Alpha Interferon Inhibits Human Herpesvirus 8 (HHV-8) Reactivation in Primary Effusion Lymphoma Cells and Reduces HHV-8 Load in Cultured Peripheral Blood Mononuclear Cells

PAOLO MONINI, FRANCESCA CARLINI, MICHAEL STÜRZL, PAOLA RIMESSI, FABIANA SUPERTI, MARINA FRANCO, GIANNA MELUCCI-VIGO, AURELIO CAFARO, DELIA GOLETTI, CECILIA SGADARI, STEFANO BUTTO, PATRIZIA LEONE, PASQUALINA LEONE, CHIARA CHIOZZINI, CATERINA BARRESI, ANTONELLA TINARI, ANGELA BONACCORSI, MARIA R. CAPOBIANCHI, MASSIMO GIULIANI, ALDO DI CARLO, MASSIMO ANDREONI, GIOVANNI REZZA, AND BARBARA ENSOLI

Laboratory of Virology, Laboratorio di Ultrastrutture, and Centro Operativo AIDS, Istituto Superiore di Sanità, Rome, Italy, and Institute of Virology, University “La Sapienza,” Rome, Italy, and Section of Microbiology, Department of Diagnostic and Experimental Medicine, University of Ferrara, Ferrara, Italy, and Institute of Molecular Virology, GSF-National Research Center for Environment and Health, Neuherberg, and Institute of Virology, Technical University of Munich, Munich, Germany

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Infection by human herpesvirus 8 (HHV-8) is associated with the development of Kaposi’s sarcoma (KS). Since regression of KS can be achieved by treatment of the patients with alpha interferon (IFN-α), we analyzed the effects of IFN-α or anti-IFN-α antibodies (Ab) on HHV-8 latently infected primary effusion lymphoma-derived cell lines (BCBL-1 and BC-1) and on peripheral blood mononuclear cells (PBMC) from patients with all forms of KS and from at-risk subjects. IFN-α inhibited in a dose-dependent manner the amplification of HHV-8 DNA in BCBL-1 cells induced to lytic infection with tetradeoxycytidylic acid (TPA). This effect was associated with the inhibition of the expression of HHV-8 latently infected nuclei and kaposin genes that are induced early and several hours, respectively, after TPA treatment. In addition, IFN-α inhibited virus production and/or release from BCBL-1 cells. Inhibition of latently infected nuclei and kaposin genes by IFN-α was also observed in BC-1 cells induced with n-butrate. Conversely, the addition of antih-IFN-α Ab to TPA-induced BCBL-1 cells resulted in a larger number of mature enveloped particles and in a more extensive cytopathic effect due to the neutralization of the endogenous IFN produced by these cells. IFN-α was also produced by cultured PBMC from HHV-8-infected individuals, and this was associated with a loss of viral DNA during culture. However, the addition of antih-IFN-α Ab or antitype I IFN receptor Ab promoted the maintenance of HHV-8 DNA in these cells that was associated with the detection of the latency-associated kaposin RNA. Finally, the addition of IFN-α reduced the HHV-8 load in PBMC. Thus, IFN-α appears to have inhibitory effects on HHV-8 persistent infection of PBMC. These results suggest that, in addition to inhibiting the expression of angiogenic factors that are key to KS development, IFN-α may induce KS regression by reducing the HHV-8 load and/or inhibiting virus reactivation.

Kaposi’s sarcoma (KS) is a tumor of vascular origin that is particularly common and aggressive in human immunodeficiency virus (HIV)-infected individuals (AIDS-KS) but is also found as other epidemiologic forms including African or endemic KS, classic KS (C-KS) and posttransplantation KS (PT-KS) (9, 25, 67). KS develops as multiple lesions arising at independent sites in the skin and, in the most aggressive forms, also in visceral and lymphatic organs (26). The lesions are characterized by a rich inflammatory-cell infiltrate (particularly evident in early lesions), angiogenesis, slit-like vascular spaces, extravasated erythrocytes, and the presence of perivascular and interstitial spindle-shaped cells that are considered to be the tumor cells of KS (KS cells) (22, 30, 48, 63, 75).

