Cytomegalovirus Reactivation in Patients with Sepsis in an Intensive Care Unit in Portugal

Reativação do Citomegalovírus em Doentes com Sépsis numa Unidade de Cuidados Intensivos em Portugal

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ABSTRACT

Introduction: In the last few years, cytomegalovirus reactivation has been considered an aggravating factor for septic patients in Intensive Care units. The main objectives of this study were to determine cytomegalovirus reactivation in patients with a diagnosis of sepsis admitted to an intensive care unit, and whether this reactivation was related to the evolution of the patient's clinical condition.

Material and Methods: The detection of cytomegalovirus DNA was performed by real-time polymerase chain reaction and the concentration of nine cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL- TNF-α, and INFγ) were determined by a Multiplex ELISA technique.

Results: Eight of 22 septic patients (36.3%) from the Intensive Care Unit of the Hospital da Luz had cytomegalovirus reactivation. No association was found between cytomegalovirus reactivation and gender, age, length of Intensive Care unit stay, duration of mechanical ventilation, and patient death. No significant differences were found in cytokine concentrations in patients with and without reactivation. However, patients with cytomegalovirus reactivation had a longer hospital stay from Intensive Care unit entry to hospital discharge or patient death (p = 0.025).

Discussion: Despite the low sampling rate, the present study suggests that reactivation is a frequent event in patients diagnosed with sepsis and may be related to prolonged hospital stay in these patients.

Conclusion: The overall analysis of the results obtained and the literature review do not support the concept that cytomegalovirus monitoring should be implemented in routine practice, but it seems prudent to wait for further randomized trials using antiviral prophylaxis, before assuming a definitive attitude towards the role of cytomegalovirus in sepsis.

Keywords: Critical Care; Cytomegalovirus; Portugal; Sepsis; Virus Activation

RESUMO

Introdução: A reativação do citomegalovírus tem sido considerada um factor de agravamento nos doentes diagnosticados com sépsis nas unidades de Cuidados Intensivos. Os principais objetivos deste estudo consistiram na determinação da reativação do *Cytomegalovirus* em doentes internados numa unidade de Cuidados Intensivos com diagnóstico de sépsis, e se essa reativação estaria relacionada com a evolução do quadro clínico do doente.

Material e Métodos: Na presente investigação foram estudados 22 doentes, internados com o diagnóstico de sépsis na Unidade de Cuidados Intensivos do Hospital da Luz. A deteção do ácido desoxirribonucleico do citomegalovírus foi realizada por técnica de *polymerase chain reaction* em tempo real e as concentrações de nove citocinas (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, e INFγ) foram determinadas através de uma técnica de ELISA *Multiplex*.

Resultados: A reativação ocorreu em oito doentes (36,3%). Não foram encontradas relações entre a reativação do citomegalovírus e o sexo, idade, tempo de permanência na unidade de Cuidados Intensivos, duração da ventilação mecânica e morte do doente. Também não foram encontradas diferenças significativas nas concentrações das citocinas nos doentes com e sem reativação. Contudo, os doentes com reativação do citomegalovírus apresentaram um maior tempo de internamento no hospital desde a entrada na unidade de Cuidados Intensivos até a alta hospitalar ou morte do doente (p = 0,025).

Discussão: Apesar da amostra de pequena dimensão, o presente estudo indicia que a reativação é um evento frequente nos doentes diagnosticados com sépsis e que pode estar relacionada com o prolongamento do tempo de permanência no hospital destes doentes.

Conclusão: A análise conjunta dos resultados obtidos e da revisão da literatura não apoiam o conceito de que a monitorização do citomegalovírus deva ser implementada na prática clínica, mas parece prudente aguardarem-se por mais ensaios randomizados utilizando profilaxia antiviral, antes de se assumir uma atitude definitiva relativamente ao papel do citomegalovírus na sépsis.

