Herpes simplex virus ICP0 and ICP4 immediate early proteins strongly enhance expression of a retrovirus harboured by a leptomeningeal cell line from a patient with multiple sclerosis

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Introduction

By culturing cells from the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS), we have recently isolated adherent cells that proliferate spontaneously in vitro but can seldom be cultured for more than 30 passages. These cells of leptomeningeal origin were isolated from a patient with definite MS, and named LM7 and found to harbour a retrovirus different to known human immunodeficiency (HIV) and human T cell-lymphotropic (HTLV) viruses (Perron et al., 1989). However, the expression of this retrovirus is rather restricted in these cells. A similar retrovirus can also be detected in blood and brain cell cultures from other MS patients (Perron et al., 1991a), but it has not been possible to culture these isolates continuously until now. However, the sera of a few MS patients, from which these new isolates were obtained, have been tested with purified ‘LM7 virions’ by Western blotting and shown to contain specific antibodies against LM7 retroviral proteins (Perron et al., 1991c, 1992).

Parallel to these studies which proved to be intrinsically limited by the low expression of LM7 virus, we tried to find means to enhance virion production in the culture supernatant. Specific reverse transcriptase (RT) activity released into the culture fluids could be enhanced with the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA), but only by several fold (Perron et al., 1989). However, by superinfecting the cultures with herpes simplex virus type 1 (HSV-1) we obtained potent stimulation of LM7 virus expression.

HSV-1 is known to trigger retrovirus expression when superinfecting cells latently infected with HIV-1 (Mosca et al., 1987a), and certain HSV-1 immediate early (IE) gene products (protein ICP0 in particular) have been shown to be responsible for the trans-activation of retroviral long terminal repeat (LTR) regulatory sequences (Laurence, 1990; Mosca et al., 1987b). Consequently, we also studied the effect of transfecting plasmids expressing three HSV-1 IE proteins (ICP0, ICP4 or ICP27) and the parental plasmid pAT153 into LM7 and control cells.

We present results showing that: (i) superinfection of LM7 cells with HSV-1 results in a sharp increase in RT activity in culture supernatants with retrovirus-like particles being identifiable by electron microscopy. These effects were not observed with a plasmid expressing ICP27 or with the parental plasmid in LM7 cells, nor with any of these four plasmids in control cells. These results show that HSV IE trans-activating proteins strongly enhance the expression of the latent retrovirus present in LM7 cells. The possible role in vivo of herpesviruses as ‘triggering’ cofactors in the retrovirus hypothesis for multiple sclerosis aetiology is also discussed.
produce a confluent monolayer in culture flasks that can be dissociated instead of 5% FCS.

HOS cells were cultured in the same basic medium as cells were cultured in RPMI 1640 medium, penicillin (200 U/ml), streptomycin (200 mg/ml), L-glutamine (2 mmol/ml), with trypsin EDTA for passage.

MRC5 cells (human foetal lung fibroblasts; Bio-Mérieux) and Vero cells were cultured in RPMI 1640 medium, penicillin (200 U/ml), streptomycin (200 mg/ml), L-glutamine (2 mmol/ml), with 5% heat-inactivated foetal calf serum (FCS).

MRC5 cells, containing 10% heat-inactivated newborn calf serum instead of poly(C).oligo(G)12-18. The reaction was stopped by adding 75 μl of a cold solution containing 12.5% H2O saturated with sodium phosphate, 12.5% H2O saturated with sodium pyrophosphate and 20% TCA. After 30 min to 1 h at 4 °C, the tubes were filled with a 5% TCA solution, and the contents were filtered and washed five times with 5% TCA onto a cellulose acetate membrane (Sartorius ref. 11106 25 N; pore size 0.45 μm, diameter 25 mm) under depression in a 1225 sample collector (Millipore ref. XX2702550). Before removal, the membranes were rinsed again with 20 ml 5% TCA to reduce the background as much as possible. The membranes were then immersed in scintillation fluid (Ready-Safe, Beckman) and the RT activity was measured as c.p.m. and d.p.m. in a β-counter.

The d.p.m. value above background (inherent to the technique and measured using control cultures in similar conditions) is proportionate to the amount of tritiated nucleotide incorporated into nucleotide templates by the RT present in retrovirus particles.

EM of ultrathin sections of cultured cells. Cells with a marked c.p.e. were fixed in situ with a buffered solution containing 2.5% glutaraldehyde and kept overnight at 4 °C. The cells were then stained with osmic acid, dehydrated and embedded in epoxy resin. After the sheet of polymerized resin had been removed with liquid nitrogen, areas containing sufficient cells were cut from the plastic bottom of culture flasks, stuck to a resin lamp and cut with a microtome into ultrathin sections, 70 nm thick. These sections were then placed on 300-mesh grids and stained with uranyl acetate and lead citrate. The preparations were observed with a transmission electron microscope (Philips CM10).