All epidemiologic forms of KS have the same histological features, and are all associated with infection by human herpesvirus 8 (HHV-8) (3, 11, 14, 20, 53, 71). HHV-8 is a novel herpesvirus that is also found in primary effusion lymphoma (PEL) and Castelman’s disease (13, 77). In individuals at risk for KS, HHV-8 infection is highly predictive of disease development (33, 55, 65, 88). HHV-8 is present in a latent form in KS spindle cells and lesional endothelial cells but yields a lytic infection in lymphocytes and monocytes infiltrating KS lesions (10, 17, 61, 79, 81). In addition, HHV-8 can infect circulating B cells, monocytes/macrophages, T cells, and KS-like spindle cell progenitors that are increased in number in the blood of patients with all forms of KS (34, 38, 51, 74, 76). These cells infiltrate KS lesions and can recruit the virus into tissues (10, 30, 61, 75, 87). Although restricted to a small proportion of the inflammatory cells infiltrating the lesions, HHV-8 lytic infection may play an important role in KS initiation or progression by producing or inducing factors with paracrine chemotactic and growth effects (6, 12, 40, 54, 59, 80) and/or by transmitting the virus to the spindle and endothelial cells present in the lesions (10, 30, 75, 80). However, therapy with antiviral drugs, which blocks HHV-8 lytic infection (39, 50), has had variable results and has failed to induce remission in late-stage KS (15, 56).
RNA samples were then added 3 and 2.2 M formaldehyde; incubated at 65°C for 15 min; and chilled on ice. To the mM sodium acetate (pH 4.5), 10 mM EDTA (pH 8.0), 50% (vol/vol) formamide, buffer containing 40 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 10 mM EDTA, and highly purified sonicated salmon sperm DNA (50 µg/ml). Aliquots of 5 µl were used for PCR amplification with primers KS1 and KS2 (BCBL-1 supernatants, 30 to 35 cycles) or primers v-cycA and v-cycB (PBMC extracts, 45 cycles). To ensure that the same relative amount of cDNA was analyzed, the same extract dilutions were amplified with primers for β-globin or β-actin (see below).

RT-PCR analysis. Total RNA purified with the RNeasy Mini Kit was digested at 37°C three times in a buffer containing 10 mM sodium acetate (pH 4.5), 0.1 mM EDTA, 100 µM dNTPs, 50 µg/ml RNAse-free DNase (Boehringer, Mannheim, Germany), and 200 U of RNase-free DNase at 37°C and further purified with the RNeasy Kit as suggested by the manufacturer. cDNA was synthesized from total RNA (10° to 10° cells) with the reverse transcription system kit (Promega) by incubating the reaction mixtures with hexanucleotide random primers for 10 min at room temperature, 30 min at 42°C, and 30 min at 53°C. After heat inactivation of reverse transcriptase (RT), 1/3 of each reaction mixture was subjected to 45 cycles of PCR for VP23 or T0.7 whereas amplification of β-actin was performed with 1/15 of the RT reaction mixture in 40 PCR cycles. Primers for β-actin were 5°-CAC CAT TCC TCT CCG CATTA-3° and 5°-GTC TGC CAA GAT CAG TCG CA-3°, respectively, used for T0.7 amplification with the same cycling conditions. To ensure the integrity of the reaction mixtures, β-actin and globin sequences were amplified with primers v-cycA and v-cycB (PBMC extracts, 45 cycles). To ensure that the same relative amount of cDNA was analyzed, the same extract dilutions were amplified with primers for β-globin or β-actin (see below).

Analysis of free virus in BCBL-1 supernatants. BCBL-1 cells were cultured for 24 h in the presence or absence of IFN-α (25 IU/ml) and induced with TPA (20 ng/ml) for 5 days. Aliquots of supernatants corresponding to 3° to 10° cells were adjusted to 60 µl and digested twice with 20 U of RNase-free DNase at 37°C for 90 min in a buffer containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 1 mM MgCl2, and 0.5 mM CaCl2. The reaction was ended by adding EDTA (pH 8.0) to a final concentration of 50 mM and by rapid heating at 95°C for 10 min. The supernatants were adjusted to 100 µl by adding 40 µl of water containing polyoxyethylene 10-lauryl ether (1%, vol/vol) and digested with proteinase K (0.1 mg/ml) for 2 h at 65°C. After heat inactivation at 95°C for 10 min, the supernatants were diluted 1:100 in a buffer containing 10 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, and highly purified sonicated salmon sperm DNA (50 µg/ml), and 5 µl was used as starting dilution for semiquantitative PCR analysis as described above.

