Human herpesvirus-6 dysregulates monocyte-mediated anticryptococcal defences

Claudio Cermelli,¹ Valeria Cenacchi,¹ Francesca Beretti,¹
Francesco Pezzini,¹ Dario Di Luca² and Elisabetta Blasi¹

Correspondence
Claudio Cermelli
c.cermelli@unimo.it

¹Department of Hygiene, Microbiology and Biostatistics, University of Modena and Reggio Emilia, Via Campi 287, I-41100 Modena, Italy

²Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy

In order to investigate the interplay occurring between pathogens in the course of double infections, an *in vitro* model was set up in which the monocytic cell line THP-1 was exposed to *Cryptococcus neoformans* (Cn) and human herpesvirus 6 (HHV-6). Cn and HHV-6, both highly neurotropic, can cause serious diseases of the central nervous system and have monocytes, among other cell types, as target cells, causing alteration of their secretion pattern. Here, it was shown that unlike THP-1 cells exposed to cell-free virus inocula, THP-1 exposed to HHV-6-producing lymphocytes exhibited augmented phagocytosis against Cn. The phenomenon occurred after 24 h of monocyte/lymphocyte co-culture and was independent of direct cell-to-cell contact. Moreover, in the presence of HHV-6, THP-1 cells expressed enhanced secretory responses but reduced capability to counteract fungal infection: the enhanced ingestion by monocytes was followed by facilitated fungal survival and replication. These data provide initial *in vitro* evidence that HHV-6 may dysregulate monocyte-mediated anticryptococcal defences with an overall pro-cryptococcus result.

Received 3 January 2006
Accepted 17 February 2006

INTRODUCTION

Clinical cases of double infections by fungi and viruses are increasing, especially in immunocompromised hosts (Marti Cabane & Alvarez Rubio, 2004; Weinberg *et al.*, 2005; Fonseca *et al.*, 2003; Kobayashi *et al.*, 2003; Karino *et al.*, 1998). To date, the biomolecular events that characterize the outcome of polymicrobial diseases remain poorly investigated and little is known of the mutual interactions occurring between pathogens, and their concomitant synergistic or antagonistic effects.

Because of the role of macrophages as the first-line defence against micro-organisms, the mechanisms involved in phagocytosis and microbial killing have been thoroughly investigated (Langermans *et al.*, 1994; Nathan 1987; Vázquez-Torres & Balish, 1997). In addition, evidence exists of the variety of strategies through which micro-organisms can elude macrophage-mediated host defences (Rathman *et al.*, 1996; Russell 1994; Tardieux *et al.*, 1992); the relevance of such event(s) to pathogen persistence and eventually to disease outcome remains a matter of debate.

Cryptococcus neoformans (Cn) is a basidiomycetous yeast causing asymptomatic pulmonary infections in

immunocompetent individuals, while in immunodeficient hosts, especially AIDS patients, it frequently disseminates from the lungs, causing severe and often recurrent meningoencephalitis.

Human herpesvirus-6 (HHV-6) is the aetiological agent of exanthema subitum, also known as roseola infantum, a benign febrile infantile disease with high fever and cutaneous rash (Salahuddin *et al.*, 1986; Yamanishi *et al.*, 1988). Virus primary infection is usually asymptomatic; rarely, it may evolve as encephalitis, hepatitis, mononucleosis-like syndrome or fatal haemocytophagic syndrome (Ward, 2005). As with the other members of the *Herpesviridae* family, after primary infection, HHV-6 persists latently in the infected host. Peripheral blood lymphocytes and monocyte/macrophages, together with central nervous system (CNS) cells, support viral latency. Virus reactivation may occur and cause disease, especially in immunocompromised hosts. HHV-6 is highly neurotropic, and virus reinfections and/or reactivations are often associated with pathologies of the CNS, such as encephalitis, meningitis, radiculitis and demyelinating diseases (De Bolle *et al.*, 2005). Moreover, by infecting immune cells, HHV-6 can directly cause immune dysfunctions, with a dramatic alteration of the secretion pattern that, in turn, can induce an imbalance of the immune response (Flamand *et al.*, 1995; Arena *et al.*, 1999, 2002; Milne *et al.*, 2000; Yoshikawa *et al.*, 2002; Caruso *et al.*, 2003).

Abbreviations: Cn, *Cryptococcus neoformans*; CNS, central nervous system; CPE, cytopathic effect; HHV-6, human herpesvirus-6; IFA, immunofluorescence assay; IHC, immunohistochemistry.

Multiple reasons support the rationale for investigating HHV-6 and *Cn*. First of all, both pathogens are highly neurotropic and represent important causes of CNS disease (Dewhurst, 2004). Second, both pathogens have monocytes, among others, as target cells, and both have the capability to affect macrophage functional parameters. In particular, *Cn* has been found as an intracellular pathogen within macrophages (Levitz, 2001). Moreover, it must be considered that both pathogens can interfere with the host defence pathways. Different mechanisms allow *Cn* to avoid macrophage-mediated defences, significantly impairing *Cn* ingestion and/or enhancing intramacrophage survival and replication of the pathogen (Tucker & Casadevall, 2002; Levitz, 2001). In addition, *Cn* can alter the cytokine secretory response of macrophages (Blasi *et al.*, 1995, 2001a; Kawakami *et al.*, 2000; Goldman *et al.*, 2001; Mucci *et al.*, 2003; Uicker *et al.*, 2005).

