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## Evaluation by polymerase chain reaction of cytomegalovirus reactivation in intensive care patients under mechanical ventilation

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**Abstract** *Objective:* The study was undertaken to determine if critically ill patients under mechanical ventilation could reactivate latent cytomegalovirus (CMV) in either lung or blood.

*Design:* Prospective study in critically ill patients.

*Setting:* The study was performed in a multidisciplinary intensive care unit in a university hospital.

*Patients:* 23 non-immunocompromised, mechanically ventilated patients who were anti-CMV immunoglobulin G-positive. Ten immunocompromised patients with active CMV infection and 16 asymptomatic CMV seropositive non-immunocompromised patients constituted the positive and negative control groups.

*Measurements and results:* The presence of CMV in blood and bronchoalveolar lavage (BAL) was evaluated by both viral cultures and polymerase chain reaction (PCR). Thirty-seven blood and 22 BAL samples were in-

vestigated. Sequential samples were evaluated in 8 patients. For PCR, a 290 bp fragment in the first exon of the immediate early 1 gene was amplified. In order to exclude inhibitors of PCR amplification, a 268 bp fragment of the  $\beta$ -globin gene was concurrently amplified in all samples. Viral cultures of blood and BAL were negative in all 23 non-immunocompromised, mechanically ventilated patients. Moreover, no CMV DNA could be amplified in blood or BAL samples, whereas a  $\beta$ -globin amplification was observed in all samples. *Conclusion:* In a series of 23 critically ill patients under mechanical ventilation who were seropositive for CMV, no reactivation of CMV in blood or lung was demonstrated.

**Key words** Cytomegalovirus · Polymerase chain reaction · Reactivation · Critically ill patients

### Introduction

Cytomegalovirus (CMV) infection – especially CMV pneumonia – is a major cause of illness and death in immunocompromised patients [1]. Infection with CMV is usually asymptomatic in the immunocompetent host [2], but there are a few reports of infection or reactivation of CMV with viral detection in blood in patients sepsis [3, 4]. The incidence of such events in blood has been reported to range between 20 and 96% in patients with sep-

sis. After primary infection, the virus persists in the host as a latent infection, which can periodically reactivate to cause active infection [2]. The mechanism of reactivation is unknown but enhanced tumor necrosis factor levels may reactivate CMV [4]. White blood cells, especially monocytes, may harbor the latent virus in healthy seropositive individuals [5, 6], and CMV is known to be transmitted by blood transfusion [7]. It has also been shown that the lung could be a site of CMV latency in humans [8]. The control of CMV replication by the host involves mechanisms of both natural and acquired immunity [9, 10]. The natural

killer cells are the principal effector cells regarding natural immunity, and B-cells and T-cells (especially CD8 T-cells) are the main components of the acquired immunity. These cell types are interconnected through accessory cells [9, 10]. Critically ill patients frequently demonstrate profound immunologic abnormalities as a result of either their illness or its treatment [11]. The present study was therefore designed to investigate the hypothesis that critically ill, mechanically ventilated, non-immunocompromised patients could reactivate latent CMV either in blood or lung.

We used an *in vitro* gene amplification technique, the polymerase chain reaction, which has been applied to the detection of CMV DNA in peripheral blood leukocytes (PBLs) [12–16] and bronchoalveolar lavage (BAL) specimens [17–19]. PCR amplification of a 290-bp fragment of the exon 1 of the major immediate early gene was used to test the DNA extracted from blood or BAL cells collected in critically ill, mechanically ventilated, non-immunocompromised patients [20]. Conventional viral culture and rapid culture by the shell vial technique were performed simultaneously [21]. The results obtained failed to demonstrate CMV reactivation in blood or lung from this series of 23 patients.