Analysis of free virus in BCBL-1 supernatants. BCBL-1 cells were cultured for 24 h in the presence or absence of IFN-α (25 IU/ml) and induced with TPA (20 ng/ml) for 5 days. Aliquots of supernatants corresponding to 3° to 10° cells were adjusted to 60 µl and digested twice with 20 U of RNase-free DNase at 37°C for 90 min in a buffer containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 1 mM MgCl2, and 0.5 mM CaCl2. The reaction was ended by adding EDTA (pH 8.0) to a final concentration of 50 mM and by rapid heating at 95°C for 10 min. The supernatants were adjusted to 100 µl by adding 40 µl of water containing polyoxyethylene 10-lauryl ether (1%, vol/vol) and digested with proteinase K (0.1 mg/ml) for 2 h at 65°C. After heat inactivation at 95°C for 10 min, the supernatants were diluted 1:100 in a buffer containing 10 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, and highly purified sonicated salmon sperm DNA (50 µg/ml), and 5 µl was used as starting dilution for semiquantitative PCR analysis as described above.
crystal violet. The internal laboratory standard containing 100 IU of IFN-α2b per ml was calibrated against a reference standard of IFN-α (NIH Ga 025-902-530) and was included in each titration (37).

**Anti-HHV-8 serologic testing.** BCBL-1 cells were treated for 48 h with 20 ng of TPA per ml. A 10-μl volume of suspension (4 x 10^6 cells/ml) was smeared on the coverslips, rapidly air dried, and fixed in acetone-methanol solution for 10 min. Fixed smears were incubated successively in two steps of 30 min each at 37°C with the serum samples diluted 1:5 (in duplicate) and with fluorescein-labeled, affinity-purified goat Ab to human immunoglobulin G (Kierkgaard & Perry Laboratories). Titer determinations were done by fivefold serial dilutions. All the microscopic examinations were conducted by two different investigators on coded samples in a blinded fashion. An inverse titer of 5 was considered positive in the presence of a bright cytoplasmic staining. No correlation was found between Epstein-Barr virus and HHV-8 Ab titers by this assay (65). Serum samples from 8- to 12-month-old babies and HIV-seronegative KS patients were used as negative and positive controls, respectively.

**ISH.** Cultured PBMC were harvested, centrifuged, washed twice, resuspended in phosphate-buffered saline, seeded onto Silan-coated slides, air dried, and fixed in 4% buffered formaldehyde as described previously (81). In situ hybridization (ISH) was performed as described previously, under high-stringency conditions (10) with strand-specific 35S-labeled VP23 RNA hybridization probes (specific activity, 10^7 cpm/g) transcribed from plasmid p557-19 encompassing HHV-8 ORF 26 (10) or plasmid pBluescript-T0.7 encompassing the kapaposin gene (81).

**Transmission electron microscopy.** BCBL-1 cells were washed in phosphate-buffered saline, fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M; pH 7.2) for 20 min at room temperature, and postfixed in 1% OsO4 in cacodylate buffer for 1 h at room temperature. Fixed cells were dehydrated through a graded series of ethanol solutions and embedded in Agar 100 (Agar Aids, Cambridge, UK) for 20 min at room temperature. Fixed cells were dehydrated through a graded series of ethanol solutions and embedded in Agar 100 (Agar Aids, Cambridge, UK). Titer determinations were done by fivefold serial dilutions. All the microscopic examinations were conducted by two different investigators on coded samples in a blinded fashion. An inverse titer of 5 was considered positive in the presence of a bright cytoplasmic staining. No correlation was found between Epstein-Barr virus and HHV-8 Ab titers by this assay (65). Serum samples from 8- to 12-month-old babies and HIV-seronegative KS patients were used as negative and positive controls, respectively.