Supernatants containing shed cells were centrifuged at 1500 r.p.m. for 10 min, and the pellets were resuspended in a buffer containing 2.5% glutaraldehyde and processed as above except for centrifugation at 1500 r.p.m. for 10 min between each step to pellet the cells for fixation and dehydration. These pellets were then placed in Beem capsules and cut directly after resin polymerization.

Transfection with plasmids expressing HSV-1 IE genes. The plasmids containing individual HSV-1 IE genes and plasmid pAT153 were kindly provided by Dr Chris Preston (Institute of Virology, Glasgow, U.K.). Plasmid pJR3 encoding ICPO is a PstI–Sacl (nucleotides 118663 to 125066) restriction fragment of HSV-1 cloned into the pUC9 vector. The plasmid XhoC encoding ICPO is a XhoI fragment of HSV-1 (cut at nucleotides 123028 and 133520) cloned into vector pAT153. Plasmid HpaSV encoding ICPO27 is a KpnI–HpaI (nucleotides 121989 to 117009) fragment of HSV-1 cloned into the pAT153 vector (McGeoch et al., 1988; Perry & McGeoch, 1988).

Transfection was performed with Transfectam (Sepracor) as follows. The principle of this transfection process is a specific interaction between a cationic lipopolyamine and plasmid DNA. Cells were cultured in six-well plates (Falcon) and transfected at 50% confluence.

Methods

Cell cultures. LM7 and LM11 cells (human leptomeningeal cells obtained from a patient without MS, in similar conditions as for LM7 cells) were cultured in RPMI 1640 medium with penicillin [200 units (U)/ml], streptomycin (200 μg/ml), L-glutamine (6 μmol/ml), 1% pyruvate, heparin (50 U/ml), fibroblast growth factor (Boehringer Mannheim, purified from bovine brain; 50 ng/ml) and 20% heat-inactivated foetal calf serum. The principle of this transfection process is a specific interaction between a cationic lipopolyamine and plasmid DNA. Cells were cultured in six-well plates (Falcon) and transfected at 50% confluence.

HSV-1 infection of LM7, LM11, HOS, MRC5 and Vero cells. Culture flasks (75 cm²) containing nearly confluent cells were infected at a multiplicity of 3 p.f.u./cell with a wild defined and serotyped strain of HSV-1 isolated in our laboratory (Grenoble) from a herpetic vesicle on the lip of a patient seronegative for human retroviruses and with no other apparent disease. After 3 h of incubation at 37 °C, the medium was changed and the flasks were further incubated at 37 °C and observed twice a day. When marked c.p.e. was observed, the supernatants were collected and further processed for RT assay and electron microscopic (EM) examination of pellets from shed cells.

RT activity. All steps were performed with sterile material and solutions to avoid possible interference with bacterial enzymes during the 37 °C incubation step. Supernatants were collected before and just after c.p.e. was observed in the cultures, or when changing the medium (twice per week) in the absence of detectable c.p.e., and stored at –80 °C. For the RT assay, supernatants were thawed, cleared of cell debris by centrifugation at 10000 r.p.m. for 30 min and then centrifuged for 2 h at 35000 r.p.m. (100000 g) at 4 °C. The pellets were resuspended (concentrated 200-fold) in 0.05 M-Tris–HCl pH 8.5, homogenized, and 20 μl was added to a cocktail containing 5 μl 0.5 M-Tris–HCl, 0.04 M-EDTA pH 8.2, 2 μl 0.1 M-NaCl, 5 μl 0.03 M-MgCl₂, 23 μl double-distilled H₂O, 10 μl 2% NP40, 2 μl poly(C).oligo(G)12-18 or 2 μl poly(C).oligo(G)12-18 (10 U OD/ml; Pharmacia), 5 μl [³H]GTP (1 mCi/ml; NEN). Glass tubes (5 ml) containing the cocktails were incubated at 37 °C for 75 min. For evaluation of DNA-directed DNA polymerase activity, the cocktails contained the same concentration of poly(dC).oligo(dG)12-18 instead of poly(C).oligo(G)12-18. The reaction was stopped by adding 75 μl of a cold solution containing 12.5% H₂O saturated with sodium phosphate, 12.5% H₂O saturated with sodium pyrophosphate and 20% TCA. After 30 min to 1 h at 4 °C, the tubes were filled with a 5% TCA solution, and the contents were filtered and washed five times with 5% TCA onto a cellulose acetate membrane (Sartorius ref. 11106 25 N; pore size 0.45 μm, diameter 25 mm) under depression in a 1225 sample collector (Millipore ref. XX2702550). Before removal, the membranes were rinsed again with 20 ml 5% TCA to reduce the background as much as possible. The membranes were then immersed in scintillation fluid (Ready-Safe, Beckman) and the RT activity was measured as c.p.m. and d.p.m. in a β-counter.

