC fraction resulted in the detection of maximally 14 CEC (sample 1). Although less CEC were detected per cytopspot, the recoveries of CEC per ml blood were comparable in the samples containing approximately 10 CEC per ml blood.

Dextran sedimentation of granulocytes with subsequent cytocentrifuge preparation (routinely performed to monitor CMV antigenemia [29]) and immunocytochemical staining specific for endothelial cells was performed to detect numbers of CEC in PB. The same blood samples were processed as used for FACS sorting and cytocentrifugation of MNC fractions. The number of CEC per ml blood obtained by dextran sedimentation (Table 3) did not correlate with the number of CEC per ml blood FACS sorting onto slides or on cytopsots of MNC fractions (Table 2).

| TABLE 2: Detection of CEC in PB from CMV patients by dextran sedimentation. |
|---------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Blood sample             | Amt of blood analyzed (ml) | No. of granulocytes / ml of blood (10^6) | No. of CEC detected | No. of CEC / ml blood | CMV-antigenemia |
| Sample 1                 | n.d. \(^2\)      | n.d.            | n.d.            | n.d.            | 3410            |
| Sample 2                 | 0.1              | 5.9             | 0               | 0               | 3500            |
| Sample 3                 | 1.0              | 0.5             | 0               | 0               | 1571            |
| Sample 4                 | 1.0              | 0.6             | 1               | 1               | 52              |

\(^1\) Samples 1 to 4 are the same as in table 1. However, CEC were isolated by dextran sedimentation.  
\(^2\) The amount of blood analyzed refers to the final number of MNC analyzed. MNC were originally obtained from the indicated blood volumes.  
\(^3\) n.d.: not determined.  
\(^4\) Values are the numbers of pp65-positive granulocytes per 50,000 polymorphonuclear leukocytes.

Discussion

We present a three-step method to isolate and quantify cytomegalic endothelial cells from peripheral blood samples. For the development of this quantitative method we used in vitro cultured EC pre-added to different steps of the isolation procedure and cell losses during the individual isolation steps were determined. Because EC were detected only in the MNC fraction, we anticipated that these cells would behave like MNC during isolation and staining.
procedures. As expected, the losses of MNC and EC were comparable. Thus, determination of MNC losses before isolation and just before sorting indicated a loss factor for EC showing that the numbers of EC in blood were approximately twice the number of detected EC. After FACS sorting and binding of sorted cells to adhesion slides almost all cells were recovered on the slide. After cytocentrifugation of the MNC fraction approximately 40% of all cells were lost. These inevitable losses were due both to adherence of cells to the centrifugation cups and to cells being drawn into the papercards. As shown in Table 1 cell losses were negligible after binding to adhesion slides. So, determination of MNC counts prior to FACS sorting indicates losses of EC during the whole three step quantification procedure.

Because functional and morphological properties of CMV-infected endothelial cells are seriously disturbed [1,2,5,9,21,22,25,26,28], isolation characteristics of CEC from patients could differ from uninfected EC. Especially expression of surface antigens is influenced by infection of EC with CMV [2,9,21,25]. Therefore, we verified the expression of the antigen of MoAb E1/1 2.3 on CMV-infected EC and confirmed staining of EC with MoAb E1/1 2.3. Although a decreased expression of this EC antigen after infection of EC with CMV was observed (Fig. 1), the MoAb could still be used as a tool for immunoaffinity methods. In addition, little or no crossreactivity with other blood cells should occur. Some monocytes stained weak positive with E1/1 2.3; however this background did not disturb our quantification because the isolated cells were also evaluated afterwards by fluorescence microscopy for the cytomegalic morphology and the fluorescent intensity.

The present study with infected EC, including late-stage infected EC with the owl’s eye appearance, showed a slight reduction in the recovery, which could be due to a decreased expression of the antigen for Moab E1/1 2.3 (Fig. 1). Another reason for the reduction in the recovery could be increased fragility of EC after CMV infection (30% more cell death of CMV-infected EC compared to uninfected EC).

For the development of a quantitative method to detect CEC in PB using FACS sorting the MNC fraction with EC was stained with MoAb E1/1 2.3, followed by FITC conjugated rabbit-anti-mouse Ig. Because an additional staining step involves additional washings, the recovery of EC out of the MNC fraction could be improved by using primary antibodies which are conjugated directly to a fluorescent dye, e.g. an E1/1 2.3-FITC conjugate.

According to our results obtained by in vitro added EC (approximately 50% recovery for both methods), the isolation of CEC from peripheral blood from patients by FACS sorting or by cytocentrifugation resulted in an equal yield of CEC per milliliter of blood for each method. However, after isolation of CEC from patients by FACS sorting, a five-fold greater level of CEC could be detected than by cytocentrifugation and immunofluorescent staining. The increased detection level of CEC after FACS sorting was the result of the higher number of cells that could be processed and the almost complete removal of the MNC. Consequently, a high signal (CEC) to noise (MNC) ratio was obtained, allowing for easy quantification of the sorted CEC after their binding to adhesion slides.
Another aspect of the comparison between FACS sorting and cytocentrifugation is the influence of clinical symptoms like leukopenia [30] or lymphocytosis [4]. Both symptoms can occur in transplant recipients after rejection therapy or due to CMV infection and result in altered MNC levels in blood. Alteration in the MNC levels will alter the number of CEC in PB per number of MNC. Until now CEC have been correlated with MNC numbers present on cytosots instead of blood volume [8-10,17]. However, CEC in PB are likely to originate from the venous vessel wall and might therefore be more indicative of vascular damage. To correct for high levels of CEC in patients caused by low MNC numbers, we argue for the relation between CEC and blood volume.

Regarding a total body blood volume of approx. 5 liters, the detected concentrations of 10 CEC/ml PB actually represented 50,000 endothelial cells. No data are available about kinetics of the clearing of the circulating EC. Data obtained from CEC in PB after angioplasty showed comparable concentrations of EC in arterial as well as venous blood [6,20], suggesting that released endothelial cells might circulate. In the case of CMV-infected cytomegalic altered endothelial cells (size 30-40 µm) it is unknown whether these cells will recirculate or become trapped in capillary vessels, thus evoking disturbances in organ function.

In conclusion, a reliable three step method based on FACS sorting was developed to isolate and quantify CEC from PB of patients with an active CMV infection. This method resulted in greater sensitivity than analysis of the MNC fraction on cytosots as described by us earlier [9]. Furthermore, we argue to relate this CEC count to the blood volume analyzed in order to make the results more comparable.

Endothelial cells in peripheral blood are described for several pathophysiological conditions involving endothelial damage; thus our quantitative method could be applied to study the released cells caused by vascular damage. In addition, our method is useful for the detection of different kinds of rare cells circulating in the blood stream.

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**References**