**FACS analysis.** PBMC from a healthy volunteer were cultured as described above in the presence or absence of photheomagglutinin (PHA) (3 μg/ml) and interferon-γ (IFN-γ; 10 U/ml), anti-IFN-α Ab (100 IU/ml), or MAb 64G12 (2.5 μg/ml). Floating and adherent cells were harvested after 16 h and 2 and 7 days of culture and analyzed by fluorescence-activated cell sorting (FACS) with mouse MAbs conjugated with fluorescein isothiocyanate or phycoerythrin. Ungated cells were analyzed for size and antigen expression. Cells stained with fluorescein isothiocyanate- or phycoerythrin-conjugated isotype-matched Ab directed against irrelevant epitopes served as negative controls.

**RESULTS**

**Induction of early and late viral genes by TPA treatment of BCBL-1 cells.** The effect of IFN-α on the HHV-8 life cycle was initially evaluated in BCBL-1 cells. These cells are latently infected with HHV-8 and can be induced to undergo viral lytic replication by treatment with TPA (64).

Before analyzing the effect of IFN-α on HHV-8 reactivation in these cells, the studies focused on identifying the viral genes induced early or late after TPA treatment. To this end, the kinetics of expression of HHV-8 genes including ORFs K12 and K7, encoding kaposin and mut-1 RNA, respectively, (66), were examined by Northern blot hybridization. Kaposin RNA is expressed in latently infected cells but is upregulated upon induction of the HHV-8 lytic cycle, while mut-1 is a lytic viral nuclear RNA (70, 81, 82, 91). Both Kaposin RNA (T0.7) and mut-1 nuclear RNA (T1.1) were induced by TPA in BCBL-1 cells. Specifically, the levels of T0.7 RNA were unchanged in the first 18 h and increased at 29 h and up to 72 h after induction (Fig. 1A). By contrast, expression of T1.1 RNA was induced very early (at 30 to 60 min postinduction) and continued to rise during the following 12 h (Fig. 1A). Thus, T1.1 RNA was induced very early by TPA whereas T0.7 RNA, which behaves as a latency gene in uninduced cells, was up regulated by TPA with kinetics consistent with that of a late gene.

**Effects of IFN-α2b on HHV-8 latent and lytic infection of BCBL-1 cells.** To analyze the effect of IFN-α on HHV-8 infection, the same number of BCBL-1 cells was induced with TPA in the presence or absence of IFN-α2b or anti-IFN-α Ab and analyzed for T1.1 and T0.7 gene expression by Northern blot hybridization. Expression of both T1.1 and T0.7 was inhibited in a dose-dependent manner by IFN-α2b, whereas anti-IFN-α Ab had little or no effect (Fig. 1B).

To confirm that this effect was not due to interference of IFN-α with the TPA activation pathway, similar experiments were performed with another PEL cell line (BC-1) that was induced to undergo HHV-8 lytic infection by treatment with n-butyrate, as described previously (70). Expression of T1.1 and T0.7 was also inhibited by IFN-α2b in these cells as evaluated by normalizing the levels of T0.7 and T1.1 RNA to the hybridization bands obtained by reprobing the membranes to an 18S RNA probe (Fig. 1C).

The effect of IFN-α was then determined on viral DNA amplification in BCBL-1 cells by Southern blot hybridization. As shown in Fig. 1C, IFN-α2b, but not anti-IFN-α Ab, inhibited viral DNA amplification by 70 to 80%. Specifically, upon TPA induction, cells cultured in the absence of IFN-α2b showed a two- to fourfold increase in viral DNA load (Fig. 1C). Since under these conditions only 10 to 15% of the cells express lytic antigens (reference 61 and data not shown) or yield viral progeny (see below), this corresponds to about a 20- to 40-fold amplification of viral DNA in the responsive cells, most of which is blocked by IFN-α. Thus, IFN-α inhibits the early phases of HHV-8 reactivation and impairs downstream steps of viral lytic replication, including late T0.7 gene expression and viral DNA amplification.

To evaluate the effect of IFN-α on latent HHV-8 infection, the viral DNA load in uninduced BCBL-1 cells cultured in the presence or absence of 5 or 50 IU of IFN-α2b per ml was analyzed. Cell cultures maintained in the presence or absence of IFN-α2b were counted and plated at the same cell density (3 x 10^5 cells/ml) for seven passages. At various passages, the cells were harvested, viable cells were counted by trypan blue dye exclusion, and equal amounts of DNA and total RNA were analyzed by Southern or Northern blotting for HHV-8 load and expression, respectively. No significant inhibitory effects of IFN-α on viral DNA load were observed. However, it should be noted that any change in viral DNA load in the small fraction of cells undergoing spontaneous reactivation would probably be masked by the viral DNA present in the large number of latently infected cells. Nevertheless, a mild dose-dependent inhibition of T1.1 gene expression, particularly at late cell passages, and a mild dose-dependent inhibition of cell growth were observed (data not shown).