The clinical evidence of simultaneous infections by fungi and HHV-6 (Vuorinen *et al.*, 2004), and the finding that HHV-6 viraemia is significantly associated with invasive fungal infections in liver transplant recipients (Rogers *et al.*, 2000), raise the question of whether and to what extent the wide-spectrum immunomodulating activity of HHV-6 may affect the outcome of fungal disease. By an *in vitro* model, we evaluated the consequences of HHV-6 infection on macrophages as antifungal defence elements. The effects of HHV-6 infection were measured as the capability to alter macrophage-mediated effector and secretory functions, namely phagocytosis and killing of *Cn*, and secretion of selected cytokines.

METHODS

***Cn*.** We used a clinical isolate of *Cn* var. *neoformans* (serotype D) (laboratory name: 1526), obtained from the cerebrospinal fluid of a patient with meningitis (Blasi *et al.*, 2001b) and cultured on Sabouraud plates at room temperature.

HHV-6. The prototype strain U1102 of HHV-6 variant A (Downing *et al.*, 1987) was used for this study. The viral stock was obtained by infecting JJHAN cells with HHV-6; when more than 50% of cells showed the typical cytopathic effect (CPE), cells were concentrated to 10^7 ml⁻¹ and then frozen and thawed three times. The cell lysate was then centrifuged at low speed (800 r.p.m.) for 5 min to remove cell debris and stored in small aliquots at -20 °C (cell-free inocula).

Cell lines. The human acute monocytic leukaemia cell line THP-1 and the human T cell lymphoblast line JJHAN were maintained with RPMI medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (complete medium), at 37 °C and 5% CO₂. Both cell lines were split twice a week in fresh medium.

Protocols for THP-1 cell infection by HHV-6. We used two protocols to infect THP-1 cells with HHV-6. Protocol I was as follows. THP-1 cells were infected with cell-free HHV-6 inocula, obtained as described above, at an m.o.i. of 0.01 tissue culture infecting dose (TCID₅₀) per cell. After 2 h incubation at 37 °C, cells were centrifuged, washed with PBS and resuspended in complete medium to a final concentration of 10^6 cells ml⁻¹. Mock infection was carried out using a cell lysate from uninfected JJHAN cells that was processed in the same way as infected lysates. Protocol II was as

follows. To expose THP-1 cells to HHV-6, cells were co-cultured with uninfected JJHAN cells (THP1/JJHAN^{mock}) or with HHV-6-infected JJHAN cells (THP1/JJHAN^{HHV-6}) at a ratio of 1:1 and at a final cell concentration of 10^6 cells ml⁻¹, and seeded in wells of a 24-well cell-culture plate. At different time-points, co-cultures were employed in the assays described below. Co-cultures were also carried out in transwell plates in which the two cell populations were cultured in separate compartments; in this case, 5×10^5 THP-1 cells were seeded in the lower chamber and 5×10^5 JJHAN cells (either HHV-6- or mock-infected) were seeded in the upper chamber.

Immunofluorescence assay (IFA). The percentage of HHV-6 infected cells was evaluated by IFA according to a protocol reported previously (Cermelli *et al.*, 1992), using a HHV-6 hyperimmune human serum.

Molecular analysis. Total RNA was extracted with EUROzol (Euroclone), as recommended by the manufacturer. DNA contamination was eliminated by three cycles of digestion with 40 U RNase-free DNase at room temperature for 30 min. The complete absence of DNA contaminants was checked by PCR amplification of 200 ng total RNA without previous retrotranscription. Retrotranscription was carried out on 2 µg total purified RNA, using dNTP, random hexamer primers and 20 U of murine leukaemia virus reverse transcriptase. The presence of virus transcripts was analysed by nested RT-PCR of regions corresponding to the U42 (immediate-early) and U22 (late) genes of HHV-6, as previously described (Caruso *et al.*, 2002).

Phenotypical analysis. Phenotypical analysis of THP1/JJHAN co-cultures was carried out by means of haematoxylin/eosin and May-Grunwald/Giemsa staining according to a standard protocol, and by immunohistochemistry (IHC) microscopy using anti-CD3 mAb clone PS1. The secondary antibody was biotinylated anti-mouse IgG. After incubation with biotin-streptavidin conjugated peroxidase, diaminobenzidine was used to visualize the reaction.

Cytokine quantification. THP1/JJHAN^{mock}, THP1/JJHAN^{HHV-6} and THP1/JJHAN^{HHV-6}/*Cn* co-cultures were carried out for 1 and 2 days; then, cell-free supernatants were tested for IL-12 (Euroclone human IL-12) and IFN- α content (Human IFN- α ELISA kit, R&D Systems) according to the manufacturers' instructions.

Phagocytic activity. The phagocytosis assay was performed as previously described (Blasi *et al.*, 1990). THP-1 cells alone (10^6 cells ml⁻¹) or co-cultured with JJHAN at a ratio of 1:1 (THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6}) were infected with *Cn* (10^7 cells ml⁻¹; effector:target = 1:10) in 24-well plates and then incubated for 2 h. The excess of micro-organisms was removed by centrifugation of the cell suspension on a Ficoll cushion at 300 g for 10 min. The cells at the interface were recovered and washed. Fungal uptake was directly evaluated in May-Grunwald/Giemsa-stained cytospin preparations. A minimum of 200 macrophages were scored, and any cells containing one or more yeast were counted as phagocytic. The phagocytosis index was calculated as the total number of phagocytosed yeasts divided by the total number of phagocytic cells.