Palavras-chave: Activação Viral; Citomegalovírus; Cuidados Intensivos; Portugal; Sépsis

INTRODUCTION

Human cytomegalovirus (CMV) is a virus in the family Herpesviridae, subfamily Betaherpesvirinae and genus Cytomegalovirus, also known as human herpesvirus 5 (HHV-5). Following primary infection, CMV can remain in a latent state with expression of a small number of viral genes with no cell damage.[1] However, immunosuppression related to human immunodeficiency virus (HIV) or organ transplantation can reactivate CMV from its latent state,[2,3] in addition to activate cells with latent CMV, producing an increase of circulating cytokines due to an inflammatory process (sepsis, burns, trauma, surgery), which can reactivate this and even other viruses of the Herpesviridae family, even though CMV seems to be associated with the progression of the acute process.[4-7] A favourable environment for CMV reactivation is developed in these patients by a strong pro-inflammatory response, followed by an anti-inflammatory one, since

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inflammatory mediators released by immune system cells are responsible for the activation of the NF-κB complex and subsequent activation of the CMV IE gene promoter.[1,8,9] Due to these reasons, patients admitted to Intensive Care Units (ICU) have recently been recognised as patients at risk of CMV infection. Indeed, previous publications have described a high prevalence of CMV infection in these patients, in addition to relating the presence of the virus with severe outcomes during hospitalisation, such as an increased length of stay, duration of mechanical ventilation, susceptibility to nosocomial infections and, in some cases, death.[5,10,11]

The main objectives of this study included the identification of CMV reactivation in patients admitted to an Intensive Care Unit diagnosed with sepsis and whether this reactivation was related to the patient's clinical progression throughout the hospital stay. This was assessed through the Acute Physiology And Chronic Health Evaluation II (APACHE II) classification, the length of stay at the ICU and in hospital, the need and duration of mechanical ventilation and patient's death. It was also aimed to determine whether the viral load influenced clinical status and to assess the concentration of inflammatory mediators during the patient's stay in hospital and the relationship with CMV reactivation.

MATERIAL AND METHODS

Patients

The research took place between October 2012 and July 2013 at *Hospital da Luz*, Lisbon - Portugal. All patients admitted to the Intensive Care Unit at *Hospital da Luz* diagnosed with sepsis and who have agreed to participate were included in the study.[12] Immunosuppressed patients (HIV/AIDS infection or patients on immunosuppressive therapy) and pregnant women were excluded.

The following clinical data were collected: patient's age and gender, APACHE II score, ICU admission and discharge date, need and duration of mechanical ventilation and date of hospital discharge or, in some cases, date of patient's death.

Attending physicians did not have any access to the results and these were used for research purposes only.

The study was approved by the Ethics Committee of *Hospital da Luz* and a signed informed consent has been obtained from each patient. Samples that were collected for routine analyses requested by the attending physician were used for this study; therefore, no additional samples were required.

Samples

Samples were collected weekly, starting on the fourth day upon admission. Sampling included serum for serology and cytokines and whole blood in EDTA K3 for nucleic acid amplification polymerase chain reaction (PCR).

Analytical Methods

Serology: CMV antibody detection and quantification were obtained with ELFA methodology (Enzyme Linked Fluorescent Assay, VIDAS, bioMérieux).

PCR: Whenever a patient had IgG anti-CMV antibodies, CMV DNA was detected and eventually quantified by PCR technique. CMV DNA extraction was performed according to the supplier's protocol using 200μL of whole blood (JetQuick® Genomic DNA purification kit, Genomed, Löhne, Germany). The CMV DNA search was performed with a real-time PCR technique directed to the UL83 gene of CMV (CMV HHV6,7,8 R-gene™, ref:69-100, Argene, Biomérieux, Marcy-l'Etoile, France).

A single positive PCR result was considered as diagnosis of 'reactivation'. For the purpose of cytokine detection and quantification, cytokine analysis was performed in all patients in whom more than one sample was collected, using a Multiplex ELISA technique for 9 cytokines - IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, and INFγ (Q-PlexTM Array Chemiluminescent, Quansys, Logan, Utah).

