Human Herpesvirus-6 Enhances Natural Killer Cell Cytotoxicity Via IL-15

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Abstract

The marked tropism of human herpesvirus-6 (HHV-6) for natural killer (NK) cells and T lymphocytes has led us to investigate the effect of HHV-6 on cellular cytotoxicity. We describe here how HHV-6 infection of peripheral blood mononuclear cells (PBMC) leads to upregulation of their NK cell cytotoxicity. The induction of NK cell activity by HHV-6 was abrogated by monoclonal antibodies (mAbs) to IL-15 but not by mAbs to other cytokines (IFN-α, IFN-γ, TNF-α, TNF-β, IL-2, IL-12) suggesting that IL-15 secreted in response to viral infection was responsible for the observed effect. Furthermore, NK activation by HHV-6 was blocked with mAb to CD122, as well as by human anti–HHV-6 neutralizing antibodies. Using RT-PCR, we were able to detect IL-15 mRNA upregulation in purified monocyte and NK cell preparations. IL-15 protein synthesis was increased in response to HHV-6. Finally, addition of IL-15 to PBMC cultures was found to severely curtail HHV-6 expression. Taken together, our data suggest that enhanced NK activity in response to viral infection represent a natural anti-viral defense mechanism aimed at rapidly eliminating virus-infected cells. (*J. Clin. Invest.* 1996. 97:1373–1381.) Key words: human herpesvirus 6 • interleukin-15 • NK cells • exanthem subitum • anti-viral

Introduction

Natural killer (NK)[1] cells are lymphocytes capable of killing tumor cells and virus-infected cells independently of MHC restriction (1, 2). Their importance in viral immunosurveillance in vivo is evidenced by the fact that patients lacking NK cells are subject to multiple infections by herpesviruses (3, 4). One herpesvirus gaining medical interest since its discovery in 1986 (5) is the human herpesvirus-6 (HHV-6). This virus' growing importance in immunobiology is partly attributable to its ability to infect CD4+ T lymphocytes, the main cellular target of HIV-1; interestingly, HHV-6 has been shown to interact with HIV-1 (6–9). Infection of T cells by HHV-6 leads to the downregulation of CD3 molecule (10), upregulation of CD4 molecule (11) and inhibition of both IL-2 synthesis (12) and cellular proliferation (12–14). HHV-6 can also infect other immunocompetent cells such as B and CD8+ T lymphocytes, monocytes/macrophages, megakaryocytes and NK cells (11, 15–18). It has been reported that NK cells rapidly internalize HHV-6 (i.e., within 1 h) but signs of productive infection are only observed if cells are cultured for several weeks prior to infection, a time at which no residual NK cytotoxic activity is observed (18). Infection of NK cells by HHV-6 also leads to upregulation of CD4 molecules on the cell surface which correlates with susceptibility of these cells to HIV-1 infection (18).

NK cells are known to represent an important natural defense mechanism in controlling viral infection (1–2), and the ability of NK effectors to lyse autologous HHV-6-infected PBMC has been reported (19). However, there are no studies on the consequences of HHV-6 infection on NK cell activity per se. One group has reported that during the acute febrile phase of HHV-6 infection there was a significant increase in NK cytotoxicity when compared to the NK activity observed during convalescence (20). Our objective was to learn more about, and evaluate the potential effect of, HHV-6 infection on NK cell activity. Our results clearly show an enhancement of NK activity during the early phase of infection (24–48 h); this enhancement is abrogated by neutralizing antibodies to IL-15 as well as by anti-CD122 mAbs which react with the β subunit of the IL-2 receptor. IL-15 is a recently described cytokine of 14 kD first isolated from a simian epithelial cell line (21). Another group has also isolated a cytokine from the human leukemic Hut102 cell line, designated IL-T, which resembles closely IL-15 (22). IL-15 shares biological activities with IL-2 such as stimulation of T cell proliferation, induction of cytotoxic T cells and lymphokine activated killer (LAK) cell generation (21–23). IL-15 mediates its action through binding to the CD122 molecule as well as to the common gamma chain receptor (21, 23, 24). The present results show for the first time that the early events of immune cell activation such as NK cell cytotoxicity observed following infection by HHV-6, a human lymphotropic herpesvirus, are mediated via IL-15 induction, and also confirm that this cytokine is indeed a potent stimulator of NK activity. Our data may also explain the increase in NK activity during the acute febrile phase of HHV-6 infection (exanthem subitum) reported by other investigators (20).

Methods

Cell lines and viruses. HSB-2, K562 and CEM NKr cell lines were purchased from the American Type Culture Collection (ATCC) (Rockville, MD) cells were obtained through the National Institutes of Health AIDS Research and References Reagent Program (Bethesda, MD). These cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics

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1. *Abbreviations used in this paper:* CD, "cluster of differentiation," a leukocyte surface marker; HHV-6, human herpesvirus-6; IFA, immunofluorescence assay; NK, natural killer; P.I., Post-Infection; RT-PCR, reverse transcriptase polymerase chain reaction; TBS, Tris buffer saline; TCID 50, tissue culture infections dose 50.

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(i.e., complete medium). HHV-6 (GS strain) (5) was propagated in HSB-2 cells as previously described (25). Virus was concentrated from culture supernatant by high speed centrifugation. The viral preparation used in this study had a titer of 10^6 50% tissue culture infectious dose ($TCID_{50}$)/ml.

Peripheral blood mononuclear cell (PBMC) isolation. PBMC were obtained from healthy donors after centrifugation of heparinized venous blood over Ficoll-Hypaque (Pharmacia, Montreal, Canada) gradients and cultured in complete medium. Various cell populations were depleted from PBMC by first incubating the cells with either anti-CD3, -CD4, -CD8, -CD21 (Ortho Diagnostics, Raritan, NJ), anti-CD14 (Immunotech Inc., MA) or anti-CD16 (Becton Dickinson, CA) for 30 min at room temperature followed by addition of low toxicity baby rabbit complement (C') (Gibco Life Technologies, Mississauga, Canada) for 1 h at 37°C. Cells were washed and effectiveness of depletion assessed by flow cytometry (for CD3, CD4, CD8, CD21), cytomorphology (CD14) and cytotoxic activity (CD16). Consistently, $< 2\%$ contaminating CD3+, CD4+, and CD8+ and less than 1% CD21+ and monocytes were present in selectively depleted cultures. NK activity of CD16-depleted cultures was $< 2\%$ when tested in standard ^{51}Cr-release assay using K562 target cells. In some experiments, monocytes were isolated by elutriation. These monocyte preparations were $> 90\%$ pure as determined by CD14 marker reactivity.

NK cell cytotoxicity assay. NK cell activity was determined by standard ^{51}Cr-release assay (20). Briefly, K562 target cells (10^6) were labeled with 100 μCi of sodium chromate (DuPont, Canada) for 60 min at 37°C. After four washes, the target cells (10^4) were mixed with effector PBMC at a 1:20 ratio in V-bottomed wells and incubated for 16 h at 37°C in a CO_2 incubator. Plates were centrifuged and the radioactivity of supernatant (0.1 ml) was measured using a gamma counter (LKB, Sweden). Data are expressed as percent specific release after calculation using the following formula:

$$\frac{(\text{cpm experimental} - \text{cpm spontaneous})}{\text{cpm maximum} - \text{cpm spontaneous}} \times 100$$

In some experiments, NK cytotoxicity against the NK cell activity resistant CEM NKr cell line was also determined as described above.