Anticryptococcal activity. Anticryptococcal activity was evaluated by means of the colony forming unit inhibition assay. THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6} co-cultures (10^5 cells ml⁻¹) were infected with *Cn* (10^6 cells ml⁻¹; effector:target = 1:10) in 96-well microplates. After 2, 3, 4 and 6 h incubation at 37 °C, Triton X-100 was added at 0.2% final concentration. Serial dilutions from each well were seeded on Sabouraud agar plates in triplicate. After 48–72 h incubation at room temperature, c.f.u. were counted. Control cultures consisted of *Cn* without effector cells. The results, derived from the arithmetical means of the c.f.u. values, were

expressed as percentage anticryptococcal activity, according to the formula:

percentage inhibition

$$= 100 - (\text{c.f.u. experimental cultures} / \text{c.f.u. control cultures}) \times 100.$$

Statistical analysis. Each experiment was performed three times and each sample was in duplicate. The results were analysed by the Mann–Whitney test. The data reported in figures and tables are the means of values from three different experiments (the SD was mostly less than 10 %).

RESULTS

Susceptibility of THP-1 cells to HHV-6 infection

Viral infection of THP-1 cells was carried out employing a lysate of HHV-6-infected JJHAN cells, according to Protocol 1 (Methods). Microscope observation of the cultures up to 10 days post-infection did not show evidence of any CPE. Moreover, IFA performed 1, 2 and 6 days after infection showed that approximately 1 % of THP-1 cells were positive for virus antigen expression (Table 1). In parallel, RT-PCR analysis was carried out on RNAs extracted from HHV-6-infected THP-1 cells; as shown in Fig. 1, single-round PCR did not give a positive signal, while nested PCR showed amplification bands for U42 (transcribed during the immediate-early phase of infection) and U22 (transcribed in the late phase of infection). The absence of amplification bands in RNA samples without previous retrotranscription confirmed that the bands corresponded to viral transcripts and not to contamination from residual DNA. Since the detection limit of our single-round PCR was 10^3 copies (Caruso *et al.*, 2002), we were able to assume that less than 1 % of cells had one copy of the transcript (Table 1, Fig. 1), thus confirming the IFA results.

In order to assess whether HHV-6 infection could influence macrophage effector functions, phagocytosis assays were carried out comparing control THP-1 cells with THP-1 cells infected with lysates of HHV-6-infected cells. THP-1 cells exposed to HHV-6 did not display any difference in their

Fig. 1. Molecular analysis of THP-1 cells infected with cell-free HHV-6 inocula. THP-1 cells were infected with cell-free HHV-6. Total RNA was extracted from HHV-6-infected THP-1 cells, and RT-PCR amplification was performed with reactions specific for HHV-6 U42 (immediate-early gene), HHV-6 U22 (late gene) and cellular β -actin. Lanes 1 and 2 show the results of single-step PCR after retrotranscription of THP-1 cells harvested, respectively, 24 and 48 h post-infection. Lanes 3 and 4 show the results of nested PCR of THP-1 cells 24 and 48 h post-infection, respectively. Lanes 5 and 6 show, as control for the absence of contamination from residual DNA, the results of nested amplification of RNA extracted from THP-1 cells, without previous retrotranscription. β -Actin RNA was amplified by single-step PCR: lanes 1 and 2, PCR results of THP-1 cells, 24 and 48 h post-infection; lanes 3 and 4, negative controls (RNA without retrotranscription). The expected size of amplicons is shown to the right of the figure.

phagocytic activity against *Cn* in comparison with unexposed THP-1 cells (data not shown).

Phenotypical analysis of THP1/JJHAN co-cultures

Since direct exposure of THP-1 cells to HHV-6 inocula did not result in a productive infection, we set up a model of co-culture between mock- or HHV-6-infected JJHAN and THP-1 cells at a 1:1 cell ratio (THP1/JJHAN^{mock} and

Table 1. Susceptibility of THP-1 cells to HHV-6 infection

THP-1 cells (10^6 ml^{-1}) were infected with a cell-free HHV-6 inoculum at an m.o.i. of 0.01 tissue culture infecting dose (TCID₅₀) per cell, and then harvested at different times for IFA and PCR, as described in Methods. NT, Not tested.

Day	CPE	IFA-positive cells (%)	HHV-6 DNA detection			
			Single-round PCR		Nested PCR	
			U22 gene	U42 gene	U22 gene	U42 gene
1	—	<1	—	—	+	+
2	—	1	—	—	+	+
6	—	1	NT	NT	NT	NT

THP1/JJHAN^{HHV-6}, respectively). To verify whether the initial 1:1 monocyte:lymphocyte ratio was maintained over time, May–Grunwald/Giemsa, haematoxylin/eosin and IHC staining of the co-cultures was performed after 1 and 2 days of co-culture. A comparable percentage of lymphocytes and monocytes was observed under both HHV-6-infected and uninfected conditions (Table 2): in both THP1/JJHAN co-culture groups about 50 % of cells were consistently CD3 positive. No differences were observed at later times of co-culture (data not shown). On the other hand, both HHV-6-infected and uninfected JJHAN cells remained 100 % CD3 positive. Interestingly, THP1/JJHAN^{HHV-6} showed a decrease in the percentage of mitotic figures (from 20 to 15 %), the appearance of apoptotic cells (from 0 to 9 %), and an increase in the percentage of giant cells (from 2 to 6 %) compared with THP1/JJHAN^{mock} (Table 2).