Statistical analysis

Data analysis was carried out by using SPSS software (version 16.0). In addition to descriptive statistical methods (mean and standard deviation) and Shapiro Wilk test, parametric (Student's t-test, paired sample t-test, Pearson's correlation) and non-parametric tests (Mann-Whitney's U-test, Fisher's exact test, Wilcoxon's test, Spearman's correlation) were also used. Differences between the variables were considered significant with p-value < 0.05.

RESULTS

Patients diagnosed with sepsis

A total of 740 patients were admitted to the ICU within the study period; 27 patients were diagnosed with sepsis. However, five patients were excluded, including one due to withdrawal of informed consent, two due to immunosuppression and two with negative CMV serology. A total of 59 samples were collected from the remaining 22 patients with positive anti-CMV IgG, (mean = 2.73; range 1-5), including patients aged 44-85, mostly male (n = 15). Three patients were diagnosed with septic shock and presented with at least one organ failure (eight patients). An average 21.86 APACHE II score has been found (range 14-34), with an average 11.93 days ICU stay (range 1.10-52.40) and a 21.82 mean hospital stay (range 5-50 days). Sixteen patients required mechanical ventilation with a mean duration of 10.07 days (range 0.17-43.92). Three patients from 22 died during their stay in the ICU, while one patient died after being discharged to other hospital services (Table 1).

Active CMV infection

CMV DNA was analysed in 59 samples from 22 patients and was detected in 12 (12/59), corresponding to eight patients. A 36.4% rate of reactivation has been found in these patients, i.e., 8 out of 22 patients had at least one positive PCR result and were therefore diagnosed with CMV reactivation. The detection occurred on average 16 days upon admission to the hospital (range 3-39) and in the first

sample (three patients). A CMV reactivation was found upon discharge from the ICU to other hospital departments (three patients). When considering CMV reactivation restricted to the patients staying at the ICU, it would have occurred on average after 9.2 days of stay (range 3-22). Three from the eight patients diagnosed with CMV reactivation were discharged and three probably died with CMV active infection, as CMV DNA was found in the last samples that were collected from these patients.

When data obtained from patients with CMV reactivation throughout their hospital stay were compared (Table 1), significant differences were only found in the length of hospital stay, which was longer in patients with CMV reactivation when compared to patients with no reactivation (30.25 vs. 17 days, $p = 0.025$).

The data collected from patients with CMV DNA level < 1,000 copies per mL (n = 5; 62.5%) during reactivation were compared to those collected from patients with CMV DNA level > 1,000 copies per mL (n = 3; 37.5%), in order to determine whether viral load had an impact on the patient's outcome (Table 2). However, no significant differences were found, similarly to the analysis of the peak of viral load during hospitalisation (Table 3).

Cytokine detection and quantification

Only six of the nine cytokines that were analysed in 16 patients were detected in all the patients: IL-1β (> 0.3 pg/mL), IL-4 (> 0.2 pg/mL), IL-6 (> 0.6 pg/mL), IL-8 (> 0.4 pg/mL), IL-10 (> 0.3 pg/mL) and TNF-α (> 1.4 pg/mL). IL-2 was not detected in any of the samples and its concentration was always below the method detection limit, while the concentrations of IL-1α have showed values above the method detection limit in only half of the samples, even though below the quantification limit, preventing their use in the comparison between patients diagnosed with *vs.* without CMV reactivation.