Other antibodies and cytokines. Neutralizing mAbs to human IL-2, IL-12, interferon-α (IFN-α), tumor necrosis factor α and β were purchased from R&D Systems (Minneapolis, MN). Neutralizing mAb to IFN-γ was purchased from Genzyme (Boston, MA) and to CD122 from Becton-Dickinson. mAb to IL-15 and recombinant simian IL-15 were a gift from Immunex (Seattle, WA).

Cell Treatment. One million PBMC or PBMC depleted of various cell subpopulations were either treated (in duplicate) with mock-infected culture supernatant or infected with HHV-6 for 2 h at 37°C after which cells were washed with Hank's buffer to remove unadsorbed virus and resuspended in 1 ml of complete medium. After 20 h at 37°C, cells were adjusted to 4×10^6/ml and 0.05 ml of this suspension (in triplicate) were added to 10^4 ^{51}Cr-labeled K562 cells and NK activity was determined as described above. In some experiments, cell-free supernatant from mock- and HHV-6–treated PBMC were collected at various time points and added (at 25% final volume) to untreated PBMC before mixing with K562 cells.

NK activity of PBMC was also tested in presence of rIL-15 (50 ng/ml), anti–IL-15 (10 μg/ml) and anti-CD122 (10 μg/ml).

Virus neutralization and inactivation. HHV-6 was inactivated by heat (56°C, 1 h) or by ultra-violet (UV) light irradiation (2×10^6 μJ). Effectiveness of inactivation treatments was determined by testing infectivity of viral preparations by immunofluorescence assays (IFA) on HSB-2 cells using mAb specific for HHV-6 gp110 (ABI, Columbia, MD). Consistently, $< 1\%$ of cells were found positive for HHV-6 gp110 4 d after treatment with inactivated preparations while 40% of cells were positive for gp110 after treatment with infectious virus. As a specificity control, HHV-6 preparation neutralized with a reference serum was used (12, 16). The HHV-6 antibody-positive serum used was from a patient with acute lymphocytic leukemia from whom the GS isolate was obtained. GS serum had a IgG titer (to HHV-6) of 320, as measured by IFA and neutralized 1000 TCID50 of HHV-6 at a 1:20 dilution. This serum (with gold labeling) binds to the surface of HHV-6 (not shown). In control experiments, we found that this serum sample adsorbed with Epstein-Barr virus, human cytomegalovirus and herpes simplex virus lost its neutralizing activity to these viruses but still retained activity against HHV-6.

Immunofluorescence and DNA slot blot. The effects of rIL-15 on HHV-6 expression was determined by IFA and DNA slot blot techniques. PBMC were depleted of CD8+ or CD56+ or both cell populations using magnetic beads coated with anti-CD8 or CD56 mAb and then stimulated with PHA (1 μg/ml) and IL-2 (5 U/ml) for 2 d. Cells were infected with HHV-6 for 2 h, then washed with PBS and resuspended in complete medium containing IL-2 (5 U/ml) with or without rIL-15 (10, 50, 100 ng/ml) or anti–IL-15 antibodies (10 μg/ml). On day 7 after infection, the cells were harvested, washed with PBS and processed for IFA. Live cells were stained with anti–HHV-6 2D6 mAb (ABI, Columbia, MD) followed by fluoresceinated goat anti–mouse IgG antibodies (Sigma Chemical Co., St. Louis, MO). Five thousand cells were analyzed by flow cytometry (Facscan, Becton Dickinson, CA). On day 7 after infection, genomic DNA was extracted using the Easy DNA kit (Invitrogen, San Diego, CA) and processed for slot blot hybridization. 0.5 μg, 0.1 μg, and 0.02 μg of DNA from each sample were denatured with NaOH (0.1 N) and applied to nylon membranes. Membranes were probed with the HHV-6 HinDIII fragment ZVH14 and detection was carried out by chemiluminescence according to the manufacturer's guidelines (Amersham, Arlington Heights, IL).

Immunodetection of IL-15 production by monocytes. Elutriated monocytes (91% CD14+) were either treated with mock-infected fluid or with HHV-6 for 1 h, washed with PBS and resuspended in complete medium (20 million/ml) for varying time periods (i.e., from 2 to 18 h) and then supernatants were collected and stored frozen until used. 200 ul of undiluted, 1/2 and 1/4 diluted samples were applied to nitrocellulose membrane using a dot blot apparatus (Life Technologies, Grand Island, NY). Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 (TBS-T) for one hour, washed with TBS-T and incubated overnight at 4°C with anti–IL-15 mAb (Genzyme, Cambridge, MA) (5 μg/ml). Detection was made by chemiluminescence (Amersham) using a peroxydase-labeled goat anti–mouse serum. The relative IL-15 protein level was determined by laser densitometry.

IL-15 mRNA analyses by RT-PCR. PBMC, elutriated monocytes, and positively selected cell populations were treated either with mock fluid or HHV-6 for varying time periods and then total RNA was extracted, treated with DNase and subjected to RT-PCR as previously described (12). Ten percent of the PCR reaction was electrophoresed on a 2% agarose gel, transferred to nylon membranes and hybridized with specific ^{32}P end-labeled oligonucleotide probes complementary to an internal region of the amplicons. The following oligonucleotides were used in PCR reactions: IL-15A (5′-ATGAGAATTTCGAAACCACATTTG-3′), IL-15B (5′-CCATTAGAAGACAAACTGTTCTTTGC-3′), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin (see Refs. 12, 25). The following hybridization probe was used for IL-15: IL-15P (5′-ATGTCTTCATTTTGGGCTGTTTCAGTGCAG-3′). After hybridization and washes, membranes were exposed to films. The housekeeping genes GAPDH and actin were used as RNA controls to evaluate the amount of RNA used for amplification. The concentration of RNA among the various samples was corrected by calculating the optical density values of the actin and GAPDH bands. To calculate the fold increase in IL-15 RNA transcripts, the ratio (IL-15/housekeeping gene) obtained from HHV-6-stimulated cells was compared to the ratio (IL-15/housekeeping gene) for mock-treated cells, as previously described (12, 25).

