In immunocompromised patients, e.g., organ transplant recipients and human immunodeficiency virus-infected patients, cytomegalovirus (CMV) may cause symptomatic infections involving several organs (30). Patients with 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 were shown by an increased intestinal permeability to lactulose (3). Although mechanisms of CMV-induced pathophysiology in patients are not clear, we think endothelial cells (EC) are involved. Results from histochemical studies of CMV-infected lung and gastrointestinal tissues show that EC are important targets for virus, together with epithelial cells, fibroblasts, and smooth muscle cells (23).

Another important finding is the occurrence of cytomegalic EC (CEC) in peripheral blood (PB), as described by Grefte et al. (9, 10); CEC appear during or shortly after the peak in CMV pp65 antigenemia in patients with active CMV infection. These CEC in PB might be correlated with the severity of CMV disease and organ involvement (8, 17), although we were unable to 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 into the relationship between CEC counts in PB and organ involvement. In addition, with this method further studies toward characterization of CEC should be possible.

In addition to CMV infections, EC or EC 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 EC in blood have been described. One procedure makes use of Ficoll-Hypeaque density centrifugation followed by cytocentrifuge preparation of cells on slides and subsequent immunocytochemical staining of EC. This strategy was described for EC in the mononuclear cell (MNC) fraction of PB from patients after heart catheterization (20). Also, CMV-infected EC were detected in 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).

For the development of a quantitative method, we isolated EC from whole blood by density centrifugation, followed by EC-specific staining and subsequent FACS of the MNC fraction onto adhesion slides. The FACS method was compared to centrifugation of the MNC fraction onto slides, followed by EC-specific staining. Experiments were performed with noninfected EC or human CMV-infected EC; with these cell populations, no differences in recovery between the two methods were observed.

We report FACS as a method with improved sensitivity for studying the kinetics of the occurrence of CEC in PB during CMV infection and for further characterization of the isolated CEC in PB of CMV patients.

MATERIALS AND METHODS

Antibodies. EC-specific antibodies were E1/1 2.3, a mouse monoclonal antibody directed to a 90-kDa cell surface antigen (18), and a polyclonal rabbit antibody against vWF (Dakopatts A/S, Glostrup, Denmark). Antibodies directed against exon 2 of the major immediate early gene were E13 (15) (fluorescein isothiocyanate [FITC]) (Biosoft, Paris, France) and C10/C11, a mixture of two antibody against vWf (Dakopatts A/S, Glostrup, Denmark). Antibodies directed to a 90-kDa cell surface antigen (18), and a polyclonal rabbit antibody against vWF (Dakopatts A/S, Glostrup, Denmark). Antibodies directed against exon 2 of the major immediate early gene were E13 (15) (fluorescein isothiocyanate [FITC]) (Biosoft, Paris, France) and C10/C11, a mixture of two mouse monoclonal antibodies directed to CMV pp65 (29).

Cell culture. Human umbilical vein EC were isolated from human umbilical cord veins (13, 16). Briefly, EC were harvested from umbilical cords by using chymotrypsin and were grown on 1% gelatin in endothelial growth medium (RPMI 1640, 20% pooled human serum or 20% foetal calf serum, 50 µg of EC growth factor per ml, 5 U of heparin per ml, 2 mM glutamine, 100 U of penicillin...
per ml, and 0.1 mg of streptomycin per ml). EC were used at passage two or three.

CMV-infected EC. The endotheliotropic CMV clinical isolate TB42 (24) was used to infect EC cultures. Viral infection of EC was achieved by seeding tryptophan-CMV-infected EC together with uninfected EC at a ratio 1:1 in culture flasks. After 6 days, more than 95% of the EC were infected, as determined by immunostaining of cytospun cells (Cytospin II, Shandon, Astmoor, United Kingdom). Cells were analyzed by immunofluorescence staining for the CMV major immediate early viral protein.

Patrients. Blood samples were obtained from two patients: three blood samples from one patient at different time points and one blood sample from the other patient. The samples were drawn from a cubital vein via venapuncture after 10 min of venous stasis (by tourniquet) and gentle rubbing of the forearm. The samples were from two kidney transplant recipients with an active CMV infection, as diagnosed by positive CMV antigenemia (29).