Responsiveness of latently and lytically infected BCBL-1 cells to IFN-α was determined by RT-PCR analysis of the MxA gene, whose expression is triggered specifically by type I IFNs and is associated with a strong antiviral response (5, 72, 78) (Fig. 2). MxA expression was undetectable in cells cultured in the absence of IFN-α but was induced at low levels by 1 IU of IFN-α2b per ml and efficiently stimulated at higher IFN-α concentrations (Fig. 2). Therefore, IFN-α did not inhibit latent HHV-8 infection in BCBL-1 cells, although the cells were responsive to exogenous IFN-α.

In addition, a faint RT-PCR band was evident in cultures induced by TPA. When increasing doses of IFN-α2b were added to TPA-induced cells, MxA expression was stimulated in a synergistic fashion and strong RT-PCR signals were observed at all IFN-α concentrations used (Fig. 2). These results suggest that the transduction pathways activated by IFN-α and TPA are not antagonistic and that HHV-8 reactivation and replication is inhibited by the strong antiviral response elicited by IFN-α.

**Inhibition of virus production and release from BCBL-1 cells by IFN-α.** BCBL-1 cells induced for 5 days with TPA release large numbers of HHV-8 particles into the medium (39, 44). To analyze the effect of IFN-α on virus production,
BCBL-1 cells were cultured for 24 h with or without IFN-α (25 IU/ml) and subsequently induced for 5 days with TPA. At the end of the induction, supernatants were harvested and extensively digested with pancreatic DNase I. After digestion, the supernatants were analyzed by limiting-dilution PCR to determine the relative amount of DNase-resistant (encapsidated) HHV-8 DNA. DNase-resistant DNA was about 25 times more abundant in TPA-induced BCBL-1 cells than in uninduced cells (Fig. 3f and e, respectively) and about 5 times less abundant on November 9, 2017 by guest http://jvi.asm.org/ Downloaded from
FIG. 4. Thin-section electron micrographs of BCBL-1 cells induced with TPA (20 ng/ml) for 48 h in the absence (A and B) or presence (C and D) of anti-IFN-α Ab (100 IU/ml). Cells in various stages of particle maturation and cytopathic effects are shown. (A) Cell induced with TPA in the absence of anti-IFN-α Ab, showing intranuclear aggregation of virus-specific electron-opaque material (arrows). (B) Cell induced with TPA in the absence of anti-IFN-α Ab, showing HHV-8 particles in different stages of maturation (nucleocapsids [arrowheads] and complete virions [arrows]). (C) Cell induced with TPA in the presence of anti-IFN-α Ab, revealing capsids in various stages of packaging of viral DNA (arrowheads) and numerous complete virions at the cell surface. (D) Extensively lysed cell induced with TPA in the presence of anti-IFN-α Ab, in which nucleocapsids with typical hexagonal outlines and variable DNA cores in the nucleus and in the cytoplasm can still be observed. Bars, 1 μm.
FIG. 4—Continued.
dant in TPA-induced cells cultured in the presence of IFN-α (Fig. 3g and f, respectively). Thus, IFN-α caused a 80% reduction of virus release and/or production.

Production of endogenous IFN-α by BCBL-1 cells and improvement of viral particle morphogenesis and virus yield in the presence of anti-IFN-α Ab. Since most virally infected cells produce IFN activity, BCBL-1 cells were analyzed for the production of endogenous IFN. Serially diluted supernatants from BCBL-1 cells were added to human lung A549 cells, and after 24 h the cells were infected with EMCV to determine the capability of supernatants to inhibit viral infection (37). A small but measurable antiviral activity, ranging from 3 to 10 IU/ml and potentially including type I and II IFNs, was detected in the supernatants from both TPA-induced and uninduced BCBL-1 cells.