Results

HHV-6 increases cytotoxic activity of CD16+ cells. Patients' NK activity during the acute febrile phase of HHV-6 infection has

been shown to be increased when compared to that of NK activity of patients in convalescence (20). In an effort to better understand this phenomenon, we studied NK activity of PBMC from healthy individuals after in vitro infection by HHV-6. PBMC (10^6) from 10 individuals were infected with the GS strain of HHV-6 (10^5 TCID50) and 20 h P.I. cytotoxic activity was determined using a standard ^{51}Cr-release assay on K562 target cells. Among the PBMC donors, individuals with known low NK activity were also included. As shown in Fig. 1 *A*, an increase in cytotoxic activity was observed in all HHV-6-infected PBMC samples when compared to the cytotoxic activity of mock-infected cells. The mean difference in cytolytic activity between infected and uninfected cells was highly significant ($P < 0.001$, by Student *t* test). Although variations in cytotoxic activity between PBMC from different donors was observed as expected, all showed greater killing efficiency following infection with HHV-6, regardless of their HHV-6 antibody (i.e., seropositive or seronegative) status. Furthermore, the increase in cytotoxicity was correlated with the HHV-6 dose used. As shown in Fig. 1 *B*, at the lowest dose of HHV-6 used (i.e., 0.0025 $TCID_{50}$), no significant increase was observed while higher dose of virus caused significant enhancement of target cell lysis. Similar experiments conducted with HHV-6 strain Z29 (B type) gave identical results (not shown). To determine if this increase in cytotoxic activity is associated with a NK phenotype, we selectively depleted CD16+ cells from PBMC and tested the effect of HHV-6 on the cytolytic activity of the residual cell population. As shown in Fig. 2, infection by HHV-6 of unfractionated PBMC from 3 donors caused a significant increase ($P < 0.01$) in killing. These cells were treated with rabbit complement in order to provide appropriate controls. However, when CD16+ cells were depleted from PBMC, a significant decrease ($P < 0.01$) in spontaneous NK activity was observed. Moreover, HHV-6 infection of CD16-depleted PBMC did not result in increased cytotoxicity above that of mock-infected PBMC, suggesting that CD16+ cells are directly associated with the increased killing of K562 cells observed following infection by HHV-6. Addition of HHV-6 directly to K562 cells in the absence of effector cells did not provoke lysis of target cells ruling out the possibility of direct cytotoxicity by HHV-6 (data not shown).

Detection of NK stimulatory factor in supernatants of HHV-6–infected PBMC cultures. Increased NK activity associated with infection of PBMC could be due to direct activation of NK cells by HHV-6 or via the induction and release of NK stimulatory factor(s). To test these hypotheses, PBMC from two donors were infected with HHV-6 and supernatants were collected at 0, 5, 10, 15, and 20 h post-infection (PI). PBMC were washed extensively after virus adsorption in order to prevent supernatant contamination by residual viral particles. Supernatants collected at various times after infection were added (25% final volume) to resting PBMC of the corresponding individual donor and analyzed for their ability to stimulate NK activity. As shown in Fig. 3, a significant ($P < 0.05$) increase in NK activity was observed after addition of supernatant (5 h) from donor 1, when compared to that of supernatant at time 0, which represents the baseline value. Supernatants collected 10 h post-infection were capable of stimulating NK cells of both donors efficiently ($P < 0.02$). Longer incubation post-infection did not significantly increase NK activity above that at 10 h after infection. Since it is known that productive infection of resting PBMC by HHV-6 is not an efficient process

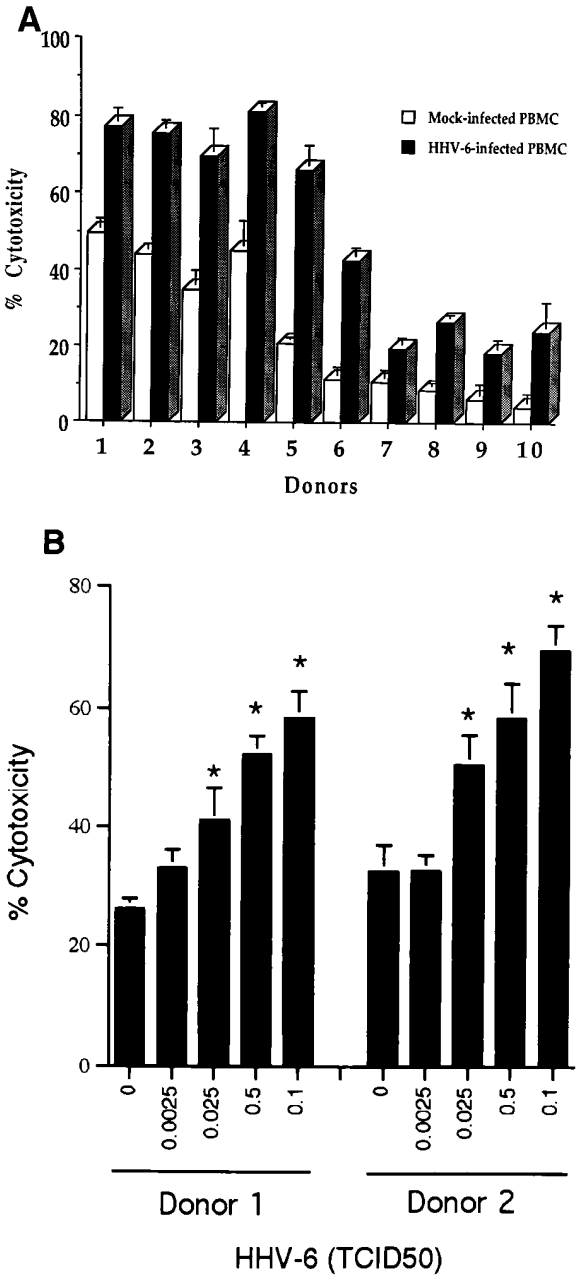

Figure 1. Enhancement of cytotoxic activity of PBMC by HHV-6. (*A*) PBMC from 10 healthy individuals were obtained as described in Methods. PBMC (10^6) were either treated with mock-infected culture supernatant or infected with HHV-6 (10^5 TCID50) and 20 h P.I. cytotoxicity was determined by standard 16-h ^{51}Cr-release assay on K562 cell line at a 20:1 effector to target ratio. Data, expressed as percent cytotoxicity, represent mean±SD obtained from triplicate experimental values for each donor. Comparison of mean differences between infected and noninfected PBMC gave a *P* value of < 0.001. Donors 3 and 8 represent HHV-6 seronegative individuals. (*B*) PBMC from two donors were treated with varying doses of HHV-6 and cytotoxicity evaluated as above. $*P < 0.05$.

(26), it is very unlikely that the presence of viral particles in supernatant was responsible for the NK stimulation observed. Moreover, addition of supernatant from HHV-6–treated PBMC to K562 cells, in the absence of effector cells, did not cause any

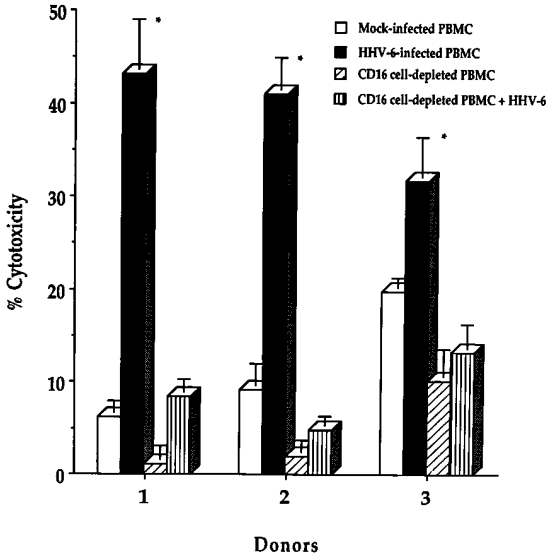

Figure 2. Cytotoxic activity is associated with a CD16 phenotype. Unfractionated PBMC or PBMC depleted of CD16+ cells were either treated with mock-infected culture supernatant or infected with HHV-6 (10^5 TCID50) and 20 h P.I. cytotoxic activity was determined as described in Methods. Data, expressed as percent cytotoxicity, represent mean±SD calculated from six experimental values for each donor. $*P < 0.01$.