## Patients and methods

### Patients

Between January and August 1993, 23 non-immunocompromised patients admitted to the intensive care unit were included in this study. We have evaluated anti-CMV immunoglobulin G (IgG)-positive patients who had: (a) an expected duration of hospitalisation for at least 5 days; (b) a need for mechanical ventilation; (c) a previous surgical procedure, or a sepsis syndrome, or a pulmonary injury. Upon entry into the study, the hospital records of each patient were reviewed, and the following clinical variables were noted: age, sex, severity of underlying medical conditions stratified according to the criteria of McCabe and Jackson as fatal, ultimately fatal, and non-fatal [22], first-day Acute Physiology and Chronic Health Evaluation II score, which is an index of the severity of illness of patients admitted to an ICU [23], white blood cell count, serum creatinine, bilirubin and aspartate aminotransferase, partial pressure of oxygen fractional inspired oxygen (FIO<sub>2</sub>) ratio, pulmonary impairment determined by the Lung Injury Score described by Murray et al. [24], duration of admission, and final clinical outcome. The criteria of the sepsis syndrome have been previously reported [25] and include: (1) clinical suspicion of infection; (2) temperature <35.5°C or >38.3°C; (3) tachycardia >90 beats min; (4) mechanical ventilation; (5) inadequate organ perfusion or dysfunction as expressed by one of the following: alteration in mental status, hypoxemia (partial pressure of oxygen in arterial blood <72 mmHg at FIO<sub>2</sub>=0.21), elevated plasma lactate or oliguria (urine output <30 ml or 0.5 ml/kg for at least 1 h). Septic shock was defined with the above criteria plus: a decrease in systolic arterial pressure (<90 mmHg) despite adequate vascular filling; and need for vasoactive drugs (dopamine >5 µg/kg per min, dobutamine, epinephrine, or norepinephrine).

Twelve patients received packed red blood cells during the month prior to inclusion in the study. In order to exclude exogenous transmission of CMV, packed red cells were filtered through a SQ40 PALL filter (PALL Biomedical France, Saint-Germain-en-Laye,

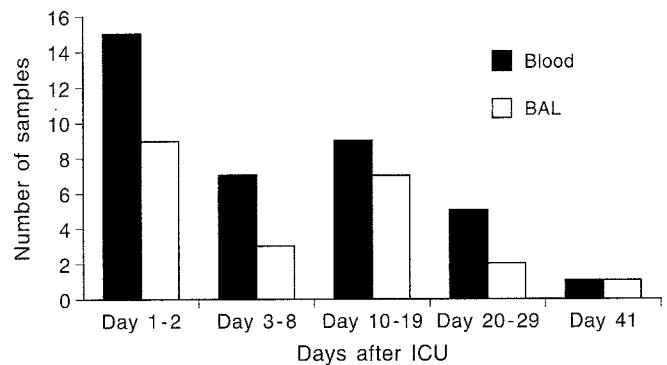


Fig. 1 Distribution of blood and BAL samples after ICU admission

France). According to the manufacturer, such a filter removes  $\geq 95\%$  of the leukocytes in a unit of normal blood.

Ten additional patients with kidney ( $n=5$ ) or bone marrow ( $n=5$ ) transplantation and active CMV infection were also studied. Blood and BAL cultures were positive for CMV. These patients constituted the positive control group.

Sixteen asymptomatic non-immunocompromised patients who were CMV-seropositive constituted the negative control group. In all of these patients, the search for CMV by viral cultures in BAL fluid, blood or urine was negative.

### Serology

The presence of specific IgG anti-CMV antibodies was tested in serum using an enzyme-linked immunosorbent assay (CMV IgG Wellcome (Dartford, Kent, UK)).

### Specimen collection

#### BAL procedure

Among the 23 patients studied, 14 patients underwent a BAL procedure. BAL ( $n=22$ ) was performed at a mean of 10 days (range, 1–41 days) after ICU admission for diagnosis of new or persistent lung infiltrates ultimately related to pneumonia ( $n=17$ ), pulmonary edema ( $n=3$ ), and atelectasis ( $n=2$ ) (Fig. 1). Therefore, in 4 patients, BAL samples were evaluated on  $\geq 2$  occasions. Briefly, after wedging the bronchoscope into a subsegmental bronchus of either the right middle lobe or the lobe with maximal infiltration on chest roentgenograms, sterile 0.9% sodium chloride was infused in 20- or 50-ml aliquots, for a total of 150 to 200 ml, and gently aspirated after each instillation. Total cell counts were evaluated using a hemocytometer, and differential counts were performed on cytocentrifuge preparation using a Cytospin 2 centrifuge (Shandon Southern Products, Cheshire, UK); stained with Wright-Giemsa stain. Cytocentrifuge preparations of these cell populations showed a mean ( $\pm$ SEM) of 16.6 $\pm$ 4% alveolar macrophages, 80 $\pm$ 4.5% neutrophils, 3.3 $\pm$ 0.9 lymphocytes, and 0% eosinophils. The mean ( $\pm$ SEM) total cell count was 535.8 $\pm$ 127.4 cells  $\pm 10^6$ /ml.

