PRIMARY EFFUSION LYMPHOMA CELLS UNDERGOING HUMAN HERPESVIRUS TYPE 8 PRODUCTIVE INFECTION PRODUCE C-TYPE RETROVIRAL PARTICLES

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Primary effusion lymphomas (PELs) are invariably infected by the human herpesvirus 8 (HHV8) that is present in most PEL cells as latent virus but replicates in a subset of permissive cells to produce infectious progeny. Here we show that productively infected PEL cells release C-type retrovirus-like particles encoding an Mn++-dependent RT activity, which is typical of endogenous retroviruses. Strikingly, C-type particles are produced only in cells showing advanced HHV8 morphogenesis. Phorbol esters, which induce productive HHV8 replication and morphogenesis in PEL cells, increase RLP production. Phosphonoacetic acid, a blocker of HHV8 late gene expression, inhibits the production of C-type particles, whereas neutralizing anti-αIFN antibodies, which are known to increase HHV8 assembly, increases C-type particle production. These data suggest that factors expressed in advanced stages of HHV8 reactivation support endogenous C-type particle morphogenesis in PEL cells.

Primary effusion lymphomas (PELs) are rare lymphomas that most frequently arise in the setting of HIV infection. PELs develop upon the clonal expansion of a post-germinal center B cell precursor, which leads to malignant effusions localized at the serous cavities in the absence of an identifiable tumor mass (1). PEL cells are invariably infected by the human herpesvirus 8 (HHV8) and, at a lower frequency, also by the Epstein Barr virus (EBV) (1). HHV-8 is a member of the gamma herpesviruses associated with human tumors and reactive proliferative diseases including PEL, Kaposi's sarcoma, and Castleman disease. Although both HHV8 and EBV are present in PEL cells as latent episomes expressing a limited subset of genes, a small fraction of cells becomes, at any time, permissive for HHV8 replication both *in vivo* and *in vitro* (2). Evidence indicates that HHV8 productively-infected cells may contribute to PEL growth by producing factors with paracrine activity, including viral cytokines and chemokines. However, the exact role, if any, of HHV8 replication in the pathogenesis of PEL is still unclear (3).

Here we show that upon HHV8 reactivation

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PEL cells produce late-viral gene products or virus-induced host factor(s) promoting retrovirus-like particle morphogenesis, which, in turn, may contribute to PEL pathogenesis.

MATERIALS AND METHODS

Cells cultures

BCBL-1 cells, (AIDS Research and Reference Reagent Program National Institutes of Health) were cultured in RPMI 1640, 10% fetal calf serum (FCS), 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells were split at a density of 3×10^5/ml twice a week. HHV-8 lytic replication was induced in exponentially growing BCBL-1 cells by 20 ng/ml of phorbol 12-myristate 13-acetate (TPA; Sigma, Milan, Italy) for 48-72 hours. The earliest phase of HHV8 DNA replication was blocked by treatment of TPA induced BCBL-1 cells with 500 μM phosphonacetic acid (PAA; Sigma). BCBL-1 cells were split at a density of 500,000 cells/ml to obtain identical cultures, which were then treated or not with IFN-α neutralizing antibody (Ab) (sheep, polyclonal Ab; BioSource, Milan, Italy) 24 hours before TPA induction.

BC-1 cells (a kind gift from Patrick Moore, Columbia University, New York, N.Y.) were cultured in RPMI 1640 containing 15% FCS.

Transmission electron microscopy (TEM)

For TEM examination of BCBL-1 cells, cells were washed in PBS and fixed in 2.5% cacodylate-buffered (0.1M, pH 7.2) glutaraldehyde for 20 min at room temperature and post-fixed in 1% OsO_4 in cacodylate buffer for 1 h at room temperature. Fixed specimens were dehydrated through a graded series of ethanol solutions and embedded in Agar 100 resin (Agar Aids, Cambridge, U.K.). Ultrathin sections were collected on 200-mesh grids and then counterstained with uranyl acetate and lead citrate.