To evaluate the effect of this endogenous IFN-α on viral particle assembly and virus yield, BCBL-1 cells were cultured for 24 h in the presence or absence of anti-IFN-α Ab (100 IU/ml). The cells were then induced with TPA for 48 h in the presence of anti-IFN-α Ab and analyzed by transmission electron microscopy. Viral particles were found in both Ab-treated and untreated cells, and the percentage of cells undergoing viral lytic replication was similar under the two experimental conditions (10 of 92 and 15 of 92 cells in the absence and presence of anti-IFN-α Ab, respectively). However, considerable differences were observed in viral particle maturation, cytopathic effect, and number of viral particles per cell (Fig. 4).

Specifically, numerous productively infected cells from untreated cultures showed only the early events of viral maturation, i.e., intranuclear aggregation of virus-specific electron-opaque material (Fig. 4A). These features were never observed in anti-IFN-α Ab-treated cultures, in which viral morphogenesis was in a more advanced phase (Fig. 4C and D), although individual viral particles in every stage of maturation were observed in both untreated (Fig. 4B) and treated (Fig. 4C and D) cells. Moreover, extracellular virions were more numerous in treated cells (Fig. 4C) than in untreated cells (Fig. 4B) and viral replication was frequently associated with a more extensive cytopathic effect in treated cells (Fig. 4D).

Since anti-IFN-α Ab had no effects on HHV-8 gene expression (Fig. 1) or DNA replication, these results suggest that endogenous production of IFN-α can inhibit viral particle morphogenesis.

**TABLE 1. Detection of HHV-8 DNA and HHV-8 serologic test results in PBMC from patients with KS or at risk for KS**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients</th>
<th>HHV-8 DNA (no. positive/no. analyzed) (%)</th>
<th>Anti-HHV-8 Ab (no. positive analyzed) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS-KS</td>
<td>16</td>
<td>5/16 (30)</td>
<td>15/16 (100)</td>
</tr>
<tr>
<td>C-KS</td>
<td>2</td>
<td>2/2 (100)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>PT-KS</td>
<td>1</td>
<td>0/1 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>HIV*</td>
<td>6</td>
<td>1/6 (15)</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>PT</td>
<td>3</td>
<td>0/2 (0)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>8/27 (31)</td>
<td>22/23 (95)</td>
</tr>
</tbody>
</table>

* PCR for HHV-8 detection was performed with primers specific for HHV-8 ORF 26 (VP 23) or ORF 13 (v-cycD) amplification.

ND, not determined.
Production of IFN-α by cultured PBMC from HHV-8-infected individuals causes a decrease in the viral load that is counteracted by anti-IFN-α Ab. To analyze the effect of IFN-α on HHV-8-infected primary cells, sera and PBMC from 28 patients with KS or at risk for KS (16 with AIDS-KS, 3 with C-KS, 1 with PT-KS, 2 PT, and 6 HIV+), were analyzed by an immunofluorescence assay or by PCR for the presence of anti-HHV-8 Ab or HHV-8 DNA, respectively (Table 1). All KS patients (15 of 15) and PT patients (2 of 2), and 80% of the HIV+ men (4 of 5) had anti-HHV-8 Ab, whereas a total of 31% of these patients (8 of 26) also contained detectable levels of HHV-8 DNA (Table 1). However, culture of these PBMC for 6 to 7 days resulted in the loss of viral DNA from these cells (see below). To verify whether this was associated with the production of endogenous IFN, supernatants of PBMC from 12 individuals (6 with AIDS-KS, 1 with C-KS, and 5 HIV+) were analyzed for the production of endogenous IFNs. All supernatants protected A549 cells from EMCV infection (IFN titers of 4 [range, 3 to 10] IU/ml), indicating that PBMC from these patients produced measurable levels of IFNs during culture.