#### Peripheral blood leukocytes

Blood samples ( $n=37$ ) were collected from all the 23 patients at a mean of 5 days (range 1–41 days) for the first sample and at a mean of 17 days (range 6–29 days) for the others (Fig. 1). Finally, in 8 patients sequential samples were taken.

### Viral isolation

Both conventional viral culture (up to 21 days) and rapid culture by the shell vial technique were used for BAL fluid and blood, as previously described [21].

### BAL and blood sample preparation

PBLs were isolated from 5 ml of ethylenediaminetetraacetic acid (EDTA) blood. The buffy coat was aspirated and treated with lysis buffer (0.9 mM ammonium bicarbonate, 0.13 M ammonium-<sup>15</sup>N chloride) to lyse red cells. The cell pellet ( $10^6$  cells) was stored at  $-20^{\circ}\text{C}$ .

BAL samples were centrifuged at 1000 rpm for 10 min at  $4^{\circ}\text{C}$ . The supernatant was removed and the cell pellet was resuspended in the lysis buffer at a concentration of  $10^6$  cells/ml. Aliquots of  $10^6$  cells were stored at  $-20^{\circ}\text{C}$  until the sample was processed for testing.

### DNA extraction

DNA was extracted by a rapid extraction method [26]. Briefly, the cells were resuspended in 50  $\mu\text{l}$  of solution A (100 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM  $\text{MgCl}_2$ ), and in 50  $\mu\text{l}$  of solution B (10 mM Tris-HCl (pH 8.3), 2.5 mM  $\text{MgCl}_2$ , 1% Nonidet-P40, 1% Tween 20). The cells were digested by incubation with proteinase K (200  $\mu\text{g}/\text{ml}$ ) at  $60^{\circ}\text{C}$  for 1 h. Proteinase K was then inactivated by placing the tube in a  $95^{\circ}\text{C}$  waterbath for 10 min. The DNA released with this procedure can be amplified directly.

### Control human CMV (HCMV) viral DNA

The usual fibroblast (MRC5) cell line was infected in vitro with a wild-type strain of HCMV isolated from a patient with acute CMV infection. The presence of CMV was confirmed by the observation of the characteristic cytopathic effect. The cells were scraped and harvested 5 to 7 days after being infected and the DNA was extracted as described above.

### Conditions for polymerase chain reaction (PCR) amplification

PCR was performed according to the method described previously [19]. For PCR amplification, we chose to amplify a 290-bp segment located in the exon 1 of the HCMV immediate early gene [20]. This region of the genome was selected since it is markedly conserved between the various human CMV strains [27]. Two primers were used: 5' primer sequence, 5'-ATCGCCTGGAGACGCCATCC-3'; 3' primer sequence, 5'-CGTCACCAATAGGGGAGTGG-3'. These oligomers were purchased from Genset and were synthesized on a DNA synthesizer as recommended by the manufacturer (Genset, Paris, France). Amplification reactions were performed according to the procedure of Saiki et al. [28]. Ten  $\mu\text{l}$  of DNA samples were routinely amplified for 30 cycles (DNA Thermal Cycler, Perkin-Elmer-Cetus) using 2.5 U/100  $\mu\text{l}$  of Taq DNA polymerase (Perkin-Elmer-Cetus) in 1.5 mM  $\text{MgCl}_2$  with reaction times of 1 min at  $55^{\circ}\text{C}$  for annealing, 2 min at  $72^{\circ}\text{C}$  for extension, and 1 min at  $94^{\circ}\text{C}$  for denaturation.