For negative staining, suspensions of ultracentrifuged viral particles were placed on Formvar-carbon coated 400 mesh copper grids for 10 s, wicked with filter paper and placed on a drop of 2% sodium phosphotungstate, pH 7.0, for 10 s, wicked again and air-dried.

Ultrathin sections and negatively stained preparations were observed with a Philips 208 electron microscope at 80 kV.

Reverse Transcriptase (RT) activity

The supernatants from retrovirus producing or control cells were collected, clarified and ultracentrifuged. The pelleted virus was then permeabilized with Triton X 100 (0.1%), and aliquots of the lysates were assayed for RT activity in the presence of 10 mM $MgCl_2$ or $MnCl_2$ as already described (4).

RNA extraction and cDNA syntesis

BCBL-1 cells were collected and total RNA was purified with the RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany). RNA Poly(A) was isolated from total RNA with the Dynabeads mRNA Purification kit (Dynal, Oslo, Norway).

cDNA was synthesized from 500 ng of Poly(A) RNA in a reaction mixture containing 20 mM Tris/HCl, pH 8.4, 10 mM DTT, 50 mM KCl, 2.5 mM $MgCl_2$, dNTPs mixture 0.5 mM each, 10 U RNasin (Promega, Madison, Wisconsin), 30 pmol hexanucleotide random primers and 20 U MLV RT (Roche Diagnostics, Basel, Switzerland). The reaction was incubated for 1 hour at 42°C and heat inactivated at 95°C for 5 min.

RT PCR and dot blot analysis

Conserved sequences of the retroviral *pol* gene were amplified from cDNA using degenerate oligonucleotide primers described previously (5-6). These primers correspond to highly conserved regions present in the *pol* genes of endogenous and exogenous retroviruses. The amplified *pol* products were radioactively labelled and hybridized to a filter loaded with fragments of HERV *pol* sequences amplified from chromosomal DNA as previously described (5-6).

Immunofluorescence assay (IFA)

BCBL-1 cells were spotted onto multitest slides (ICN, Aurora, Ohio), air dried and fixed in 4% paraformaldehyde. After permeabilization with PBS containing 0.2% Triton X-100, the cells were incubated in 100 mM glycine solution for 10 min and then in PBS containing 10% FCS for 20 min. The cells were then incubated for 1 h at 37°C with anti-viral-interleukin-6 (vIL-6) rabbit polyclonal antibody (Advanced Biotechnologies Inc., Columbia, Md) or anti-viral-glycoprotein (K8.1) monoclonal antibody (Advanced Biotechnologies Inc., Columbia, Md) and washed with PBS. Slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma), washed with PBS and treated with VECTASHIELD® Mounting Medium with DAPI (Burlingame, CA) as a counterstaining dye for DNA. Cells were visualized using an Axioskop 2 plus microscope (Zeiss, Jena, Germany) using the AxioVision 3.06 software (Zeiss) under 20× original magnification.

RESULTS

PEL cells undergoing HHV8 morphogenesis produce retrovirus-like particles (RLPs)