CMV antigenemia. The CMV antigenemia assay was performed according to the method described by Van der Bij et al. (29). Briefly, peripheral blood leukocytes were dextran sedimented, followed by lysis of remaining erythrocytes with NH,Cl. After two washes, the leukocytes were counted and cytopsots 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 were counted, the number of negative cells were counted by automated image analysis (Quantimet, Leica, Rijswijk, The Netherlands), 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 sedimented twice in a Nagotte (Omninlabo, Etten-Leur, The Netherlands) hemocytometer before the cells were added to 1 ml of whole blood or 10^6 MNC. Blood obtained by venapuncture from healthy donors was collected in siliconized tubes (Vacutainer; Becton Dickinson, Meylan, France) containing EDTA or heparin. Cell differentiation of whole blood samples was performed on a Coulter STKS (Coulter Electronics, Hialeah, Fla.). The MNC fraction with or without added EC was isolated by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) (d = 1.077 g/cm^3) density gradient centrifugation and washed twice with RPMI 1640. Cells were counted in a Coultercounter (Cell-Dyn 610; Abbott Diagnostics, Irving, Tex.).

FACS. EC were added to whole blood or to MNC or were first stained with E1/1 2.3, quantified, and added immediately prior to FACS. 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 and 5% human pooled serum, and subsequently labelled with FITC-conjugated rabbit anti-mouse immunoglobulin G on ice for 30 min. Cells were washed twice and collected in Hanks' balanced salt solution and 5% human pooled serum. FACS was performed on a Coulter Elite equipped with a gated amplifier and upgraded with enhanced system performance. A sortgate was set by measuring MNC and E1/1 2.3-labelled EC, whereby the gate was selected for log forward scatter/log side scatter and FITC-positive cells. Cells were triggered for sorting by a positive FITC signal. The FITC-positive cells were sorted onto adhesion slides (Bio-Rad, Munich, Germany) and fixed with 1% paraformaldehyde in phosphate-buffered saline. Afterwards, the adhered cells were stained with DAPI (4',6-diamidino-2-phenylinodole hydrochloride) (Boehringer Mannheim, Almere, The Netherlands), 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 cytospun 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. Recovery was determined by counting 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.

RESULTS

EC recovery after FACS. To develop a quantitative method, we determined EC recoveries for the different steps during isolation and we correlated EC losses to losses of MNC. The FACS method was composed of three key steps: density centrifugation, EC-specific staining, and FACS of EC out of the MNC fraction onto adhesion slides. Cell losses after the density centrifugation step were 17.6% ± 15% (mean ± standard deviation) of MNC and also of 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 EC, was 30% ± 25% due to washing of the cells during the staining procedure. Losses due to FACS onto

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

adhesion slides and adhesion of the sorted EC were negligible. Loss percentages of MNC and EC isolated from whole blood were similar at 30% ± 25% and 38%, respectively (EC recovery, 62% ± 17.2%). During FACS purification itself, virtually no EC were lost (EC recovery, 98% ± 3.6%), whereas most of the blood MNC (>99%) were removed. EC added directly to adhesion slides were recovered at a rate of 94% ± 11.4%. Thus, recovery of EC added to whole blood was 53% ± 16.5%, caused by losses due to isolation of the MNC fraction and EC-specific staining.

EC recovery after cytocentrifugation of MNC fractions. Similarly, the effects of every step on specific losses of added EC were 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%. 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 cytopsots were analyzed for quantification of EC among the MNC on a 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 (intraspot variance, 23.5%) than between different samples (intrasample variance, 6.0%). Thus, EC isolated from whole blood by FACS sorting or cytocentrifugation of MNC resulted in similar recovery percentages of pread EC from whole blood, 43% ± 4.3% and 53% ± 16.5%, respectively.