Cytokine concentrations were compared in patients diagnosed with *vs.* without CMV reactivation in samples collected at admission and two weeks later, as CMV reactivation occurred on average within the second week of hospitalisation. No statistically significant differences were found between both groups at admission (Table 3) *vs.* at two weeks (Table 4). Cytokine concentrations decreased in patients without CMV reactivation, except IL-4 (mean of 2.93 and 2.96, at admission and at two weeks, respectively) and IL-8 (80.11 and 83.63). Only two patients with decreased concentrations showed statistically significant differences in IL-6 (p = 0.023) and IL-10 (p = 0.043). No statistically significant differences were found in patients diagnosed with

Table 1 – Clinical status and CMV reactivation

		Total number of patients	Reactivation		*p*-value
			Yes (n = 8)	No (n = 14)	
Gender	Male	15	5	10	1
	Female	7	4	3	
Age: Mean (range)		68.82 (44; 85)	72.75 (53; 85)	66.57 (44; 82)	0.330**
APACHE II score at study inclusion		21.86 (14; 34)	21 (14; 30)	22.36 (16;34)	0.607***
Presence of septic shock		3	1	2	
Length of hospital stay [Mean (days) (range)]		21.82 (5; 50)	30.25 (10; 44)	17 (5;50)	0.025***
Length of stay at the ICU [Mean (days) (range)]		11.93 (1.10; 52.40)	11.48 (2.50; 30.50)	12.20 (1.10; 52.40)	0.973**
Mechanical ventilation	Yes	16	7	9	0.351*
	No	6	1	5	
Duration of mechanical ventilation [Mean (days) (range)]		10.07 (0.17; 43.92)	8.22 (0.17; 2633)	11.52 (0.63; 43.92)	0.681**
Patient's death (n = 20)	Yes	7	3	4	1*
	No	13	5	8	

*: Fisher's test; **: Mann-Whitney U test; ***: Student's *t*-test

Table 2 – Clinical status and CMV viral load

		CMV DNA level > 1,000 copies/mL (n = 3)	CMV DNA level < 1,000 copies/mL (n = 5)	*p*-value
APACHE II score		20.33 (14; 26)	21.40 (14; 30)	0.824*
Mechanical ventilation	Yes	2	5	0.375*
	No	1	0	
Duration of mechanical ventilation [Mean (range)]		14.56 (2.79; 26.33)	5.68 (0.17; 11.29)	0.277**
Length of stay at the ICU [Mean (range)]		13.70 (3.50; 30.50)	10.14 (2.50; 21.90)	0.727**
Length of hospital stay [Mean (days) (range)]		26.33 (17; 32)	32.60 (10; 44)	0.447**
Patient's death	Yes	2	1	0.464*
	No	1	4	

*: Fisher's exact test; **: Student's t-test

CMV reactivation, even though three cytokine concentrations decreased and three increased.

DISCUSSION

This is the first study carried out in Portugal on the relationship between sepsis and CMV infection, to the best of our knowledge.

The results obtained in this study are in line with other studies describing the frequent CMV reactivation in patients admitted to intensive care units. Considering the evidence of active infection (positive PCR) as reactivation, a 36.4% reactivation rate has been found in this study (8/22), in line with those described by other studies on CMV reactivation in patients with sepsis (8.5 - 45%).[13,14] The wide-ranging results obtained from different studies could be explained by different reasons, namely different seroprevalences of CMV in the study population (some studies were restricted to CMV seropositive patients, while seronegative patients were included in others), different clinical severity in patients from different populations, different CMV testing frequencies (from a single collection to two collections per week, therefore with an increasing detection rate with the number of collections), techniques (antigen tests and/or culture and/or PCR, the latter being the most sensitive) and sampling (blood alone or in combination with respiratory samples and/or urine). However, 30-40% rates were found in most of the studies with a similar design, suggesting that around one third of the patients admitted to the ICU with sepsis will develop a CMV reactivation, as long as they stay in the hospital long enough to allow the development of a CMV reactivation.