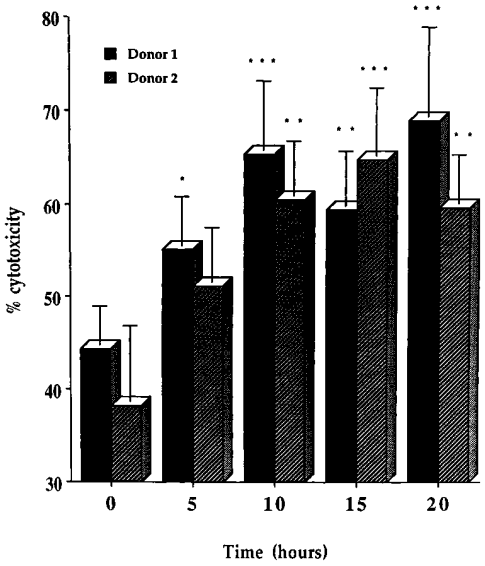

Figure 3. HHV-6 induces a soluble factor responsible for NK cell activation. PBMC from two donors were obtained as described in Methods. Cell pellets (10^6 cells/pellet), in duplicate, were treated with HHV-6 (10^5 TCID50) and immediately washed with PBS ($t = 0$) or left undisturbed for 2 h at 37°C before being washed and resuspended in 1 ml of complete medium. At various times (5, 10, 15, 20 h) cells were centrifuged and supernatants collected and stored at −80°C until used. Ability of supernatants from various time points to stimulate NK activity was determined by adding them (at 25% final volume) to resting (20 h) PBMC at time of mixing with K562 cells. T = 0 represent baseline values of NK activity. Data, expressed as percent cytotoxicity, represent mean±SD calculated from six experimental values for each time point. $*P < 0.05$, $**P < 0.02$, $***P < 0.01$.

significant lysis, thus excluding the possibility of direct cytotoxicity by soluble factors.

Having determined that HHV-6 induced a NK stimulatory factor within 10 h of infection, we became interested in identifying which cell population(s) is(are) responsible for the production of such factor. Supernatant from HHV-6–infected (20 h P.I.) PBMC (of four donors), which were depleted of selected cell populations such as CD3+, CD4+, CD8+, CD14+, CD21+, and CD16+ cells were therefore tested for their ability to stimulate NK activity. Results from these experiments suggested that NK stimulatory factor production cannot be ascribed to a sole cell subset (data not shown) and is presumably the result of the response of different cell types to viral infection.

Infectivity of HHV-6 is not required for NK cell stimulation. After the above evidence that NK cell stimulation by HHV-6 is a result of a cell secreted factor, as a next step we tested if viral infectivity was necessary for induction of this factor. The results summarized in Table I indicate that UV- or heat-inactivated HHV-6 is still able to stimulate NK activity as efficiently as the infectious virus. However, neutralization of HHV-6 by human antibodies abolished the ability of HHV-6 to stimulate NK activity. These results suggest that thermostable protein(s), recognized by the neutralizing antibodies, is(are) involved in the triggering of the NK stimulating-factor.

In light of the above observations on the NK activity-enhancing effect of HHV-6 we were also interested in knowing whether this virus can modulate NK cytotoxicity against a NK activity resistant cell line such as CEM-NKr. Results presented in Table I (bottom panel) indicate that no increase in killing against the CEM target was observed following infection of PBMC by HHV-6.

IL-15 is responsible for NK cell activation by HHV-6. It has recently been reported that human PBMC, upon stimulation with IL-15, become capable of efficiently lysing different target cells (21). However, the effect of IL-15 on NK activity against the NK sensitive K562 cell line has not been reported. Addition of recombinant simian IL-15 to PBMC cultures greatly enhanced the lytic power of NK cells towards K562 target cells (Fig. 4). However, pre-incubation of IL-15 with neutralizing mAb to IL-15 eliminated this cytotoxicity-enhancing activity of the IL-15 (not shown). Interestingly, the addition of anti IL-15 to PBMC cultures resulted in loss of NK activity while anti IFN-γ had no effect (data not shown).

Knowing that HHV-6 induced a soluble factor capable of

Table I. Effect of Infectious and Inactivated HHV-6 on NK Cell Stimulation

PBMC treatment	Donor 1	Donor 2
Percent cytotoxicity against K562 target cells		
Mock	23.1±2.8	34.9±3.6
HHV-6	58.9±7.9*	69.7±5.6*
Neutralized HHV-6	28.1±1.6	48.2±5.2
UV-irradiated HHV-6	49.8±2.9*	68.3±4.0*
Heat-inactivated HHV-6	44.5±4.3*	68.1±3.9*
Percent cytotoxicity against CEM NK^r target cells		
Mock	11.9±0.7	5.3±0.9
HHV-6	12.1±3.8	8.2±3.2

$*P \leq 0.05$

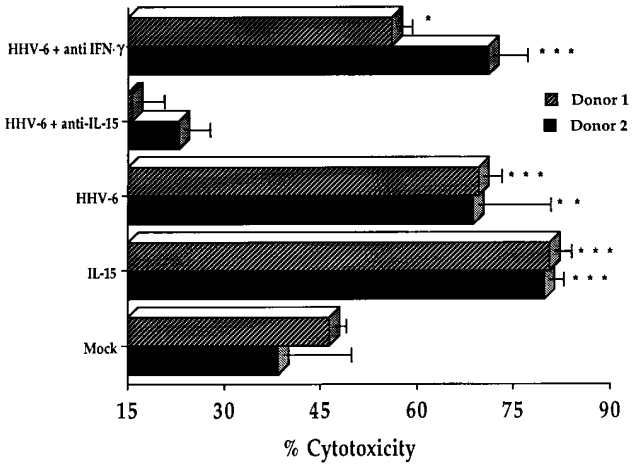

Figure 4. Inhibition of HHV-6-induced NK cell activity by mAb to IL-15. PBMC from two donors were either treated in duplicate with mock-infected culture medium, IL-15 (50 ng/ml), HHV-6, HHV-6+ anti–IL-15 (10 μg/ml) or HHV-6 + anti IFN-γ (10 μg/ml) and 20 h after the NK cytotoxic activity was determined as described in Methods. Data, expressed as percent cytotoxicity, represent mean±SD obtained from six experimental values. $*P < 0.05$, $**P < 0.02$, $***P < 0.01$.