While studying HHV8 morphogenesis in a PEL

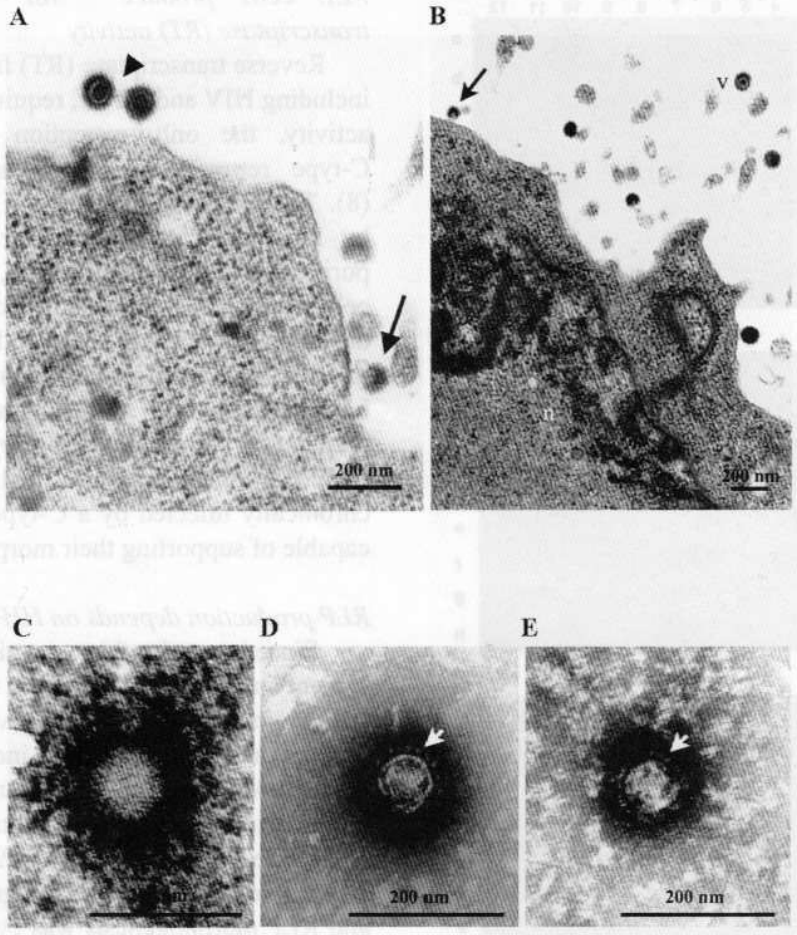

Fig. 1. ***A*** *and* ***B)*** *Electron micrographs of thin sectioned BCBL-1 cells producing both HHV8 and retrovirus-like particles:* ***A)*** *HHV8 mature particles (arrowhead) and RLPs gemming (arrow) at the plasma membrane exhibiting a distinctive morphogenesis similar to C-type retroviruses;* ***B)*** *RLPs (arrow) in a cell showing advanced HHV8 morphogenesis (n, HHV8 nucleocapsids; v, HHV8 mature virions).* ***C, D,*** *and* ***E)*** *Negative staining of supernatants from PEL cell lines. Two kind of virus particles were observed: typical herpesvirus* ***C)*** *and RLPs from BCBL-1 cells* ***D)*** *and from EBV-negative KS-1 PEL cells* ***E)*** *of about 90 nm exhibiting peplomers (white arrows) corresponding to retroviruse surface spikes.*

cell line negative for EBV (BCBL-1 cells), we detected a fraction of cells producing RLPs. RLPs were visible by thin-sectioning TEM as particles budding at the plasma membrane, and exhibited a distinctive morphogenesis similar to C-type retroviruses (Fig. 1A, B). RLPs were about 85 nm in size, and in most of them surface spikes and outer clear zone were not detectable (Fig. 1A, B). Strikingly, RLPs were present only in cells showing advanced HHV8 morphogenesis (Fig. 1B).

To extend these observations, negatively stained cleared supernatants from exponentially growing BCBL-1 cells were analyzed by TEM, as previously described (7). In addition, an HHV8/EBV co-infected PEL cell line (BC-1 cells) and a purified HHV8 preparation from the EBV-negative KS-1 PEL cell line (Advanced Biotechnologies Inc., Columbia, MD) were analyzed. Two different viral particles were present in the supernatants from all cell lines, namely, herpesvirus particles (Fig. 1C) and enveloped icosahedral particles (Fig. 1D, E). The latter showed a retrovirus morphology, and their size (about 90 nm) (Fig. 1D, E) corresponded to the RLPs previously observed by thin-sectioning TEM