The d.p.m. value above background (inherent to the technique and measured using control cultures in similar conditions) is proportionate to the amount of tritiated nucleotide incorporated into nucleotide templates by the RT present in retrovirus particles.

Effect cannot be obtained with another IE HSV protein, ICP27 with the parental plasmid or with any plasmid in control cells.

EM of ultrathin sections of cultured cells. Cells with a marked c.p.e. were fixed in situ with a buffered solution containing 2.5% glutaraldehyde and kept overnight at 4 °C. The cells were then stained with osmic acid, dehydrated and embedded in epoxy resin. After the sheet of polymerized resin had been removed with liquid nitrogen, areas containing sufficient cells were cut from the plastic bottom of culture flasks, stuck to a resin lamp and cut with a microtome into ultrathin sections, 70 nm thick. These sections were then placed on 300-mesh grids and stained with uranyl acetate and lead citrate. The preparations were observed with a transmission electron microscope (Philips CM10).

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Fig. 2. Pseudo-crystalline arrangements of HSV-1 nucleocapsids in the nucleus of an infected cell. (a) The nucleus is very processed and contains stacks of aligned HSV-1 nucleocapsids. (b) Higher magnification shows the quasi-geometrical disposition of parental nucleocapsids, with the presence of a few empty capsids. The bar markers represent 1 µm.

with 2 µg plasmid DNA/well. The plasmids were diluted in 250 µl sterile 0.3 M-NaCl solution, mixed immediately before transfection with the same volume of double-distilled water containing 5 µl of Transfectam stock solution per µg of plasmid DNA. This Transfectam-plasmid mixture was then placed in the culture wells, previously washed with serum-free RPMI 1640 (twice for 5 min each), mixed with 1 ml of serum-free RPMI left in the wells and distributed evenly over the cell monolayer surface. Plaques were then incubated for 6 h in a 5% CO₂ incubator at 37 °C. The medium containing the Transfectam-plasmid suspension was removed, frozen at -80 °C and replaced with 5 ml normal medium, and the plaques were incubated further. The supernatant from each well was then collected, frozen at -80 °C and replaced with 5 ml of fresh medium at 24, 48 and 72 h post-transfection. Each plasmid was tested in three different wells. The frozen culture supernatants were thawed, centrifuged and tested for RT activity as described above.

The expression of HSV IE proteins in transfected cells was visualized by an indirect immunofluorescence assay using a monoclonal antibody (MAb) or an antipeptide rabbit serum (data not shown). These antibodies were kindly provided by Drs Anne Cross, Roger Everett and Howard Marsden (Institute of Virology, Glasgow, U.K.). Two MAbs (10.176 and 10.462) against ICP4 were used. Rabbit antibodies against ICP27 were made against a branched peptide representing amino acids 1 to 16 of the protein.

Results

In vitro superinfection of leptomeningeal cell cultures with HSV-1

Superinfection of LM7 cells with HSV-1 produced a c.p.e. starting 6 h post-infection, followed by complete shedding of the LM7 cell monolayer within 24 h. The detached cells were collected 24 h post-infection and pelleted from the culture supernatant. We observed by EM the co-expression of two categories of virus particles, as shown in Fig. 1: typical herpesvirus particles (arrow in Fig. 1) and numerous retrovirus-like particles with a diameter of 100 to 110 nm, a large electron-dense nucleocapsid and thin envelope structures surrounded by diffuse spike/knob projections (inset Fig. 1) were visible.

In the control cells (MRC5, Vero, HOS and LM11), infected under similar conditions with the same HSV-1 strain, no retrovirus-like particles were ever seen and only typical herpesvirus particles were observed, as shown in Fig. 2 and 3. Different aspects of the HSV-1 particle maturation process were visible and these were used as a reference for morphological comparison with the particles co-expressed in LM7 cells after super-infection.

In LM7 cells treated with TPA far fewer retrovirus-like particles were seen, but their morphology was similar to that of particles found in HSV-1-infected LM7 cells. These particles were limited to a few clusters in the adherent cell monolayer or found isolated in single cells scattered throughout the culture (Perron et al., 1989).