CMV-infected EC. The surface expression level of the antigen for monoclonal antibody E1/1 2.3 was more heterogeneous

FIG. 2. Recovery of CMV-infected and noninfected EC. One hundred noninfected EC (open bars) or CMV-infected EC (hatched bars) were added to 10^6 MNC. No differences in recovery between CMV-infected EC and noninfected EC were observed after FACS (F, P = 0.239) or cytocentrifugation followed by subsequent immunofluorescence staining (C) (P = 0.917) (unpaired t test). Experiments were performed in triplicate (FACS of CMV-infected EC, n = 3). For each sample, three or four cytopsots were analyzed.
three or four cytospots were analyzed. 

50 EC added to 10^6 MNC. Below this level, recovery decreased limited spread in recovery was possible only with a minimum of FACS as well as by cytocentrifugation and immunofluorescent to 10^6 MNC. A minimum of 5 EC was reproducibly detected by series of EC added to MNC, varying from 5,000 to 5 EC added to 0.8 to 9.0 CEC/ml of blood (sample 4, sample 1). Counting ml of blood (Table 1), 5 to 72 CEC were detected, equivalent FACS or cytocentrifugation. After FACS of a range of 4 to 8 CMV infection and compared detected CEC numbers after quantification procedure for CEC in PB, we determined CEC significant.

Deion level was sufficiently high to discriminate between FITC-positive and -negative cells (FITC fluorescence is used as the sortpulse). During the FACS procedure or cytocentrifugation of MNC, CMV-infected EC and uninfected EC behaved similarly (Fig. 2). FACS of CMV-infected EC showed an extra of MNC, CMV-infected EC and uninfected EC behaved sim-

mately (Fig. 2). FACS 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 EC added to MNC, varying from 5,000 to 5 EC added to 10^6 MNC. A minimum of 5 EC was reproducibly detected by FACS as well as by cytocentrifugation and immunofluorescent staining (Fig. 3). The quantification of EC on cytospots with limited spread in recovery was possible only with a minimum of 50 EC added to 10^6 MNC. Below this level, recovery decreased and the variation of recovery increased (Fig. 3). With the FACS procedure, about 60% of the preadded EC were recovered over the whole range of added EC tested. In addition, recovery was also constant when 50 EC were isolated from either 1 x 10^6, 5 x 10^5, or 10 x 10^6 MNC (data not shown).

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 active CMV infection and compared detected CEC numbers after FACS or cytocentrifugation. After FACS of a range of 4 to 8 ml of blood (Table 1), 5 to 72 CEC were detected, equivalent to 0.8 to 9.0 CEC/ml of blood (sample 4, sample 1). Counting of CEC on cytospots of MNC fraction resulted in the detection of maximally 14 CEC (sample 1). Although fewer CEC were detected per cytospot, the recoveries of CEC per milliliter of blood were comparable in the samples containing approximately 10 CEC per ml of blood.

Dextran sedimentation of granulocytes with subsequent cytocentrifuge preparation (routinely performed to monitor CMV antigenemia [29]) and immunocytochemical staining specific for EC were performed to detect numbers of CEC in PB. Similar blood samples were processed as used for FACS and cyto-

centrifugation of MNC fractions. The number of CEC per milliliter of blood obtained by dextran sedimentation (Table 2) did not correlate with the number of CEC per milliliter of blood by FACS onto slides or on cytospots of MNC fractions (Table 1).

**DISCUSSION**

We present a three-step method to isolate and quantify CEC from PB samples. For the development of this quantitative method, we used in vitro-cultured EC preadded 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 iso-

lation 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 and binding of sorted cells to adhesion slides, almost all cells were recovered on the slides. 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 paper cards. Cell losses were negligible after binding to adhesion slides (recovery, 94% ± 11.4%). Determination of MNC counts prior to FACS,
released EC might circulate. In the case of CMV-infected infections of EC in arterial and venous blood (6, 20), suggesting that CEC in PB after angioplasty showed comparable concentrations of EC in PB are described for several pathophysiological conditions involving endothelial damage; thus, our quantitative method could be applied to study the release of cells caused by vascular damage. In addition, our method is useful for detection of different kinds of rare cells circulating in the blood.

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