To determine whether endogenous IFN-α could account for the loss of viral DNA, PBMC from six PCR-positive patients (four with AIDS-KS, one with C-KS, and one HIV+) were cultured in the presence or absence of anti-IFN-α Ab (100 IU/ml). Anti-IFN-α Ab maintained or increased the viral DNA load in PBMC from five of these patients (Fig. 5 and data not shown). In particular, in the absence of anti-IFN-α Ab, PBMC from four patients showed a loss or dramatic reduction in the HHV-8 DNA load after culture. In contrast, in the presence of anti-IFN-α Ab, they maintained the viral DNA load or increased it (Fig. 5A and B and data not shown). Two patients positive in both serologic tests and PCR in previous bleedings (one C-KS patient and one AIDS-KS patient) tested PCR negative at the time of this analysis. However, in the C-KS patient, culture with anti-IFN-α Ab increased the DNA load to levels detectable by PCR (Fig. 5C). In addition, PBMC from the other PCR-positive C-KS patient were cultured in the presence or absence of MAb 64G12, a MAb that acts as an antagonist for the type I IFN receptor (23). Similarly to anti-IFN-α Ab, MAb 64G12 was able to maintain the HHV-8 DNA load at levels higher than those obtained with the culture medium alone (Fig. 5D). Maintenance of viral DNA was observed in both floating and adherent cells from one AIDS-KS patient (Fig. 5A) and in the floating cells from the HIV+ patient (Fig. 5B). PBMC from the other patients were collected as bulk cells.

PBMC from two of the above patients yielded enough cells to allow a parallel RT-PCR analysis and were therefore analyzed for the expression of the MxA gene. Both patients gave positive results for MxA RNA, further supporting the idea that the effects elicited by the Abs were due to the neutralization of endogenous IFN-α.

To rule out the possibility that the effects of the anti-IFN-α Ab on HHV-8 DNA load were due to the activation of PBMC by the antibody preparations, cells from a healthy donor were cultured in the presence or absence of anti-IFN-α Ab, MAb 64G12, or PHA and interleukin-2 and analyzed by FACS for the expression of activation markers. As expected, PHA induced a dramatic up regulation of all the activation markers analyzed, including HLA-DR, CD25, CD30, and CD86. By contrast, no difference in the pattern of expression of the above markers was detected in PBMC cultured for either 16 h or 2 or 7 days in the presence of either Ab preparation compared to PBMC cultured with medium alone (data not shown).

To determine the effect of IFN-α on the HHV-8 load, PBMC from two AIDS-KS patients were cultured for 3 to 6 days in the presence or absence of IFN-α2b (100 IU/ml). A 10-fold reduction in the viral load was observed in PBMC from one patient after 6 days of culture, and another 2-fold reduction was induced by IFN-α, as determined by serial dilution PCR (Fig. 6). The other patient showed a reduction of the PCR signal only after culture with 500 IU of IFN-α2b per ml (data not shown). These data suggested that, due to the production of endogenous IFN, relatively high concentrations of
FIG. 7. ISH with an antisense riboprobe for T0.7 mRNA (bright-field results on the left, dark-field results on the right) of PBMC from an AIDS-KS patient after culture with anti-IFN-α Ab. (Panels 1) PBMC from the AIDS-KS patient cultured for 7 days in the presence of anti-IFN-α. The arrow points to a cell showing T0.7 signals. Cells were negative after ISH with a VP23 antisense riboprobe (data not shown). (Panels 2). Negative control made with Jurkat cells. (Panels 3). TPA-induced BCBL-1 cells hybridized with a VP23 antisense probe as a control of the ability of ISH to detect VP23 expression under the conditions used. (Panels 4) TPA-induced BCBL-1 hybridized with the T0.7 probe. (Panels 5) PBMC from the AIDS-KS patient hybridize with a probe for β-actin mRNA. No T0.7 signals were observed in the adherent cells from the patient after culture without anti-IFN-α Ab (data not shown). PBMC were negative after ISH with a VP23 antisense riboprobe (data not shown).
IFN-α2b are required to reduce the HHV-8 load in cultured PBMC.

T0.7 gene expression in PBMC cultured in the presence of anti-IFN-α Ab. To verify the effect of anti-IFN-α Ab on HHV-8 gene expression, PBMC from two of the PCR-positive AIDS-KS patients were analyzed by RT-PCR and ISH for the expression of T0.7 and VP23 mRNA after culture with or without anti-IFN-α Ab (100 IU/ml). Expression of T0.7 but not VP23 was detected by RT-PCR and, in one patient, also by ISH (Fig. 7 and data not shown). By contrast, no T0.7 or VP23 expression was detected in cells cultured without anti-IFN-α Ab (data not shown). The same PBMC showed a dramatic decrease in or complete loss of HHV-8 DNA upon culture, which was counteracted by anti-IFN-α Ab. These results suggest that neutralization of endogenous IFN-α results in an increase in the viral load that may be associated with HHV-8 latent infection in cultured PBMC.