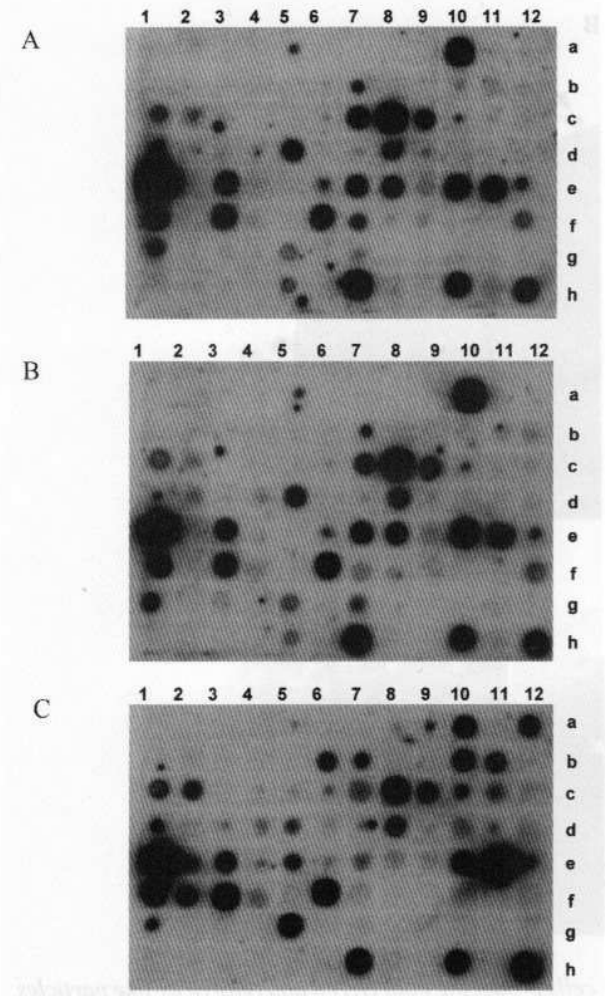

Fig. 2. ***A)*** *and* ***B)*** *Reverse dot blot hybridyzation of analysis of retroviral pol expression patterns. Poly(A)+ RNA was isolated from BCBL-1 cells either non-treated* ***A)*** *or treated* ***B)*** *with TPA (20 ng/ml, 48 h) to stimulate HHV8 replication. Using degenerate primers, a conserved region of the retroviral pol gene was amplified by RT-PCR, radioactively labeled and hybridized to a macroarray containing a representative number of defined pol fragments amplified and isolated from chromosomal DNA.* ***C)*** *As a control, reverse dot blot hybridyzation was performed using radioactively labeled pol fragments amplified from chromosomal DNA as a probe.*

in HHV8 producing cells (Fig. 1B, C). Most of the RLPs present in the cell supernatants (Fig. 1D) and in the HHV8 preparation (Fig. 1E) carried peplomers identical to retrovirus spikes.

PEL cells produce Mn^{++}*-dependent reverse transcriptase (RT) activity*

Reverse transcriptase (RT) from all retroviruses, including HIV and HTLV, requires Mg^{++} for optimal activity, the only exception being mammalian C-type retroviruses, which are Mn^{++}-dependent (8). To confirm the production of C-type particles by PEL cells, supernatants from BCBL-1 cells, purified HHV8 preparation and, as a control, Jurkat cell supernatants were analyzed for Mg^{++} or Mn^{++} dependent RT activity. None of the samples analyzed had a significant Mg^{++}-dependent RT-activity, while both BCBL-1 cell supernatants and the HHV8 preparation showed an Mn^{++}-dependent RT activity (Table I). These data indicated that PEL cells are chronically infected by a C-type retrovirus and are capable of supporting their morphogenesis.