The supernatant from an HSV-1-superinfected LM7 culture cleared of cell debris showed much higher RT activity (with the same magnesium concentration and template) compared to unstimulated LM7 cells, and no significant RT activity was found in HSV-1-infected control cells. These data are shown in Fig. 4.
Fig. 3. Normal appearance of HSV-1 particles during successive steps in virion assembly. (a) Parental nucleocapsids in the nucleus of an infected cell. (b) A nucleocapsid budding through the nuclear envelope, thus acquiring its first envelope. (c) The first envelope is now closed around the capsid. (d) Enveloped nucleocapsids as they are found in the cytoplasm. (e) A nucleocapsid with its first envelope budding through the membrane of a cytoplasmic vacuole, thus taking its second membrane (a cytoplasmic invagination is superimposed on the middle of the nucleus). The second envelope can also be formed by a similar process of budding through the plasma membrane. (f, g) Typical mature extracellular HSV-1 virions. Bar markers represent 1 μm (a) and 0.1 μm (b to g).
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Fig. 4. RT activity in the cell-free supernatants of LM7 (■) and control (■) cell cultures infected or not with HSV-1. The mean RT activity from control cells was obtained with normal MRC5, Vero, HOS and LM11 cells.

Fig. 5. Kinetics of RT activity in the cell-free supernatants of LM7 (■, ◦) and control LM11 (●, ○) cell cultures transfected with HSV-1 IE genes ICP0 (■, ●) or ICP27 (◦, ○). Results represent the RT activity calculated from the average total d.p.m. measured in the culture supernatants (about 2 ml at 6 h, and 5 ml at 24, 48 and 72 h) collected from wells containing about 2.5 x 10⁶ cells. The confidence intervals are shown for ICP0 only.

Fig. 6. Kinetics of RT activity in the cell-free supernatants of LM7 (■, ◦) and control LM11 (●, ○) cell cultures transfected with HSV-1 IE gene ICP4 (■, ●) or with the parental pAT153 plasmid (◦, ○). Results represent the RT activity calculated from the average total d.p.m. measured in the culture supernatants (about 2 ml at 6 h, and 5 ml at 24, 48 and 72 h) collected from wells containing about 2.5 x 10⁶ cells. The confidence intervals are shown for ICP4 only.

Transfection with plasmids

The kinetics of RT activity measured in the culture supernatants after transfection of cells with plasmids expressing HSV IE genes or with a plasmid alone are shown in Fig. 5 and 6.

It can be seen that there is a significant increase in specific RT activity when LM7 cells are transfected with either ICP0 or ICP4, but not with ICP27 or a plasmid alone. In the control cells, no significant increase was observed in any case. The activity detected in these cells constitutes a non-specific background signal; in unstimulated LM7 cells the small volume of supernatant used in the assays (2 to 5 ml) produces a ‘virion load’ below the level of detection by this technique. Specific RT activity in unstimulated LM7 cells usually becomes detectable with about 30 ml of supernatant.

RT induction kinetics differ for ICP0 and ICP4. With ICP0 the released activity is significantly increased at 6 h and peaks at 24 h, after which it sharply decreases and might start a new ‘cycle’ at 72 h post-transfection; with ICP4, significant activity is seen only after 24 h, but this level is maintained until 72 h (if we take the confidence intervals into account).

The expression of IE protein after transfection was confirmed by immunofluorescence assay of cells fixed at different intervals, using specific monoclonal and polyclonal antibodies in the case of ICP4 and ICP27 (Fig. 7). EM examination of ICP0-‘stimulated’ LM7 cells fixed at 16 h post-transfection revealed the presence of rather abundant retrovirus-like particles in the cytoplasm of cells adherent to the culture surface (Fig. 8). These intracytoplasmic particles have an average diameter of 90 to 100 nm, with a very dense core surrounded by a close-fitting envelope without spikes.

EM examination, after negative staining of ultracentrifugation pellets from clarified and 0.45 μm-filtered supernatants of ICP4-‘stimulated’ cultures revealed the presence of extracellular retrovirus-like particles with an average diameter of 100 nm as seen in Fig. 9.