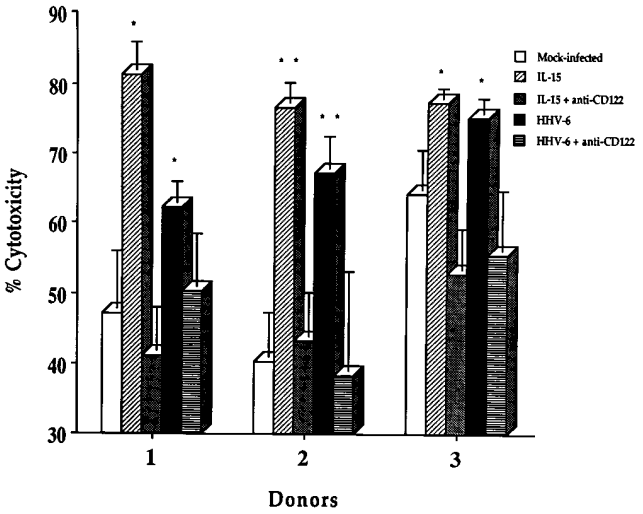

Figure 5. mAb to CD122 blocks stimulation of NK cell activity by both HHV-6 and IL-15. PBMC from three individuals were either treated with mock-infected culture supernatant, IL-15 (50 ng/ml), IL-15 + anti-CD122 (10 μg/ml), HHV-6 or HHV-6 + anti-CD122 and 20 h after cytotoxic activity of PBMC was determined as described in Methods. Data, expressed as percent cytotoxicity, represent mean±SD obtained from six experimental values. $*P < 0.05$, $**P < 0.01$.

stimulating NK activity, our efforts were deployed in identifying such factor. We therefore incubated HHV-6–infected PBMC in presence of neutralizing antibodies to cytokines, i.e., IL-2, IFN-α, IFN-γ, TNF-α, TNF-β, and IL-12 known to activate NK cells. There were no significant differences in cytotoxic killing of target cells by HHV-6–infected PBMC treated or not with these cytokine-neutralizing antibodies (data not shown). However, when anti–IL-15 antibodies were added to HHV-6 infected PBMC cultures, a striking reduction in NK activity was observed. As shown in Fig. 4, both HHV-6 and rIL-15 (50 ng/ml) increased cytotoxic activity of PBMC. Addition of antibodies to IL-15 at the time of infection considerably reduced the lysis of target cells to levels below those of untreated PBMC, presumably as a consequence of the neutralization by these antibodies of the spontaneously produced IL-15. On the other hand, addition of neutralizing antibodies to IFN-γ at the time of infection did not significantly affect the ability of HHV-6 to stimulate NK cells.

It has been reported that IL-15 interacts with the β chain (CD122) of the IL-2 receptor and that blocking of CD122 with antibodies abolishes the ability of IL-15 to stimulate cytotoxic activity (21, 23). As an additional test of the hypothesis that HHV-6 mediates NK cell activation through an IL-15–dependent pathway, we examined the effect of anti-CD122 antibodies on such activation. First, as shown in Fig. 5, no increase in cytotoxicity was observed when HHV-6–infected PBMC were incubated in the presence of anti-CD122, confirming the notion that IL-15 is indeed the cytokine involved in NK cell activation associated with HHV-6 infection. Second, we confirmed that incubation of cells with anti-CD122 blocks the effect of IL-15 on NK cell activation.

HHV-6 induces IL-15 mRNA, and IL-15 protein synthesis in purified monocytes. All evidence gathered so far suggested that IL-15 was the cytokine responsible for the enhanced NK activity observed after HHV-6 treatment. Since adherent cells obtained from PBMC were found to express high level of IL-15 messages (21), we used purified monocyte preparations to test whether HHV-6 can actually modulate IL-15 gene expression. Elutriated monocytes were treated with HHV-6 for varying time periods after which IL-15 and actin mRNA levels were determined by RT-PCR. As shown in Fig. 6 (top and middle figures), both mock-treated PBMC and monocytes constitutively expressed IL-15 messages. Upon HHV-6 stimulation, induction of IL-15 mRNA was observed as early as 2 h P.I. reaching maximal activation at 10 h P.I. with a threefold increase in IL-15 mRNA as compared to mock-treated cells. In addition, we tested whether IL-15 transcripts could be detected in cell types other than monocytes. PBMC were treated with HHV-6 for 8 h and then, with the use of antibody-coated magnetic beads, CD2+, CD19+, CD14+, and CD56+ cells were positively selected, lysed and processed for RT-PCR as described above. IL-15 transcripts were only detected in unfractionated PBMC, and in CD14+ and CD56+ cell fractions (Fig. 6, *bottom*) while no IL-15 signal was observed in T (CD2+) and B cell (CD19+) fractions (not shown).

Supernatants from mock-and HHV-6–treated monocytes were also tested for immunoreactive IL-15 as described in Methods. As shown in Fig. 7, a slight induction of IL-15 was observed three hours post HHV-6 treatment. By 6 h, a 2.5-fold increase was found and by 9 and 19 h P.I. a 3.6-fold increase in IL-15 protein level was observed in HHV-6 treated monocytes as compared to sham-treated control group.

NK and CD8+ T cell–mediated control of HHV-6 infection. Killing of HHV-6 infected targets by NK cells has been observed previously (19). To evaluate the potential antiviral role of NK cells and their activation by IL-15, we first determined the ability of HHV-6 to infect CD8- and CD56-depleted PBMC cultures. As shown in Fig. 8 *A*, the level of HHV-6 expression was slightly increased in NK-depleted cells compared to that of CD8-depleted cultures. Addition of anti–IL-15 neutralizing antibodies resulted in enhanced HHV-6 expression

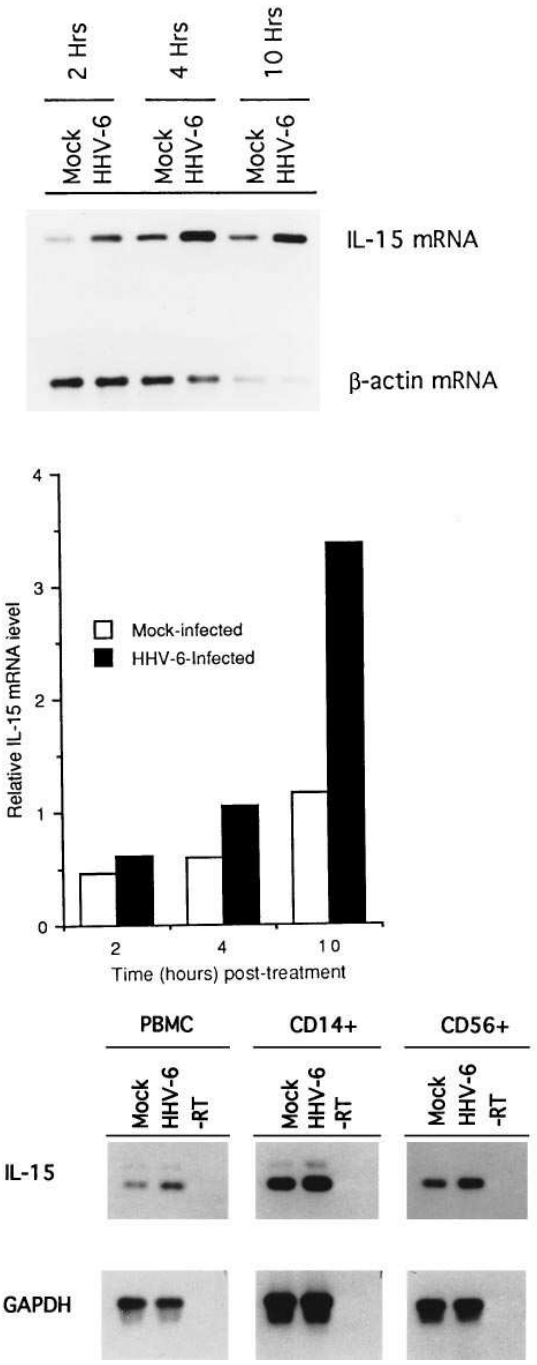

Figure 6. HHV-6 augments IL-15 transcription in monocytes. Elutriated monocytes were treated either with mock infected culture fluid or HHV-6 for time periods indicated. Total RNA was then extracted and subjected to RT-PCR for IL-15 and actin as described in Methods. The top figure represents autoradiograph of IL-15 and actin mRNA. The middle figure represents the calculated relative IL-15 mRNA levels after normalization to actin as previously described (12). The bottom figure represents the IL-15 and GAPDH transcripts in PBMC and positively selected CD14+ and CD56+ cells after 8-h treatment with HHV-6. RT- lanes represent PCR amplification controls for DNA contamination in which no reverse transcriptase was added.