THP1/JJHAN co-culture: effects on IL-12 and IFN- α production

Supernatants from THP1/JJHAN co-cultures were analysed for the levels of IL-12 and IFN- α by ELISA. All three types of co-culture showed a time-dependent increase in IL-12 content that was significantly higher for THP1/JJHAN^{HHV-6} and THP1/JJHAN^{HHV-6}/Cn in comparison with THP1/JJHAN^{mock} (Fig. 2). Moreover, in no case was IFN- α detectable, except for THP1/JJHAN^{HHV-6} co-cultures infected with Cn (28.8 pg ml⁻¹ at 1 day after infection).

THP1/JJHAN co-culture: effects on phagocytosis

THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6} co-cultures were assayed for their capability to phagocytose Cn. Thus, THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6} co-cultures were infected with Cn (effector:target = 1:10) for 2 or 4 h and then assessed for the percentage of phagocytosis, as detailed above. As shown in Fig. 3, THP1/JJHAN^{HHV-6} showed an increase in the percentage of phagocytic cells in comparison with THP1/JJHAN^{mock} (19.5 versus 10.3 %, respectively). This remarkable increase in Cn phagocytosis by THP1/JJHAN^{HHV-6} was observed regardless of the duration of the co-culture.

Fig. 2. IL-12 production by THP1/JJHAN co-cultures. THP-1 cells were co-cultured with mock- and HHV-6-infected JJHAN for 1 and 2 days; IL-12 was then measured in supernatants by ELISA, as reported in Methods. $P < 0.01$ between each type of co-culture at each time-point. Grey bars, THP1/JJHAN^{mock}; white bars, THP1/JJHAN^{HHV-6}; black bars, THP1/JJHAN^{HHV-6}/Cn.

THP1/JJHAN co-culture in transwell plates

To verify whether the direct cell-to-cell contact was necessary to induce the observed increase in phagocytosis, we carried out experiments with transwell plates. As shown in Fig. 4, in this case also, the percentage of phagocytosis increased when THP-1 cells were exposed to HHV-6-infected JJHAN in comparison with THP-1 cells exposed to uninfected JJHAN; this phenomenon occurred after both 24 and 48 h co-culture (14.8 versus 19.3 % and 6.8 versus 11 %).

THP1/JJHAN co-culture: effects on anticryptococcal activity

THP1/JJHAN co-cultures were tested for anticryptococcal activity by colony forming unit inhibition assay. THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6} co-cultures were exposed to Cn for 2, 3 and 4 h, and then tested as detailed in Methods. We found low levels of activity and no difference between THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6}

Table 2. Morphological analysis of THP1/JJHAN co-cultures

THP-1 and JJHAN cells were mixed at 1:1 cell ratio. Morphological analysis was carried out after 1 and 2 days of co-culture by haematoxylin/eosin and May–Grunwald/Giemsa staining and by immunohistochemistry with a mAb against CD3, as described in Methods.

Cell mixture	Proportion (%) of:			
	CD3+ cells	Mitotic cells	Apoptotic cells	Giant cells
THP1/JJHAN ^{mock}	50	20	0	2
THP1/JJHAN ^{HHV-6}	51	15	9	6

Fig. 3. Phagocytic activity of THP1/JJHAN co-cultures. THP-1 cells were co-cultured with mock- and HHV-6-infected JJHAN for 1 and 2 days, and then infected with *Cn*. After 2 h incubation, phagocytosis was assessed by May–Grunwald/Giemsa staining, as reported in Methods. $P < 0.01$ between the two types of co-culture at each time-point. Grey bars, THP1/JJHAN^{mock}; white bars, THP1/JJHAN^{HHV-6}.

(Fig. 5). Interestingly, when the time of exposure to *Cn* was raised to 6 h, the co-cultures showed negative levels of anticyptococcal activity that were more marked for THP1/JJHAN^{HHV-6} (−9.9 versus −22.3 %).

DISCUSSION

The aim of this study was to determine whether the macrophage-mediated immune reaction against *Cn* is somehow influenced by the co-presence of a viral pathogen, HHV-6. On the whole, our results demonstrate that macrophages exposed to HHV-6 have increased phagocytic ability but reduced anticyptococcal activity.

Fig. 4. Increase in phagocytic activity of THP1/JJHAN^{HHV-6} is independent of direct cell-to-cell contact. THP-1 cells were co-cultured with mock- and HHV-6-infected JJHAN for 1 and 2 days in transwell plates and then infected with *Cn*. After 2 h incubation, phagocytosis was assessed by May–Grunwald/Giemsa staining, as reported in Methods. $P = 0.04$ at day 1; $P = 0.01$ at day 2. Grey bars, THP1/JJHAN^{mock}; white bars, THP1/JJHAN^{HHV-6}.

Fig. 5. Kinetics of anticyptococcal activity of THP1/JJHAN. THP-1 cells were co-cultured with mock- and HHV-6-infected JJHAN overnight and then infected with *Cn*. After 2, 3, 4 and 6 h incubation, anticyptococcal activity was assessed by the colony forming unit inhibition assay, as reported in Methods. * $P < 0.01$. Grey bars, THP1/JJHAN^{mock}; white bars, THP1/JJHAN^{HHV-6}.