A median time of 13 days to CMV reactivation was found in the study (range 3-39). A median time to the first detection ranging between 4 and 28 days has been found in literature, even though this was reduced to 4-12 days with the use of PCR;[15] therefore a slightly longer time was found in

Table 3 – Cytokine concentrations in patients with / without CMV reactivation in samples obtained at admission

	CMV reactivation (n = 8)		Without CMV reactivation (n = 8)		*p*-value
	Mean (pg/mL)	Range	Mean (pg/mL)	Range	
IL-1β	22.80	12.42 - 58.16	18.10	15.89 - 20.22	0.721*
IL-4	2.66	0.20 - 4.12	2.85	2.75 - 3.00	0.798*
IL-6	96.15	8.26 - 244.59	57.98	8.57 - 116.73	0.878*
IL-8	82.10	19.55 - 197.48	80.35	20.14 - 232.56	0.959*
IL-10	9.55	6.10 - 15.65	13.54	5.81 - 55.25	0.721*
TNF-α	22.34	9.78 - 56.54	23.38	7.42 - 69.05	0.878*

*: Mann-Whitney U-test

Table 4 – Cytokine concentrations in patients with / without CMV reactivation in samples obtained at two weeks

	CMV reactivation (n = 6)		**Without CMV reactivation** (n = 8)		***p*-value**
	Mean (pg/mL)	Range	Mean (pg/mL)	Range	
IL-1β	20.22	16.76 - 29.17	18.09	17.10 - 19.80	0.491*
IL-4	3.03	2.83 - 3.29	2.90	2.72 - 3.42	0.142*
IL-6	56.18	9.02 - 114.86	20.27	7.51 - 42.88	0.282*
IL-8	123.00	24.41 - 285.16	54.11	10.21 - 94.51	0.228*
IL-10	7.39	0.30 - 15.83	4.21	0.30 - 7.66	0.218**
TNF-α	23.74	9.09 - 59.04	12.64	9.27 - 19.58	0.181*

*: Mann-Whitney U-test; **: Student's *t*-test

our study, still within the expected range for the PCR technique and considering a weekly sampling. A twice a week sampling, as used in some studies,[4,16] could have probably contributed to a slightly shorter median time, when compared to our study.

Most studies restricted to patients with sepsis have described an association between CMV infection and prolonged mechanical ventilation, in addition to the length of stay at the ICU.[10,11] In this study, significant differences were found in the length of hospital stay, with longer stay found in patients with CMV reactivation *vs.* those without reactivation. Although this association may simply be due to a longer hospital stay related to higher detection rate, a significant evidence of an association between CMV and prolonged hospital stay seems to exist.[17] Nevertheless, a relationship with prolonged ventilation was not found in our study, even though this relationship is even supported by experimental studies showing that CMV reactivation in immunocompetent mice may lead to lung injury and that this injury may even be prevented by the administration of antiviral drugs.[18]

The association with increased mortality was also not found in our study, which is in line with some studies that only included patients diagnosed with sepsis.[13] However, this association has been described in other studies and it is possible that disparities are simply related to low statistical power and/or biased patient selection.[19]

It is worth mentioning that the association found in our study, in line with other studies, do not prove the CMV's causality and this may only be a marker of severity in sepsis rather than an aggravating factor, as it happens with other members of the Herpesviridae family, which also reactivate frequently in sepsis.[7] However, the long-standing pathogenic role of CMV reactivation in immunosuppressed individuals should be considered[2,3] and the clarification of the role of this virus is therefore of the utmost relevance. Only randomised trials for CMV prevention or treatment in the context of sepsis can provide a conclusive answer to this question. In a randomised study with immunocompetent patients with sepsis, the prophylactic use of ganciclovir, when compared with placebo, did not have the desired effect of reducing the duration of mechanical ventilation, the incidence of secondary bacteraemia and fungaemia, length of ICU stay, mortality or IL-6 decrease at day 14 (the latter being one of the endpoints of the study).[20] Therefore, even though further studies are required to reach any comprehensive conclusions, the possibility that it is only a marker of severity seems to be the most reasonable conclusion at the moment.