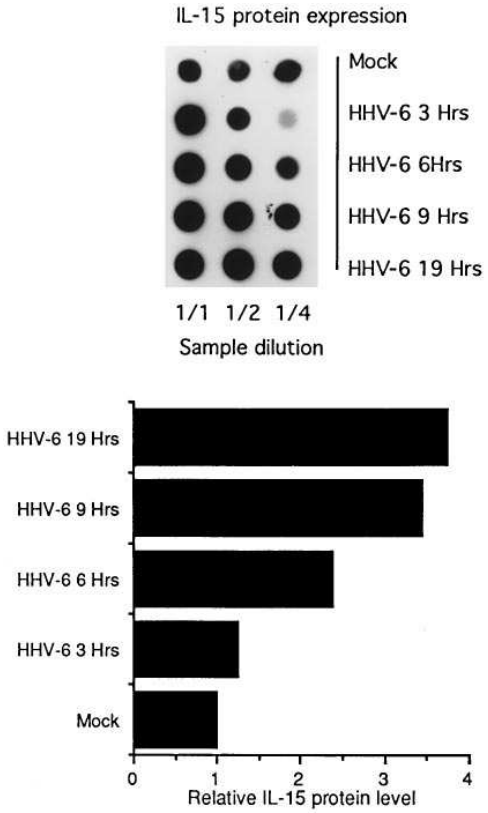

Figure 7. HHV-6 induces IL-15 protein secretion in human monocytes. Freshly elutriated monocytes (20×10^6) were treated either with mock fluid or HHV-6 for 1 h after which cells were washed twice with PBS and resuspended in one ml culture medium. At times indicated in the figure, cell-free supernatants were taken and stored frozen (−80°C) until used. IL-15 protein levels were determined using mAb to IL-15 and goat anti–mouse IgG (peroxydase labeled) as secondary antibody. Detection was done by chemiluminescence according to manufacturer's technical guidelines (Amersham). Relative protein levels were determined by laser densitometry after normalization to mock-treated control.

with the most dramatic effect observed in NK-depleted cultures (Fig. 8 *A*, *middle panels*). The addition of recombinant IL-15 (10 ng/ml) to CD8- or CD56-depleted cultures was found to severely inhibit HHV-6 infection (Fig. 8 *A*, *right panels*) suggesting that the control of HHV-6 infection is not solely dependent on one phenotypic cell population. To learn more on this aspect, we then tested the effect of IL-15 on HHV-6 infection of cultures depleted of both CD8+ and CD56+ cells. In these doubly depleted cultures, a previously effective dose of IL-15 (10 ng/ml) was found to have minimal effect on HHV-6 infection, suggesting that at least one of the cell types, i.e., either CD8+ or CD56+ cells, must be present for IL-15 to be effective in the control of HHV-6 infection. At the highest dose of IL-15 used (i.e., 100 ng/ml), a reduction in HHV-6 infection was observed, likely as a result of residual CD8+ T or NK cell in the culture.

Discussion

In general, viral infections trigger a cascade of events in the host, leading to immune cell activation which eventually re-