Initially, we addressed the susceptibility of THP-1 cells to infection by HHV-6 by employing cell-free virus inocula (Methods, protocol I). Although several conditions were employed, THP-1 infection by HHV-6 was not efficient, as documented by the absence of CPE and by the IFA results (about 1 % positive cells). Moreover, RT-PCR analysis confirmed that only a minority of cells expressed early and late HHV-6 mRNAs. The amplification of both immediate-early and late viral mRNAs indicated that productive infection of THP-1 cells took place; yet, the transcription occurred with only low efficiency, as shown by the presence of bands using nested PCR alone. Furthermore, HHV-6 infection of THP-1 cell cultures did not affect their phagocytic activity. Using a similar approach, Li *et al.* (1997) have reported a dysregulation of monocyte secretory functions by HHV-6, showing induction of IL-10 and IL-12 in HHV-6-infected THP-1 cells. Nevertheless, they do not provide direct evidence of the efficiency of the viral infection of THP-1 cells.

With the aim of enhancing the efficacy of HHV-6 infection, another approach (protocol II) was chosen. In particular, THP-1 cells were exposed to HHV-6-producing lymphocytes. This experimental approach may be particularly relevant, since it closely reproduces the situation that potentially occurs in patients, in whom infected CD4⁺ T cells are the main source of HHV-6 (Lusso *et al.*, 1988). Moreover, while this paper was in preparation, Meeuwse *et al.* (2005) published a study of the effects of HHV-6A on human astrocytes. In that experimental system also, the co-culture model was employed, since the authors could not obtain infection of astrocytes using cell-free HHV-6 inocula. In our study, THP-1 cells were co-cultured with JJHAN cells that had been infected with HHV-6 5 or 6 days earlier. Under

these conditions, the viral CPE on JJHAN cells was at its peak (usually about 50–60 % of cells were IFA positive for HHV-6 antigens). As negative controls, THP-1 cells were co-cultured with mock-infected JJHAN under the same experimental conditions. The co-cultures were subsequently exposed to *Cn* for different times; then, phagocytosis, cytokine secretory response and anticryptococcal activity were assayed. Here, we show that THP1/JJHAN^{HHV-6} displayed increased phagocytic activity in comparison with uninfected THP1/JJHAN^{mock} co-cultures; this increase occurred regardless of the duration of the co-culture, and did not require direct contact between lymphocytes and monocytes, as established by experiments in transwell plates. It is worth noting that the enhanced percentage of phagocytic activity cannot be attributed to an alteration of the monocyte:lymphocyte ratio. As shown in Table 2, phenotypic analysis of the co-cultures disclosed that the initial 1:1 ratio was consistently maintained after 24 and 48 h, in both THP1/JJHAN^{mock} and THP1/JJHAN^{HHV-6} co-cultures. Nevertheless, a reduced number of mitotic figures and an increased percentage of apoptotic cells were observed in THP1/JJHAN^{HHV-6} with respect to THP1/JJHAN^{mock}, although these differences were not statistically significant. These data were consistent with the findings that HHV-6 induces an arrest of the cell cycle and apoptosis (Inoue *et al.*, 1997; Oster *et al.*, 2005).

Since the observed upregulation of phagocytosis was independent of direct contact between lymphocytes and monocytes, it is likely that released soluble factor(s) account for the observed phenomenon. With this in mind, we tested the THP1/JJHAN supernatants for IFN- α and IL-12. We chose these cytokines for different reasons. IFN- α is an important antiviral effector, produced by virus-infected cells, and it has also been reported to reduce the adhesiveness, intracellular multiplication and invasiveness of *Shigella flexneri* in HEP-2 cells preinfected with coxsackie B1 virus (Modalsli *et al.*, 1992). IL-12 is crucial in the development of an effective immune response, favouring protective Th1-dependent resistance (Trinchieri, 1997). Moreover, we (Blasi *et al.*, 2001a) and other authors (Kawakami *et al.*, 1999) have shown that *Cn* can inhibit IL-12 production, whereas, as said above, HHV-6 can induce its production. In this work, we found that THP1/JJHAN^{HHV-6} produced higher amounts of IL-12 and IFN- α with respect to the control cultures in a time-dependent manner. The presence of *Cn* further increased IL-12 production by HHV-6-infected co-cultures. Although we cannot rule out some involvement of HHV-6-infected JJHAN cells, we favour the conclusion that, in our system, THP-1 cells are the main IL-12 producers, in accordance with an earlier report describing the IL-12 response of THP-1 cells exposed to cell-free HHV-6 inocula (Li *et al.*, 1997). The observed production of IL-12, a powerful IFN- γ inducer, together with that of IFN- α , also detected in supernatants from THP1/JJHAN^{HHV-6}, may in turn contribute to the increased phagocytosis of *Cn* by macrophages. In this respect, Siren *et al.* (2005) have recently shown that IFN- α upregulates toll-like receptor

(TLR) expression in human monocyte-derived macrophages as well as the expression of adapter molecules and kinases involved in TLR signalling, known to be critically involved in pathogen–host cell recognition events (Roeder *et al.*, 2004; Netea *et al.*, 2004).