Before analysing the potential changes caused by CMV in inflammatory molecules, it is worth mentioning that sepsis is by itself already responsible for significant changes in cytokine values, regardless of any concomitant CMV reactivation. In fact, other studies have already found significant differences in several cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α) in patients with *vs.* without sepsis.[21,22]

The relationship between CMV reactivation and some inflammatory molecules in patients with sepsis has also been the subject of several studies, both experimental, with laboratory animals and in studies with populations of patients with sepsis and with or without CMV reactivation. As regards the former, it has been demonstrated that both lipopolysaccharide from Gram negative bacteria,[8] as well as TNF-α, IL-1β (1,9) and IL-6 can reactivate the virus from its latent form.[23]

Several studies performed in patients with sepsis, still in the 1990s, allowed the first comparison of IL-6, IL-1 and TNF-α concentrations obtained during the hospitalisation of patients with sepsis and CMV reactivation *vs.* patients with sepsis but without viral reactivation. These studies showed that TNF-α and IL-1β concentrations were higher in patients with CMV reactivation.[4] Other effects have also been found, including a decreased secretion by Killer cells before CMV reactivation episodes, increased levels of IL-10 and IL-15 preceding reactivation[24] or marginally elevated IL-10 levels in reactivation.[10]

Nine inflammatory molecules were assessed in the present study, representing one of the publications with the largest number of cytokines assessed in patients with sepsis and CMV infection. When comparing cytokine

concentrations in patients with and without CMV reactivation, using samples collected at admission and at two weeks, even though no statistically significant differences were found, there is a trend towards higher concentrations of all cytokines in patients with reactivation after two weeks of hospitalisation. When analysing the two-week evolution, a decrease in the concentrations of cytokines has been found in patients without CMV reactivation and this difference was even statistically significant in two of them, namely IL-6 ($p = 0.023$) and IL-10 ($p = 0.043$), with the exception of IL-4 and IL-1β that remained stable. There was only a significant decrease in IL-6 concentration in patients with CMV reactivation, even though without reaching statistical significance. The reduction in this cytokine, as well as in IL-8, has been associated with better clinical outcome in patients with acute respiratory distress syndrome (ARDS) and an impact on mortality with actions on cytokines has even been hypothesised. In fact, high levels of IL-6 in sepsis play a relevant role in the so-called 'cytokine storm', with some studies suggesting that blocking this cytokine may have beneficial effects on clinical outcomes.[25] The decrease in IL-6 levels that was found in our study suggests that a clinical improvement may have occurred between samplings, although the number of patients involved does not allow confirming this possibility.

As regards IL-8, its induction has been described as particularly important during CMV infection, as neutrophils are attracted by IL-8 and play an important role in virus dissemination. Furthermore, IL-8 has a positive effect on CMV replication.[26] These data are in line with what was found in the present study, as the most significant differences were found in IL-8 levels, evolving in opposite directions in patients with and without reactivation (increased in patients with reactivation and decreased in patients without reactivation).

Although IL-10 is an anti-inflammatory cytokine and, as previously mentioned, there may be a justification for finding it marginally elevated in CMV reactivation,[10] this was not found in this study, although a less significant decrease was found at the second week, when compared to the decrease in patients without reactivation.

In line with another recent study,[27] it was not possible to draw any conclusions on the progression of INFγ, as the results obtained in the present study were below the limit of quantification.

It is worth mentioning that the small sample size of the present study limits the conclusions that may be drawn from it and these conclusions should only be considered as suggestive, given the limitations of the study.

CONCLUSION

The results obtained in this study reinforced the idea that CMV reactivation could be associated with prolonged hospital stay in patients with sepsis, even though no other clinical outcomes were found, in line with other studies.

The analysis of the results and literature review do not support the concept that CMV monitoring should be routinely implemented, but it seems wise to await further randomised trials using antiviral prophylaxis before reaching a definitive conclusion on the role of CMV in sepsis.

HUMAN AND ANIMAL PROTECTION

The authors declare that the followed procedures were according to regulations established by the Ethics and Clinical Research Committee and according to the Helsinki Declaration of the World Medical Association.

DATA CONFIDENTIALITY

The authors declare that they have followed the protocols of their work centre on the publication of patient data.

CONFLICTS OF INTEREST

The authors declare that there were no conflicts of interest in writing this manuscript.

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