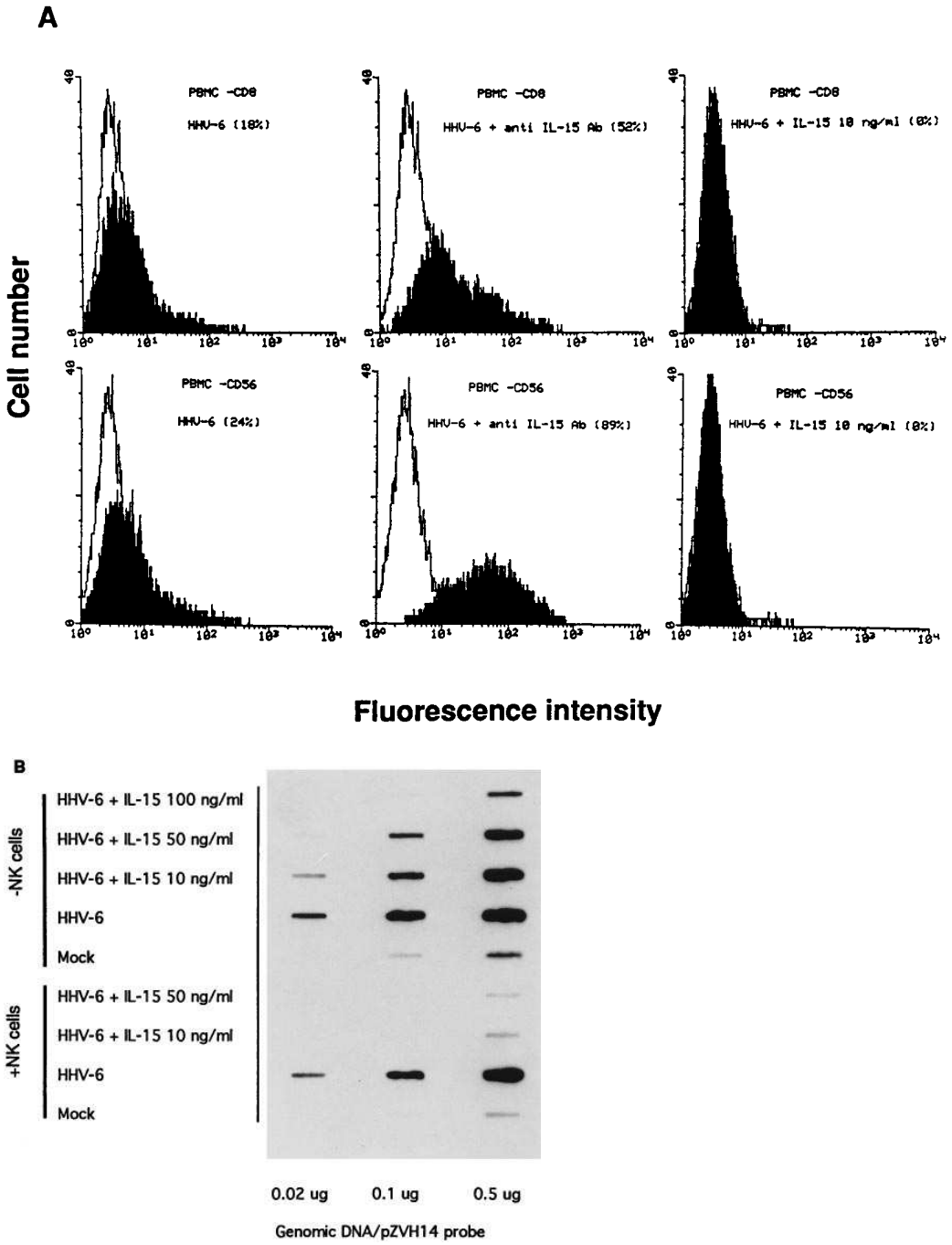

Figure 8. NK- and CD8+ T cells-mediated control of HHV-6 infection. CD8-depleted, CD56-depleted or CD8- and CD56-depleted PBMC were stimulated with PHA and IL-2 for 2 d. Cells were infected with HHV-6 for 2 h, washed with PBS and resuspended in complete medium supplemented with IL-2 (5 U/ml). Selected cultures were further conditioned with anti–IL-15 antibodies (10 ug/ml) or with rIL-15 (10–100 ng/ml). (*A*) On day 7 P.I., cells were processed for IFA as described in Methods. Top row represents HHV-6 infection of CD8-depleted PBMC while bottom row represents HHV-6 infection of CD56-depleted PBMC. Middle panels represent results of infection in presence of anti–IL-15 antibodies and right panels show results of infection in presence of rIL-15 (10 ng/ml). The percentage of HHV-6 antigen positive cells was determined by flow cytometry after analysis of 5000 cells. (*B*) CD8-depleted PBMC (lower half of figure) or CD8- and CD56-depleted PBMC (*upper half*) were infected with HHV-6 in presence or absence of rIL-15. On day 7 P.I., genomic DNA was extracted from cells and hybridized with ZVH14 probe (HHV-6 specific). Data shown are representative of two separate experiments.

sults in the control of the invading pathogens. The nonspecific arm of the immune response, represented by macrophages, NK cells, granulocytes and cytokines is thought to play an important role in limiting the early progression of infection by viruses. Mechanisms such as inhibition of virus multiplication by IFNs and lysis of infected cells by NK cells enable specific B and T lymphocytes to mount an effective response which eventually leads to viral clearance. Although most viral infections are cleared this way, some viruses have elaborated sophisticated strategies to evade immune attack by limiting viral gene expression in infected cells. Perhaps the best examples of immune evasion by viruses are those observed with Herpesviruses. These viruses are never eliminated from the host despite a strong immune response developed following primary infection. Whether it is by residing in privileged areas such as sensory neurons (e.g., HSV-1 and VZV) or through modulation of cell surface molecules (e.g., CMV and EBV), these herpesviruses persist throughout the host's life. Viral reactivation from latently-infected cells can lead to serious secondary pathogenic events especially in immunocompromised patients such as organ graft recipients, patients under anti-cancer chemotherapy, and HIV-seropositive individuals.

One herpesvirus gaining increasing medical importance is HHV-6. HHV-6's marked tropism for cells of the immune system has made this virus the source of numerous speculations with regard to its contribution to various immunodeficiency-related diseases. A previous report by Lusso et al. (18) described the infection and destruction of human NK cells by

HHV-6. Although these authors observed rapid internalization of HHV-6 particles (i.e., within 1 h PI), productive infection of NK cells could only be observed if NK cells were cultured for several weeks before infection. The direct impact of HHV-6 infection could not be studied since no residual NK activity was observed after prolonged in vitro culture of NK cells. Our objective was to learn more about HHV-6-NK cell interactions by studying the cytotoxic activity of such cells after an encounter with HHV-6. Our results clearly show that HHV-6 increases the cytotoxic activity of NK cells early after exposure to virus. Our data also show that enhancement of NK cell cytotoxicity by HHV-6 is restricted to NK-sensitive cell lines such as K562 and is not observed against naturally resistant cells such as the CEM-NKr cell line. These results suggest that HHV-6 increases the cytotoxic potential of NK cells in such a way that NK sensitive target cells are more readily recognized and killed. The mechanism by which HHV-6 stimulates NK cell activity was defined after the observation that conditioned media from HHV-6–infected PBMC could also stimulate NK cells. The secretion of a soluble factor, in response to viral infection, was responsible for the observed effect. The fact that both UV- and heat-inactivated HHV-6 preparations could also stimulate NK cells as efficiently as the infectious virus, suggested that a thermostable HHV-6 protein, after interaction with cells, triggers the secretion of immunomodulatory factor(s) capable of stimulating NK cells. It is conceivable that the binding of viral and cellular ligands was responsible for this effect. After numerous attempts to identify the NK activating factor(s) we succeeded in linking IL-15 to this phenomenon. Other cytokines such as IL-2, TNF-α, TNF-β, IFN-α, IFN-γ, and IL-12 were not found to be involved in NK cell activation in this system. IL-15 is a newly described cytokine that possesses biological properties similar to those of IL-2 such as induction of LAK, T cell growth promotion and induction of CTL activity (21–23). Furthermore, it was shown that IL-15 binds to CD122 (p75 or β chain) and that blocking of CD122 with mAb results in the inhibition of IL-15 biological activities. In our system, in the presence of mAb to CD122, HHV-6 did not stimulate NK activity, corroborating the observation that IL-15 needs to interact with CD122 to transmit its stimulatory signal to the NK cell (21, 23).

It is known that NK cells play an important role in immunosurveillance through killing of virus-infected and tumor cells. The results presented here, using HHV-6, have enabled us to better understand the network of interactions involved in sustaining and increasing NK cell activity in response to a threat such as a viral infection. Through IL-15 secretion, NK cells acquire increased killing potential, which is effective in eliminating infected cells thereby limiting viral growth and spread. Secretion of spontaneous IL-15 also contributes to maintain NK cell killing potential in vitro. Addition of antibodies to IL-15 considerably reduced the spontaneous NK activity which was correlated with an increase in HHV-6 expression. The combination of NK cell-depleted cultures together with neutralizing antibodies to IL-15 lead to the highest levels of HHV-6 expression suggesting that the cytotoxic potential and the antiviral activity of the cells involved is at least partly dependent on this cytokine. The addition of exogenous recombinant IL-15 was found to greatly influence the ability of HHV-6 to grow in PBMC, CD8- or CD56-depleted cultures. In fact, cultures treated with IL-15 were not found to support viral growth. However, the removal of both CD8+ and CD56+ cells impaired the anti-viral effectiveness of IL-15 suggesting an indirect mode of action, through cytotoxic cell activation and increased killing of infected cells. A similar, but IL-2–mediated mechanism involving effectors with a CD8+/CD16+ phenotype has been reported in the control of Epstein-Barr virus-infected/transformed cells in vitro (27).

Finally, the present data raise the question of whether other viruses are also capable of modulating NK cell activity in such a fashion (i.e., via IL-15 induction). We are currently investigating this issue.

In conclusion, the results presented here are the first to show that (*a*) HHV-6 enhances NK cell activity and that (*b*) this enhancement occurs via IL-15. Our results may also explain the in vivo observations of Takahashi et al. (20) who have reported higher NK activity in patients with acute HHV-6 infection. Our data also suggest that, although HHV-6 can interact with NK cells, these cells are still functional during the first days post-infection and, moreover, they are highly cytolytic as a consequence of IL-15 release by PBMC in response to HHV-6 infection. Secretion of IL-15 following viral infection may therefore represent a powerful host defense mechanism involved in restricting viral growth and in limiting the spread of the infectious agent.