RLP production depends on HHV8 reactivation

Since in ultrathin sections RLPs were present only in cells undergoing late stages of HHV8 morphogenesis (Fig. 1A, B), we verified whether RLP production required factors induced or expressed upon HHV8 replication by stimulating BCBL-1 cells to replicate HHV8 with 12-O-tetradecanoyl phorbol-13-acetate (TPA) (9). Upon TPA induction, HHV8 productive replication was significantly increased and RLP morphogenesis was evident in about 25% of cells replicating HHV8. To rule out TPA effects on RLP gene expression, poly-A^+ RNA from TPA-induced or non-induced cells was subjected to RT-PCR with degenerate primers capable of amplifying *pol* transcripts from most known retroviruses and analyzed in a reverse dot blot hybridization assay (5-6). Induced and non-induced BCBL-1 cells had the same macroarray hybridization pattern (Fig. 2A, B). In contrast, control genomic DNA produced a different and distinctive PCR-amplified pattern (Fig. 2C). These data confirmed that viral or host factors produced upon HHV8 replication are most likely required to support RLP morphogenesis.

Herpesvirus morphogenesis is mediated by virus-encoded assembly and regulatory factors that are expressed in the late phase of viral replication. To determine whether expression of HHV8 late genes is required for RLP morphogenesis, BCBL-1 cells were treated with TPA and the viral DNA polymerase inhibitor phosphonoacetic acid (PAA),

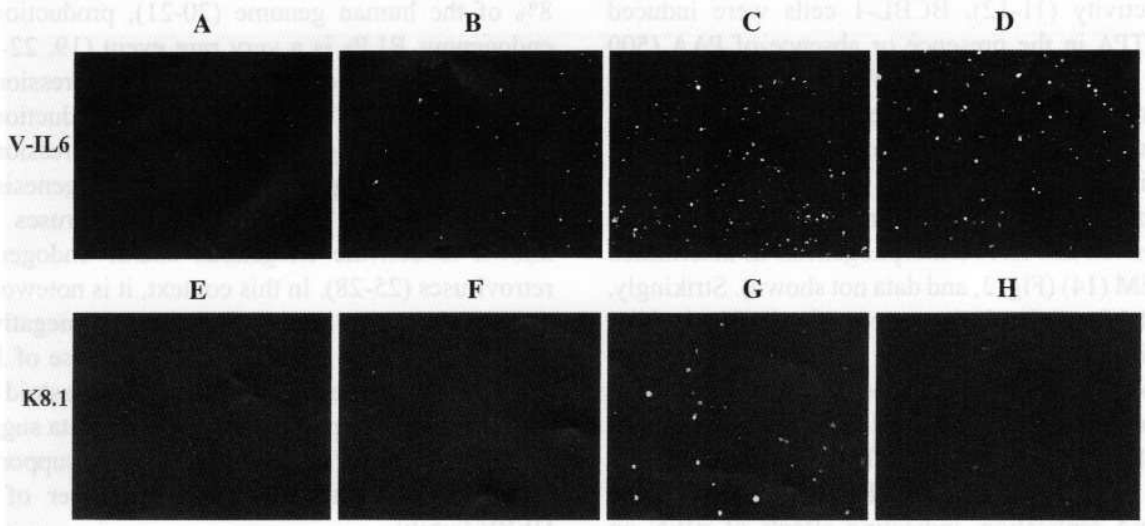

Fig. 3. *Effect of PAA on v-IL6 or K8.1 expression in BCBL-1 cells. BCBL-1 cells were induced with TPA (20 ng/ml) for 72 hours in the presence or absence of PAA (500 μM) and analyzed for v-IL6 or K8.1 expression by IFA.* ***A*** *and* ***E)*** *TPA-induced cells stained with control serum or Mab;* ***B, C*** *and* ***D)*** *cells stained for v-IL6;* ***F, G*** *and* ***H)*** *cells stained for K8.1;* ***B*** *and* ***F)*** *non-induced cells;* ***C*** *and* ***G)*** *TPA-induced cells;* ***D*** *and* ***H)*** *TPA-induced cells treated with PAA.*

Table I. *RT activity present in cell supernatants and virus preparation.*

	RT activity (cpm/ml lysate)	
Sample	**Mg^{++}-dependent**	**Mn^{++}-dependent**
Lysis buffer	22,099	9,912
Culture medium	18,102	25,578
Jurkat cell supernatant	14,966	28,396
BCBL-1 cell supernatant	50,274	406,000
ABI virus preparation*	61,502	158,690