We controlled for internal consistency standards, i.e., DNA from and HCMV-infected MRC5 fibroblast cell line was included in each PCR reaction. Negative controls contained the above PCR mixture, with distilled water replacing the template DNA to control for PCR product contamination of reagents. To minimize the risk of contamination, the PCR technique was physically separated from the laboratory area in which DNA extraction was performed. To exclude the presence of polymerase inhibitors and to control the quality of the DNA extracted, a 268-bp fragment of the human  $\beta$ -globin gene (Gene

Amplimer Betaglobin primers; Perkin-Elmer-Cetus) was amplified in parallel.

### Analysis of PCR amplification products

Amplified sequences were analyzed by direct gel electrophoresis and by slot-blot hybridization assay using an oligonucleotide probe (5'-CATTGGAACGCGGATTCC-3', purchased from Genset). For direct gel analysis, aliquots of each amplification reaction were electrophoretically separated in 2% agarose gels in Tris-acetic acid-EDTA containing 0.15  $\mu\text{g}/\text{ml}$  of ethidium bromide. DNA was visualized by ultraviolet fluorescence. DNA molecular weight marker VIII (Boehringer Mannheim Biochemica, Mannheim, Germany) was included in each gel.

Slot-blot analysis was performed as previously described [19]. Briefly, 3  $\mu\text{l}$  of the reaction mixture was denatured and the DNA was immediately applied to a nylon filter membrane (Hybond-N<sup>+</sup>; Amersham, les Ulis, France). The DNA samples were then hybridized with a homologous <sup>32</sup>P-labeled 18-base oligonucleotide probe. Autoradiography was routinely performed for 2 h at  $-80^{\circ}\text{C}$  using X-Omat autoradiograph film (Eastman Kodak, Rochester, N.Y., USA).

The sensitivity of the protocol used in this study, determined from experiments on human CMV-infected fibroblasts, has been previously reported [19]; it was concluded that the sensitivity of the protocol was one viral copy per  $10^4$  cells by slot-blot assay.

## Results

### Study population

Demographic characteristics and other markers of acute illness in the 23 mechanically ventilated patients are shown in Table 1. Anti-CMV IgG were detected in each patient's serum. Thirteen patients had undergone a previous surgical procedure which was complicated by peritonitis ( $n=3$ ), adult respiratory distress syndrome (ARDS) ( $n=2$ ), pneumonia ( $n=2$ ), mediastinitis ( $n=2$ ), coma ( $n=1$ ), myocardial infarction ( $n=1$ ), and ischemic hepatitis ( $n=1$ ), and 1 patient died the day after a surgical procedure for gastrointestinal bleeding. Ten patients had also been admitted to the ICU for a variety of disorders, including community-acquired pneumonia ( $n=3$ ), pulmonary contusion ( $n=2$ ), coma ( $n=2$ ), ARDS ( $n=1$ ), myocardial infarction with pulmonary edema ( $n=1$ ), and pyelonephritis ( $n=1$ ). Finally, 15 patients had a documented infection and 13 of these had sepsis syndrome at the time of the study. Among these 13 patients, 11 had septic shock. Overall mortality was 52%.

### PBLs and BAL cells

#### *In acute CMV infection*

The amplified DNA extracted from PBLs and BAL cells of CMV-infected patients gave a positive signal for CMV when tested by gel electrophoresis and slot-blot analysis (Fig. 2, lanes A and C, columns 2, 3).

**Table 1** Clinical and biochemical characteristics of patients (APACHE Acute Physiology and Chronic Health Evaluation, WBC white blood cell, AST aspartate aminotransaminase,  $PaO_2$  partial pressure of oxygen in arterial blood,  $FiO_2$  fractional inspired oxygen, ARDS adult respiratory distress syndrome)