DISCUSSION

IFN-α has been successfully used in the therapy of various clinical forms of KS, including AIDS-KS and C-KS, that are associated with HHV-8 infection (16, 36, 42, 57, 86). Type I IFNs are known to inhibit the replication of alpha-, beta-, and gammaherpesviruses, including herpesvirus saimiri and Epstein-Barr virus that are closely related to HHV-8 (2, 7, 35, 47, 83, 85, 89). However, no information is available on the effect of IFN-α on the life cycle of HHV-8. The results of this study show that IFN-α inhibits HHV-8 reactivation in PEL-derived cells and reduces the HHV-8 load in PBMC from patients with KS or at risk of contracting KS.

IFN-α had strong inhibitory effects on HHV-8 gene expression, DNA amplification, and viral particle release from PEL cells induced with TPA or n-butyrate. In particular, it inhibited the expression in BCBL-1 cells of the HHV-8 nut-1 gene, whose activation appears to be an early event triggered by TPA. In addition, blocking of endogenous IFN-α with neutralizing Ab resulted in a more extensive cytopathic effect and in a more efficient viral particle morphogenesis in these cells. Since anti-IFN-α Ab did not increase late (kaposin) viral gene expression or DNA amplification, these data suggest that endogenous IFN-α produced by BCBL-1 cells had inhibitory effects on virus particle maturation. In addition, HHV-8 gene expression was inhibited in n-butyrate-induced BC-1 cells. Since TPA and butyrate are known to activate different pathways (31, 46), it is likely that the effects of IFN-α were specifically directed against HHV-8 replication. Thus, as for other herpesviruses, both early and late stages of the HHV-8 life cycle may be targets of IFN-α antiviral activity (19, 52, 58, 60). Consistent with these data, low doses of human recombinant IFN-α2b induced the expression of the gene encoding MxA, which displays strong antiviral effects and is specifically triggered by type I IFNs. IFN-α, however, did not inhibit HHV-8 latent infection in BCBL-1 cells.

Anti-IFN-α Ab also had striking effects on HHV-8 infection of cultured PBMC from KS patients or individuals at risk for KS. Cultured PBMC from these individuals produced endogenous IFN-α that caused a reduction in the viral load. In fact, anti-IFN-α Ab and MAb 64G12 maintained or increased viral load during culture. This was associated with the expression of the latency-associated kaposin gene but not with the lytic VP23, suggesting that IFN-α may be able to inhibit latent HHV-8 infection in cultured PBMC. In addition, exogenous IFN-α reduced the viral DNA load in cultured PBMC. Further studies are required to determine whether these mechanisms are also operative in vivo.

HHV-8 encodes a homolog of the interferon regulatory factor (IRF) family members (32, 66, 90). Expression of cloned HHV-8 IRF (v-IRF) inhibits IRF-mediated gene expression (32, 90), suggesting that HHV-8 may use this homolog as a decoy to escape IFN-mediated antiviral effects. However, the ability of IFN to inhibit HHV-8 infection in BCBL-1 cells and PBMC indicates that, in the context of the viral genome, v-IRF may have other, as yet unknown functions.

KS onset is associated with a high HHV-8 load in PBMC (18, 38), suggesting that HHV-8-infected circulating cells play an important role in KS development. This may reflect the ability of circulating cells, including B cells, T cells, monocytes, and circulating spindle cell progenitors, to infiltrate or localize into tissues and to transmit the virus to other cell types (30, 34, 76). The inhibitory effects of IFN-α on the HHV-8 load in PBMC, its known immunomodulatory effects (reviewed in references 8 and 84), and its ability to inhibit angiogenic factors with a key role in KS development may explain its efficacy as monotherapy for all forms of KS. Since lymphocytes and monocytes infiltrating KS lesions are productively infected by HHV-8 (10, 61) and since antitherapeutic drugs have strong inhibitory effects on herpesvirus lytic infection, the use of IFN-α in combination with these antiviral drugs may be an important tool for the control of productive and latent HHV-8 infection in vivo and for the clinical management of patients with all forms of KS.

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