Smith *et al.* (2003, 2005) have shown that HHV-6 inhibits IL-12 production by dendritic cells and macrophages, suggesting an immunosuppressive effect of the viral infection. The discrepancy between these data and those of the present study can be explained by the use of different cells (primary macrophages and dendritic cells versus a monocytic cell line). As suggested by other authors (Inoue *et al.*, 1997), HHV-6 may have different effects in different cell types. Also, we cannot rule out differences in the experimental model employed. Smith *et al.* (2005) directly infected the immune cells, whereas we used infected lymphocytes as carriers of HHV-6 to macrophages. In our model, cytokine response by macrophages may be influenced not only by HHV-6 per se but also by the secretory response of HHV-6-infected T cells. In this regard, Mayne *et al.* (2001) have demonstrated a major modification in the pattern of cytokine response by lymphocytes, with an increase in pro-inflammatory cytokines and a down-regulation of anti-inflammatory cytokines in HHV-6-infected JJHAN cells. In particular, an increase of IL-18 was observed. IL-18, as well as IL-12, is a known IFN- γ inducer; moreover, Li *et al.* (1997) have proved that HHV-6-induced IFN- γ enhances IL-12 production by monocytes. Consequently, these two cytokines could have a synergistic effect, leading to the increase in phagocytic activity observed in our experimental model. Although Kawakami *et al.* (1999) have demonstrated a down-regulation of IL-12 by monocytes exposed to *Cn*, we observed the opposite outcome. First of all, different monocytic cell lines were used: in our case the human cell line THP-1 without any stimulation, while in the work of Kawakami and co-workers the murine cell line J774 was also stimulated with LPS and IFN- γ . Therefore, the two models are very different. More importantly, in our model, IL-12 induction by HHV-6 could have overcome the inhibiting effect of *Cn*, confirming the results reported by Li *et al.* (1997) of IL-12 upregulation by HHV-6 in monocytes. Moreover, the simultaneous presence of two different cell populations must be considered. Retini *et al.* (1999) have demonstrated that *Cn*-exposed monocytes increase their IL-12 production in the presence of lymphocytes.

Constitutively, THP-1 cells did not exhibit efficient killing of *Cn*. This activity was also influenced by the fungal strain employed [a more virulent *Cn* was killed even less efficiently (Blasi *et al.*, 2001b)], and it was not significantly affected by THP-1 pre-treatment with phorbol ester (data not shown). Here we show that, when assessed for anticryptococcal activity, the co-cultures were unable to counteract fungal infection at least at early time-points. Furthermore, when assessed after 6 h, *Cn* exposed to THP1/JJHAN co-cultures grew more rapidly with respect to control

yeast cells, giving rise to the observed negative values. Interestingly, such a phenomenon was more pronounced in THP1/JJHAN^{HHV-6}. While the molecular events mediating such phenomena remain to be elucidated, this study provides initial evidence of the dual role of HHV-6 on macrophage-mediated anticytotoxic defences. The net result of HHV-6 presence may be interpreted as a pro-*Cn* effect, since the fungus is better ingested, but it is also promoted in its survival and replication. Both conditions certainly favour dissemination of the pathogen and progression of the disease.

In conclusion, bearing in mind the limitations peculiar to any *in vitro* study, our co-culture cell model points to the possibility that virus infection may indeed affect the course of fungal disease. In particular, our model using lymphocytes and monocytes/macrophages could mirror the interactions between the two pathogens in the peripheral nervous system. A possible scenario suggested by these results is that *in vivo* HHV-6 reactivation may represent an additional risk factor for disseminated cryptococcosis from the periphery to the CNS. We are presently setting up an *in vitro* model of co-culture using cells of the CNS to verify that our results are confirmed in this system too.

ACKNOWLEDGEMENTS

This work was partially supported by a grant to E. B. from Ministero dell'Istruzione, dell'Università e della Ricerca PRIN2003, n. 2003062784. We thank Professor Giuseppe Barbolini (Department of Laboratory Medicine, University of Modena and Reggio Emilia) for morphological analysis of the co-cultures used in this work.