	Mean $\pm$ SEM <sup>a</sup>	Range
Number of patients	23	
Age (years)	61 $\pm$ 3.4	26–83
Sex, M/F	14/9	
APACHE II score	25 $\pm$ 2	8–50
Previous surgical procedure <sup>b</sup> (n)	13	
Severity of underlying disease (n)		
Fatal or ultimately fatal within 5 years	9	
Non-fatal	14	
Patients with chronic alcohol abuse (n)	4	
WBC count ( $10^9/l$ )	15 $\pm$ 2.1	1.3–37.7
Lymphocyte count ( $10^9/l$ )	0.97 $\pm$ 0.1	0.11–2.03
Creatinine ( $\mu$ mol/l)	174 $\pm$ 32	50–660
Bilirubin ( $\mu$ mol/l)	56.5 $\pm$ 18	5–333
AST (mU/ml)	164 $\pm$ 70	14–1130
$PaO_2/FiO_2$ ratio	191 $\pm$ 21	63–440
Murray's ARDS score	1.7 $\pm$ 0.3	0–3.8
Duration of hospitalization (days)	19 $\pm$ 3	1–45
No. who died	12	

<sup>a</sup> For quantitative variables

<sup>b</sup> Includes 6 abdominal, 4 thoracic, 2 thoraco-abdominal, and 1 orthopedic

#### In asymptomatic seropositive patients

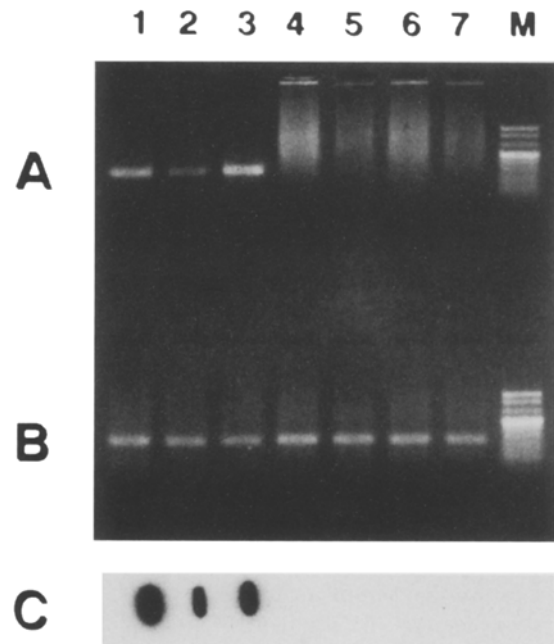
The amplification of the DNA extracted from BAL cells and PBLs of asymptomatic CMV-seropositive patients was negative for all patients when tested by direct gel electrophoresis analysis as well as by slot-blot assay (Fig. 2, lanes A and C, columns 6, 7).

#### In seropositive non-immunocompromised critically ill, mechanically ventilated patients

Amplification of the DNA extracted from BAL cells and PBLs of CMV-seropositive, critically ill, mechanically ventilated patients was negative for all patients when tested by direct gel electrophoresis as well as by slot-blot assay (Fig. 2, lanes A and C, columns 4, 5). In contrast, amplification of DNA from PBLs or BAL cells of the 268-bp fragment of the human  $\beta$ -globin gene gave positive results in all cases (Fig. 2, lane B, columns 4, 5).

## Discussion

In the present study evaluating a series of 23 patients, the search for CMV in blood or BAL was negative by either PCR or viral cultures in all of the critically ill patients seropositive for CMV.



**Fig. 2** Analysis of PCR amplification products. Lane A DNA electrophoresis on 2% agarose after ethidium bromide staining and ultra-violet analysis showing the presence of the 290-bp CMV-specific amplification product in CMV-infected MRC5 cells (column 1) and in DNA from PBLs and BAL cells from CMV-infected patient (columns 2, 3); but not in DNA from PBLs and BAL cells from a critically ill patient (columns 4, 5) and an asymptomatic CMV-seropositive subject (columns 6, 7). Column M is the DNA molecular weight marker. Lane B the 268-bp  $\beta$  globin-specific amplification product was detected in all DNA samples tested. Lane C autoradiograph after hybridization with the <sup>32</sup>P-labeled probe showing results of PCR amplification of DNA from CMV-infected MRC5 cells (column 1), from PBLs and BAL cells from CMV-infected patient (columns 2, 3), from critically ill patient (columns 4, 5) and from an asymptomatic CMV-seropositive subject (columns 6, 7)

The population studied included critically ill patients with several factors which could contribute to depressed cellular immunity and therefore potentially affect the control of CMV latency. Lymphopenia (total lymphocyte count below  $1.5 \times 10^9/l$ ) was found in 87% of the 23 patients, this has been shown to be associated with stress, inflammation, infection and severity of disease [29]. Moreover, the majority of the patients had undergone a major surgical procedure, which has been shown to be associated with altered T-cell function [11, 29]. Last, some patients, especially those with septic shock, would have detectable levels of tumor necrosis factor [30], which was possibly associated with CMV reactivation [4].