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References

1. Herberman, R.B., and J.R. Ortaldo. 1981. Natural killer cells: their role in defenses against disease. *Science (Wash. DC).* 214:24–30.

2. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187–376.

3. Fleisher, G., S. Starr, N. Koven, H. Kamiya, S.D. Douglas, and W. Henle. 1982. A non-X-linked syndrome with susceptibility to severe Epstein-Barr virus infections. *J. Pediatrics.* 100:727–730.

4. Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infection in an adolescent without natural killer cells. *N. Engl. J. Med.* 320:1731–1735.

5. Salahuddin, S.Z., D.V. Ablashi, P.D. Markham, S.F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, B. Kramarsky, and R.C. Gallo. 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science (Wash. DC).* 234:596–601.

6. Ensoli, B., P. Lusso, F. Schachter, S.F. Josephs, J. Rappaport, F. Negro, R.C. Gallo, and F. Wong-Staal. 1989. Human herpesvirus-6 increases HIV-1 expression in co-infected T cells via nuclear factors binding to the HIV-1 enhancer. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3019–3027.

7. Horvat, R.T., C. Wood, and N. Balachandran. 1989. Transactivation of human immunodeficiency virus promoter by human herpesvirus 6. *J. Virol.* 63: 970–973.

8. Horvat, R.T., C. Wood, S.F. Josephs, and N. Balachandran. 1991. Transactivation of the human immunodeficiency virus promoter by human herpesvirus 6 (HHV-6) strains GS and Z-29 in primary human T lymphocytes and identification of transactivating HHV-6 (GS) gene fragments. *J. Virol.* 65:2895–2902.

9. Lusso, P., B. Ensoli, P.D. Markham, D.V. Ablashi, S.Z. Salahuddin, E. Tschachler, F. Wong-Staal, and R.C. Gallo. 1989. Productive dual infection of human CD4+ T lymphocytes by HIV-1 and HHV-6. *Nature (Lond.).* 337:370–373.

10. Lusso, P., M. Malnati, A. De Maria, C. Balotta, S.E. DeRocco, P.D. Markham, and R.C. Gallo. 1991. Productive infection of CD4+ and CD8+ mature human T cell populations and clones by human herpesvirus 6: transcriptional down-regulation of CD3. *J. Immunol.* 147:685–691.

11. Lusso, P., A. DeMaria, M. Malnati, F. Lori, S. DeRocco, M. Baseler, and R.C. Gallo. 1991. Induction of CD4 and susceptibility to HIV-1 infection in human CD8+ T lymphocytes by human herpesvirus 6. *Nature (Lond.).* 349: 533–535.

12. Flamand, L., J. Gosselin, I. Stefanescu, D.V. Ablashi, and J. Menezes. 1995. Immunosuppressive effect of human herpesvirus 6 on T cell functions: suppression of interleukin 2 synthesis and cell proliferation. *Blood.* 85:1263–1271.

13. Flamand, L., J. Gosselin, D.V. Ablashi, R.C. Gallo, and J. Menezes. 1993. Differential effects of human herpesvirus-6 infection on cytokine synthesis by T lymphocytes. *J. Acquired Immunodef. Synd.* 6(6):697.

14. Horvat, R.T., M.J. Parmely, and B. Chandran. 1993. Human herpesvirus 6 inhibits the proliferative response of human peripheral blood mononuclear cells. *J. Infect. Dis.* 167:1274–1280.

15. Ablashi, D.V., S.F. Josephs, A. Buchbinder, et al., 1988. Human B-lymphotropic virus (human herpesvirus 6). *J. Virol. Methods.* 21:29–48.

16. Flamand, L., I. Stefanescu, D.V. Ablashi, and J. Menezes. 1993. Activation of the Epstein-Barr virus replicative cycle by human herpesvirus 6. *J. Virol.* 67:6768–6777.

17. Kondo, K., T. Kondo, T. Okuno, M. Takahashi, and K. Yamanishi. 1991. Latent human herpesvirus 6 infection of human monocytes/macrophages. *J. Gen. Virol.* 72:1401–1408.

18. Lusso, P., M.S. Malnati, A. Garzino-Demo, R.W. Crowley, E.O. Long, and R.C. Gallo. 1993. Infection of natural killer cells by human herpesvirus 6. *Nature (Lond.).* 362:458–462.

19. Malnati, M.S., P. Lusso, E. Ciccone, A. Moretta, L. Moretta, and E.O. Long. 1993. Recognition of virus-infected cells by natural killer cell clones is controlled by polymorphic target cell elements. *J. Exp. Med.* 178:961–969.

20. Takahashi, K., E. Segal, T. Kondo, T. Mukai, M. Moriyama, M. Takahashi, and K. Yamanishi. 1992. Interferon and natural killer cell activity in patients with exanthem subitum. *Pediatr. Infect. Dis. J.* 11:369–373.

21. Grabstein, K.H., J. Eisenman, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M.A. Schoenborn, M. Ahdieh, L. Johnson, M.R. Alderson, J.D. Watson, D.M. Anderson, and J.G. Giri. 1994. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Science (Wash. DC).* 264:965–968.

22. Burton, J.D., R.N. Bamford, C. Peters, A.J. Grant, G. Kurys, C.K. Goldman, J. Brennan, E. Roessler, and T.A. Waldman. 1994. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proc. Natl. Acad. Sci. USA* 91:4935–4939.

23. Bamford, R.N., A.J. Grant, J.D. Burton, C. Peters, G. Kurys, C.D. Goldman, J. Brennan, E. Roessler, and T.A. Waldman. 1994. The interleukin (IL) 2 receptor β chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proc. Natl. Acad. Sci. USA*. 91:4940–4944.

24. Giri, J.G., M. Ahdieh, J. Eisenman, K. Shanebeck, K. Grabstein, S. Kumaki, A. Namen, L.S. Park, D. Cosman, and D. Anderson. 1994. Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2822–2830.

25. Flamand, L., J. Gosselin, M. D'Addario, J. Hiscott, D.V. Ablashi, R.C. Gallo, and J. Menezes. 1991. Human herpesvirus 6 induces interleukin-1β and tumor necrosis factor alpha but not interleukin-6 in peripheral blood mononuclear cell cultures. *J. Virol.* 65:5105–5110.

26. Frenkel, N., E.C. Schirmer, G. Katsafanas, and C.H. June. 1990. T-cell activation is required for efficient replication of human herpesvirus 6. *J. Virol.* 64:4598–4602.

27. Khyatti, M., I. Stefanescu, and J. Menezes. 1993. Evidence for interleukin-2 (IL-2) mediated control of Epstein-Barr virus (EBV)-infected/transformed cells: effect exerted through effector cells with dual $CD8^+/CD16^+$ phenotype. *In* The Epstein-Barr Virus and Associated Diseases. T. Tursz, J.S. Pagano, D.V. Ablashi, G. de Thé, G. Lenoir, G.R. Pearson, editors. Colloque INSERM/John Libbey Eurotext Ltd, 225:325–331.