* *The virus preparation was purchased in 1997 and stored at -80°C. Biologically active viral stocks are not currently available from ABI.*

a molecule belonging to the family of pyrophosphate (PPi) analogues. These compounds block the earliest phase of herpesvirus DNA replication that, in turn, is required for efficient expression of late genes (10). Although PPi analogues also inhibit retroviral RT, they cannot affect the production of retrovirus particles driven by endogenous proviral sequences in chronically infected cultures, as this does not require

RT activity (11-12). BCBL-1 cells were induced with TPA in the presence or absence of PAA (500 μM), and analyzed for HHV8 early (viral IL-6, v-IL6) or late (K8.1 glycoprotein) gene expression by an immunofluorescence assay (IFA), as previously described (13). As expected, PAA did not inhibit v-IL6 expression, but it efficiently blocked both K8.1 expression and HHV8 morphogenesis as determined by TEM (14) (Fig. 3, and data not shown). Strikingly, also RLP production was completely blocked by PAA in the same cells.

We have previously shown that neutralizing anti-αIFN antibodies (anti-αIFN Ab) improve HHV8 morphogenesis in TPA-induced BCBL-1 cells without increasing HHV8 gene expression or DNA replication, indicating effects of αIFN on virus assembly (9). Therefore, if RLP and HHV8 morphogenesis were dependent on the same factor(s), cell treatment with anti-αIFN Ab should increase the production of both viruses. Thus, BCBL-1 cells were induced with TPA in the presence or absence of anti-αIFN Ab, as previously described (9). Under these conditions, the fraction of cells supporting RLPs morphogenesis was increased from 25% to 40% of cells undergoing HHV8 morphogenesis, whereas cell negative for HHV8 replication remained negative for RLP production (data not shown). These data confirmed that factors produced in late stages of HHV8 replication are involved in morphogenesis and/or assembly of both HHV8 and RLP.

DISCUSSION

The data presented here show that PEL cells produce RLPs with a distinctive C-type retrovirus morphogenesis that co-sediment with a measurable Mn^{++}-dependent RT activity, which is not present in any of the known human retroviruses (8). Since PEL cells are negative for both human immunodeficiency virus (HIV) or human T-lymphotropic virus (HTLV) (15-17), these data may suggest that PEL RLPs represent a previously unidentified human exogenous retrovirus. However, in ultrathin sections RLPs were characterized by the absence of outer clear zone and surface spikes. This is, indeed, a feature typical of human endogenous retroviruses (HERV), which rarely form mature particles (18-19).

Although HERV sequences represent about 5 to 8% of the human genome (20-21), production of endogenous RLPs is a very rare event (19, 22-25). Interestingly, our data indicate that expression of HHV8 late genes may promote the production of RLPs by providing or inducing the expression of limiting factors required for RLP morphogenesis.

HHV8, EBV and other herpesviruses are known to activate exogenous and/or endogenous retroviruses (25-28). In this context, it is noteworthy that both the size and morphology of negatively stained PEL RLPs are very similar to those of JHK retrovirus that is released by B lymphoblastoid cell lines upon EBV replication (29). These data suggest that EBV and HHV8 may provide factors supporting the morphogenesis of the same member of the HERV family.

HERV particles are released in supernatant from primary cell cultures or cultured tumor cell lines (19, 23-24). In contrast, the presence of HERV particles in specimens from normal or diseased subjects was only reported, in our knowledge, for multiple sclerosis patients (23). In this regard, it has been recently reported that plasma from patients with lymphoma or breast cancer show high endogenous retrovirus particle titers, which drop dramatically upon cancer treatment (30). These data highlight a possible role for HERV production in cancer. However, whether endogenous retrovirus morphogenesis plays any role in tumor pathogenesis remains to be determined.

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The authors disclose financial or other relationships that are relevant to the study and declare no conflict of interests.

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