The search for CMV DNA was performed by using PCR amplifying a 290-bp fragment from the first exon of the major CMV immediate early gene [20]. PCR has been reported to be successful in the detection of CMV in blood and BAL cells in CMV infection and has therefore been considered to be a particularly accurate diagnostic

method [12–18]. Of particular interest from the perspective of PCR is whether it is so sensitive that all infections, latent or active, will be detected. However, our assay did not detect CMV DNA in the blood of normal CMV seropositive healthy controls, as previously reported [19] – a result similar in other studies [12, 13, 16, 17]. Therefore, detection of CMV DNA by PCR could be related to an active CMV infection in the non-immunocompromised host. The choice of primers for amplification requires the use of highly conserved genomic sequences, such as those in the 240-kb CMV genome coding for the major immediate early antigen [27]. When testing DNA extracted from PBLs or BAL cells collected from CMV-infected patients, it was possible to detect the presence of CMV DNA in each of these patients. These patients were tested at random and could therefore be assumed to be infected by different wild strains of CMV. The choice of primers used for PCR in the present study generates the amplification of a sequence that can therefore be considered to be conserved in the various strains of CMV.

The sensitivity of the protocol used in this study has been previously reported [19] and is in accordance with that reported by other investigators [13, 15]. Therefore, the hypothesis explaining the negative findings in the present study is that the PCR assay is not sufficiently sensitive to detect very low levels of virus. However, the level of detection of the method used in the present study was shown to be one viral copy for  $10^4$  cells, and PCR is now widely accepted to be a particularly sensitive method for viral detection [12–18].

CMV viremia has been demonstrated in patients with mediastinitis following cardiac surgery [3] and in septic patients with a high level of tumor necrosis factor [4], with a frequency of 20 and 96%, respectively. It has been shown that blood transfusion may be responsible for transmission of CMV infection [7]. It is not clearly understood how CMV infection occurs after transfusion. Results favoring both reactivation and reinfection have been reported [31, 32]. Domart et al. reported the presence of CMV viremia in 23 of 115 patients (20%) with mediastinitis follow-

ing cardiac surgery [3]. Only 8 of the 22 patients tested were seropositive for CMV before surgery and the viral shedding appeared to be related to perioperative blood transfusions [33]. Since CMV transmission by blood transfusion can be prevented by using leukocyte-poor blood components [34], we systematically performed blood filtration for the patients in this study. Another study provided substantial results concerning the reactivation of CMV in septic patients with high levels of plasma tumor necrosis factor [4]. In most of the septic patients CMV was demonstrated in blood by either PCR or viral culture after centrifugation, but no signs or symptoms of CMV disease were found in these patients despite their active CMV infection. However, in this study no information on blood transfusion was reported, and some of these patients might have suffered exogenous transmission of CMV. Moreover, the severity of illness, the severity of underlying medical conditions, and the treatment given may have been different from that in our patient population.

We have also failed to demonstrate a potential reactivation of CMV in the lung. The lung has been shown to be a potential site of CMV latency [8], though the precise sites of latent CMV are not known. Alveolar macrophages do not harbor latent CMV in the lungs of asymptomatic CMV-seropositive patients; pneumocytes and/or endothelial cells can therefore be considered to be potential candidates for CMV latency [19]. Acute lung injury could interfere with the host's immune system and therefore lead to CMV reactivation. This could be of particular interest since it is known that CMV induces immunosuppression [9], which could promote pulmonary infection. However, in our study, whatever the lung disease, no CMV reactivation could be demonstrated in the lung.

In conclusion, despite the use of a very sensitive method – PCR amplification – and two methods of viral cultures, we were unable to demonstrate reactivation of CMV either in blood or lung in critically ill mechanically ventilated non-immunocompromised patients. These results suggest that, in the absence of exogenous transmission, CMV reactivation is uncommon in such patients.

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