REFERENCES

- Arena, A., Liberto, M. C., Iannello, D., Capozza, A. B. & Foca A. (1999). Altered cytokine production after human herpes virus type 6 infection. *New Microbiol* 22, 293–300.
- Arena, A., Stassi, G., Speranza, A., Iannello, D. & Mastroeni, P. (2002). Modulatory effect of HHV-6 on MCP-1 production by human monocytes. *New Microbiol* 25, 335–340.
- Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R. & Bistoni, F. (1990). immortalization of murine microglial cells by a v-*raf*/v-*myc* carrying retrovirus. *J Neuroimmunol* 27, 229–237.
- Blasi, E., Barluzzi, R., Mazzolla, R., Pitzurra, L., Puliti, M., Saleppico, S. & Bistoni, F. (1995). Biomolecular events involved in anticytotoxic resistance in the brain. *Infect Immun* 63, 1218–1222.
- Blasi, E., Colombari, B., Mucci, A., Cossarizza, A., Radzioch, D., Boelaert, J. R. & Neglia, R. (2001a). Nramp1 gene affects selective early steps in macrophage-mediated anti-cryptococcal defense. *Med Microbiol Immunol* 189, 209–216.
- Blasi, E., Brozzetti, A., Francisci, D., Neglia, R., Cardinali, G., Bistoni, F., Vidotto, V. & Baldelli, F. (2001b). Evidence of microevolution in a clinical case of recurrent *Cryptococcus neoformans* meningoencephalitis. *Eur J Clin Microbiol Infect Dis* 20, 535–543.
- Caruso, A., Rotola, A., Comar, M. & 10 other authors (2002). HHV-6 infects human aortic and heart microvascular endothelial cells, increasing their ability to secrete proinflammatory chemokines. *J Med Virol* 67, 528–533.
- Caruso, A., Favilli, F., Rotola, A., Comar, M., Horejsh, D., Alessandri, G., Grassi, M., Di Luca, D. & Fiorentini, S. (2003). Human herpesvirus-6 modulates RANTES production in primary human endothelial cell cultures. *J Med Virol* 70, 451–458.
- Cermelli, C., Moroni, A., Pietrosemoli, P., Pecorari, M. & Portolani, M. (1992). IgG antibodies to human herpesvirus-6 (HHV-6) in Italian people. *Microbiologica* 15, 57–63.
- De Bolle, L., Naesens, L. & De Clercq, E. (2005). Update on human herpesvirus 6 biology, clinical features, and therapy. *Clin Microbiol Rev* 18, 217–245.
- Dewhurst, S. (2004). Human herpesvirus type 6 and human herpesvirus type 7 infections of the central nervous system. *Herpes* 2, 105A–111A.
- Downing, R. G., Sewankambo, N., Serwadda, D., Honess, R., Crawford, D., Jarrett, R. & Griffin, B. E. (1987). Isolation of human lymphotropic herpesviruses from Uganda. *Lancet* 2, 390.
- Flamand, L., Gosselin, J., Stefanescu, I., Ablashi, D. & Menezes, J. (1995). Immunosuppressive effect of human herpesvirus 6 on T-cell functions: suppression of interleukin-2 synthesis and cell proliferation. *Blood* 85, 1263–1271.
- Fonseca, E. M., Nuno, F. J., Garcia-Alcalde, M. L. & Menendez, M. J. (2003). Infection due to herpes zoster and cryptococcus after initiating high-activity antiretroviral treatment. *Enferm Infecc Microbiol Clin* 21, 217–218.
- Goldman, D., Song, X., Kitai, R., Casadevall, A., Zhao, M. L. & Lee, S. C. (2001). *Cryptococcus neoformans* induces macrophage inflammatory protein 1 α (MIP-1 α) and MIP-1 β in human microglia: role of specific antibody and soluble capsular polysaccharide. *Infect Immun* 69, 1808–1815.
- Inoue, Y., Yasukawa, M. & Fujita, S. (1997). Induction of T-cell apoptosis by human herpesvirus 6. *J Virol* 71, 3751–3759.
- Karino, T., Nakamura, J., Fujita, K., Kobashi, Y., Yano, T., Okimoto, N. & Soejima, R. (1998). A case with chronic active EB virus infection accompanied with pulmonary candidiasis. *Kansenshogaku Zasshi* 72, 1306–1310 (in Japanese).
- Kawakami, K., Qureshi, M. H., Koguchi, Y., Nakajima, K. & Saito, A. (1999). Differential effect of *Cryptococcus neoformans* on the production of IL-12p40 and IL-10 by murine macrophages stimulated with lipopolysaccharide and gamma interferon. *FEMS Microbiol Lett* 175, 87–94.
- Kawakami, K., Koguchi, Y., Qureshi, M. H., Yara, S., Kinjo, Y., Uezu, K. & Saito, A. (2000). NK cells eliminate *Cryptococcus neoformans* by potentiating the fungicidal activity of macrophages rather than by directly killing them upon stimulation with IL-12 and IL-18. *Microbiol Immunol* 44, 1043–1050.
- Kobayashi, T. K., Ueda, M., Nishino, T., Moritani, S., Higaki, T. & Bamba, M. (2003). Cytologic detection of cryptococcosis coexisting with herpes simplex virus infection in sputum: use of liquid-based, thin-layer preparations. *Acta Cytol* 47, 103–106.
- Langermans, J. A., Hazenbons, W. L. & van Furth, R. (1994). Antimicrobial functions of mononuclear phagocytes. *J Immunol Methods* 174, 185–194.
- Levitz, S. M. (2001). *Cryptococcus neoformans*: intracellular or extracellular? *Trends Microbiol* 9, 417–418.
- Li, C., Goodrich, J. M. & Yang, X. (1997). Interferon-gamma (IFN-gamma) regulates production of IL-10 and IL-12 in human herpesvirus-6 (HHV-6)-infected monocyte/macrophage lineage. *Clin Exp Immunol* 109, 421–425.
- Lusso, P., Markham, P. D., Tschachler, E., di Marzo Veronese, F., Salahuddin, S. Z., Ablashi, D. V., Pahwa, S., Krohn, K. & Gallo, R. C. (1988). In vitro cellular tropism of human B-lymphotropic virus (human herpesvirus-6). *J Exp Med* 167, 1659–1670.