Discussion

The retrovirus harboured by LM7 cells and detected in MS patients has yet to be definitively characterized. However, as we could not obtain sufficient quantities of virions for molecular study, methods for enhancing its expression were investigated.
The present study shows that HSV-1 can infect leptomeningeal cells and undergo a complete replicative cycle leading to extensive lysis of a cultured monolayer. During HSV-1 superinfection, the expression of the retrovirus detected in LM7 cells is markedly enhanced and two types of virus particle can be observed by EM: a typical HSV-1 particle with well characterized morphology at different stages of maturation, and another with morphology similar to retrovirus-like particles seen extracellularly in TPA-stimulated LM7 cell cultures. Moreover, this production of retrovirus-like particles is associated with elevated RT activity in the culture supernatant. Since neither retrovirus-like particles nor significant RT activity can be detected in control cells infected with the same HSV-1 stock, we consider that these observations are not due to the HSV strain.
Moreover, the use of methylated cytosine template [poly(C)m] in the RT assay greatly limited the non-specific RT-like activity of cellular DNA polymerase gamma and of HSV-1 polymerase. Poly(C)m is known to be an inefficient template for DNA-directed DNA polymerases (Sarngadharan et al., 1978; Verma, 1977) and LM7 virus RT activity is best detected with it (Perron et al., 1989).

Herpesviruses can enhance the expression of HIV-1 in vitro (Laski & Troy, 1984; Laurence, 1990; Mosca et al., 1987a; Quinlivan et al., 1990). The trans-activation of HIV-1 LTRs by HSV-1 IE gene products ICP0 and, to a lesser extent, ICP4, has been demonstrated recently (Chapman et al., 1991; Mosca et al., 1987b). HSV-1 IE proteins can be detected in infected cells as early as 2 h post-infection (Roizman & Sears, 1990) and some are known to be trans-activating factors (Rice & Knipe, 1990; Roizman & Sears 1990).

Therefore we tested the effect of particular HSV-1 IE proteins in our system by transfecting LM7 and control cells with appropriate plasmids. We noted potent stimulation of retrovirus expression by ICP0 and ICP4, with a boost in extracellular retroviral RT activity as well as an accumulation of intracytoplasmic retrovirus-like particles. Such particles resemble intracellular core structures of type D retroviruses (Rhee & Hunter, 1990), or particles observed with Jaagsiekte retrovirus which is closer to type B although it has a distinctive morphology (Verwoerd, 1990).

The differences observed in the stage of maturation of particles from HSV superinfection and ICP0 transfection result from the cell fraction examined. After infection, lysis was so rapid that the majority of cells collected had been shed into the culture medium, whereas after transfection most cells were still attached and were fixed in situ. Induction kinetics must also differ, but we cannot exclude the possibility that a complete herpesvirus may have a structural helper effect on the maturation of these retrovirus particles, e.g. by modifying the properties of the cellular membrane with its own glycoproteins. Alternatively, stimulation by isolated trans-activators may not result in an adequate expression of all the retrovirus genes required for complete maturation of particles. Such mechanisms influence the formation of extracellular virions of type B retroviruses, (Bentvelzen & Hilgers, 1980; Kuff & Lueders, 1988).

Thus a trans-activating mechanism, possibly similar to that demonstrated for HSV-1 ICP0 and HIV-1, is likely to be responsible for the potent enhancement of retrovirus replication we have observed in LM7 cells. However, unlike HIV-1, ICP4 seems as efficient, if not more so, than ICP0 in stimulating the LM7 virus isolate. It also gives different kinetics of extracellular virion release, as seen from the RT activity in cell-free culture supernatants.

Considering the debate on the hypothetical role of a retrovirus in MS pathogenesis (Dalgleish et al., 1987; Johnson, 1985; Koprowski et al., 1985; Rudge, 1991), epidemiological (Fraser et al., 1981; Norrby, 1978; Perron et al., 1991b), experimental (Kastrukoff et al., 1987), and even bioclinical (Bergström et al., 1989; Mahalingam et al., 1990; Martin, 1981) studies could suggest that neurotropic herpesviruses such as HSV-1 or varicella-zoster virus may interfere in vivo with a latent retrovirus in MS patients. If so, primary herpesvirus infection (or even reactivation) would be consistent with a cofactor triggering the expression of a probably latent retrovirus. Thus it is noteworthy that relevant serological findings showing partial or occasional virus association with MS (Norrby, 1978; Perron et al., 1991b) could be explained by a triggering mechanism involving various facultative/cumulative cofactors, each having a significant role but in a limited proportion of cases. In the case of HSV-1, such ideas are also consistent with the reported
isolation of this herpesvirus from the cerebrospinal fluid of a patient during a first and acute MS attack (Bergström et al., 1989). However, we cannot speculate on a possible role for HSV-1 in MS.

Finally, this study reveals that plasmids expressing HSV ICP0 or ICP4 are valuable tools which can be used to obtain more LM7 virions from culture without producing the mixed virus populations obtained after infection with a complete HSV genome. It should help in the molecular characterization of the LM7 virus and could open a new field of investigation of herpesvirus–retrovirus interactions in humans.

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References


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