- Marti Cabane, J. & Alvarez Rubio, M. (2004). Herpes simplex esophagitis associated to *Candida albicans* in an immunocompetent host. *An Med Interna* 21, 312.
- Mayne, M., Cheadle, C., Soldan, S. S. & 7 other authors (2001). Gene expression profile of herpesvirus-infected T cells obtained using immunomicroarrays: induction of proinflammatory mechanisms. *J Virol* 75, 11641–11650.
- Meeuwssen, S., Persoon-Deen, C., Bsibsi, M., Bajramovic, J. J., Ravid, R., De Bolle, L. & Van Noort, J. M. (2005). Modulation of the cytokine network in human adult astrocytes by human herpesvirus-6A. *J Neuroimmunol* 164, 37–47.
- Milne, R. S., Mattick, C., Nicholson, L., Devaraj, P., Alcamì, A. & Gompels, U. A. (2000). RANTES binding and down-regulation by a novel human herpesvirus-6 beta chemokine receptor. *J Immunol* 164, 2396–2404.
- Modalsli, K., Bukholm, G. & Degre, M. (1992). Interferon treatment reduced adherence, invasiveness and intracellular multiplication of *Shigella flexneri* in coxsackie B1 virus-infected cells. *J Biol Regul Homeost Agents* 6, 35–45.
- Mucci, A., Varesio, L., Neglia, R., Colombari, B., Pastorino, S. & Blasi, E. (2003). Antifungal activity of macrophages engineered to produce IFN γ : inducibility by picolinic acid. *Med Microbiol Immunol* 192, 71–78.
- Nathan, C. F. (1987). Secretory products of macrophages. *J Clin Invest* 79, 319–326.
- Netea, M. G., Van der Graaf, C., Van der Meer, J. W. & Kullberg, B. J. (2004). Recognition of fungal pathogens by toll-like receptors. *Eur J Clin Microbiol Infect Dis* 23, 672–676.
- Oster, B., Bundgaard, B. & Hollsborg, P. (2005). Human herpesvirus 6B induces cell cycle arrest concomitant with p53 phosphorylation and accumulation in T cells. *J Virol* 79, 1961–1965.
- Rathman, M., Sjaastad, M. D. & Falkow, S. (1996). Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infect Immun* 64, 2765–2773.
- Retini, C., Casadevall, A., Pietrella, D., Monari, C., Palazzetti, B. & Vecchiarelli, A. (1999). Specific activated T cells regulate IL-12 production by human monocytes stimulated with *Cryptococcus neoformans*. *J Immunol* 162, 1618–1623.
- Roeder, A., Kirschning, C. J., Rupec, R. A., Schaller, M., Weindl, G. & Korting, H. C. (2004). Toll-like receptors as key mediators in innate antifungal immunity. *Med Mycol* 42, 485–498.
- Rogers, J., Rohal, S., Carrigan, D. R., Kusne, S., Knox, K. K., Gayowski, T., Wagener, M. M., Fung, J. J. & Singh, N. (2000). Human herpesvirus-6 in liver transplant recipients: role in pathogenesis of fungal infections, neurologic complications, and outcome. *Transplantation* 69, 2566–2573.
- Russell, D. G. (1994). Immunoelectron microscopy of endosomal trafficking in macrophages infected with microbial pathogens. *Methods Cell Biol* 45, 277–288.
- Salahuddin, S. Z., Ablashi, D. V., Markham, P. D. & 8 other authors (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 234, 596–601.
- Siren, J., Pirhonen, J., Julkunen, I. & Matikainen, S. (2005). IFN- α regulates TLR-dependent gene expression of IFN- α , IFN- β , IL-28, and IL-29. *J Immunol* 174, 1932–1937.
- Smith, A., Santoro, F., Di Lullo, G., Dagna, L., Verani, A. & Lusso, P. (2003). Selective suppression of IL-12 production by human herpesvirus 6. *Blood* 102, 2877–2884.
- Smith, A. P., Paolucci, C., Di Lullo, G., Burastero, S. E., Santoro, F. & Lusso, P. (2005). Viral replication-independent blockade of dendritic cell maturation and interleukin-12 production by human herpesvirus 6. *J Virol* 79, 2807–2813.
- Tardieux, I., Webster, P., Ravesloot, J., Boron, W., Lunn, J. A., Heuser, J. E. & Andrews, N. W. (1992). Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. *Cell* 71, 1117–1130.
- Trinchieri, G. (1997). Function and clinical use of interleukin-12. *Curr Opin Hematol* 4, 59–66.
- Tucker, S. C. & Casadevall, A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. *Proc Natl Acad Sci U S A* 99, 3165–3170.
- Uicker, W. C., Doyle, H. A., McCracken, J. P., Langlois, M. & Buchanan, K. L. (2005). Cytokine and chemokine expression in the central nervous system associated with protective cell-mediated immunity against *Cryptococcus neoformans*. *Med Mycol* 43, 27–38.
- Vázquez-Torres, A. & Balish, E. (1997). Macrophages in resistance to candidiasis. *Microbiol Mol Biol Rev* 61, 170–192.
- Vuorinen, T., Kotilainen, P., Lautenschlager, I., Kujari, H., Krogerus, L. & Oksi, J. (2004). Interstitial pneumonitis and coinfection of human herpesvirus 6 and *Pneumocystis carinii* in a patient with hypogammaglobulinemia. *J Clin Microbiol* 42, 5415–5418.
- Ward, K. N. (2005). The natural history and laboratory diagnosis of human herpesviruses-6 and -7 infections in the immunocompetent. *J Clin Virol* 32, 183–193.
- Weinberg, A., Bloch, K. C., Li, S., Tang, Y. W., Palmer, M. & Tyler, K. L. (2005). Dual infections of the central nervous system with Epstein-Barr virus. *J Infect Dis* 191, 234–237.
- Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y. & Kurata, T. (1988). Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* 1, 1065–1067.
- Yoshikawa, T., Asano, Y., Akimoto, S., Ozaki, T., Iwasaki, T., Kurata, T., Goshima, F. & Nishiyama, Y. (2002). Latent infection of human herpesvirus 6 in astrocytoma cell line and alteration of cytokine synthesis. *J Med Virol* 66, 497–505.