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Review

Molecular mechanisms of poliovirus persistence: key role of capsid determinants during the establishment phase

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Abstract. As viral persistence is of major medical importance, well-characterized, simple models are needed to improve our understanding of persistent infections. We have chosen to study the molecular mechanisms of viral persistence with the poliovirus (PV), because this picornavirus is one of the best characterized animal viruses, it infects the central nervous system which is a target organ for viral persistence, and it belongs to the *Picornaviridae* family of viruses, which includes several naturally persisting viruses. We have developed models of PV persistence in neuronal and epidermoid cells, and the present review will focus on the latter one because both lytic and persistent PV strains can be used to study the PV-HEp-2 cell interactions. The viral determinants of persistence have been investigated with this model, and PV determinants have proven to be of crucial importance for the establishment of persistence in HEp-2 cells. Precise determinants of PV persistence have been identified for PV serotypes 1 and 3, in capsid proteins VP1 and VP2. These determinants modify the early steps of the PV cycle, and in particular, the conformational modifications of the capsid following virus adsorption onto its receptor. These results permit us to propose several hypotheses concerning PV persistence and the early steps of the PV cycle.

Key words. Virus; poliovirus; picornavirus; mutations; capsid; persistence; receptor.

Viral persistence is of major medical importance

Many viruses are able to establish long-lasting, persistent infections in their host, and some of them are even responsible for severe pathologies [1]. Retroviruses, and most DNA viruses including in particular herpesviruses, are known to establish persistent infections in their hosts. The acquired immunodeficiency syndrome results from the persistence of the human immunodeficiency virus [2], which, in turn, favours the reactivation of many other persistent viruses, with the appearance of the corresponding pathologies. Several nonretrovirus RNA viruses also persist in their hosts [3]. For example, a common childhood infection caused by the measles virus can lead to a very rare but fatal pathology, subacute sclerosing panencephalitis, after a decade of persistent viral infection in the central nervous system (CNS). Another example is provided by the hepatitis C virus, which persists in humans very frequently after the primary infection, inducing chronic hepatitis which may finally provoke a hepatocarcinoma [4]. In fact, several different types of human cancers, including leukaemias,

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lymphomas, sarcomas and carcinomas, are associated with persistent infection by various members of the *Retro*-, *Herpes*-, *Papova*-, *Hepadna*- or *Flaviviridae* families [1]. Moreover, several chronic pathologies are probably triggered by viral persistence. It has been proposed that a persistent coxsackievirus infection could be involved in cases of chronic heart disease and in diabetes mellitus [5, 6]. Indeed, during viral persistence, housekeeping functions of the cell may not be altered, whereas specialized functions, like the secretion of insulin, may be affected, resulting in pathology at the level of the organism [7–9]. Other viruses, in particular retro-, corona- and herpesviruses, are proposed to play a role in the development of autoimmune diseases such as multiple sclerosis [10–13].

These few examples provide evidence that viral persistence is of major medical importance. Thus, well-characterized, simple models are needed to improve our understanding of persistent infections. We have chosen to study the molecular mechanisms of viral persistence with the poliovirus (PV), because this picornavirus is one of the best-characterized animal viruses; it infects the CNS, which is a target organ for viral persistence [3]; and it belongs to a family of viruses that includes several naturally persisting viruses. Furthermore, in a certain number of patients with a history of paralytic poliomyelitis, a second pathology, possibly resulting from viral persistence, has been observed to develop several decades after the acute disease [14, 15]. It is therefore interesting to investigate the capacity of PV to establish persistent infections in human cells.

General mechanisms underlying viral persistence

In vivo, the establishment of persistent infection depends on specific host factors and, particularly, on the capacity of the virus to escape immune surveillance [1, 16–20]. Indeed, antigenic variation allows the virus to avoid recognition by neutralizing antibodies, and at the same time allows the infected cell to escape from the cytotoxic T lymphocytes. In addition, suppression of the expression of cellular molecules required for immune recognition, interference with antiviral cytokines and immune tolerance all may contribute to viral persistence [21]. Moreover, certain target cells are preferential sites for viral persistence, such as lymphoid cells, and neurons of the CNS, which are relatively protected from the immune system by the blood-brain barrier and because they generally do not express the major histocompatibility class I antigens at their surface [22–24]. The nature of the virus may also be important, as nonlytic viruses establish persistent infections more easily than lytic viruses, both in vivo and in vitro.

Simplified in vitro systems, in which host factors and the immune system do not play a role, have been developed to elucidate the molecular mechanisms of viral persistence. During these infections, it has been observed that virus and cell coevolution generally occurs [25–27]. This can lead to limited viral replication, allowing cell survival. In fact, viral mutants are often selected, including antigenic variants, thermosensitive mutants or defective interfering (DI) particles. The emergence of point mutations and deletions in the genome of these mutant viruses is a common feature of in vivo and in vitro persistent infections [28–31].

Two main models of in vitro persistence have been proposed: the steady state and the carrier state. In the first case, all cells of the culture are infected under nonlytic conditions and produce small amounts of virus, whereas in the second one, a minority of cells are lytically infected in each cell generation. These models are very simplistic, representing two extremes, and more complex models have now been described [26, 27, 32]. Nevertheless, two phases are usually distinguished: the establishment phase, corresponding to the first few weeks after infection during which the virus propagates to all permissive cells in the culture, followed by the later phase of maintenance during which the production of virus and the growth of cells are more stable.

Viral determinants play an important role in persistence

The development and study of different models, both in vitro and in vivo has allowed for a better understanding of the mechanisms underlying viral persistence, and in particular for the identification of a certain number of viral determinants which play important roles during the establishment and/or maintenance phases of persistent infection. For example, a correlation between viral persistence and modified cell tropism was first clearly demonstrated in the case of the lymphocytic choriomeningitis virus (LCMV), since it was shown that a mutant virus with a leucine at position 260 of the glycoprotein has a tropism for macrophages and has the tendency to establish persistent infections in adult mice, while the nonmutant strain is neurotropic and does not persist under the same conditions [33, 34]. More recently, it has been proposed that short deletions at the termini of the genome and the addition of nontemplated nucleotides may also contribute to the establishment and maintenance of LCMV persistence [35]. In the case of persistent infections by reoviruses in vitro, the role of DI particles is well known, as is the role of certain capsid determinants which affect the disassembly of the virus [27, 36]. As for Sindbis virus, the presence of a single

determinant in the nonstructural protein-2 of this virus has been shown to be responsible for viral persistence in vitro [37].

Several members of the *Picornaviridae* family are naturally persisting in vivo. Foot-and-mouth disease virus persists in cattle after primary infection [38]. In fact, even attenuated vaccine strains are capable of persisting in vivo. In vitro carrier-state models have been developed in baby hamster kidney cells, in which the crucial importance of the cell was demonstrated [39, 40]. The majority of strains of another picornavirus, Theiler's murine encephalitis virus (TMEV), are persistent in their natural host, the mouse. In this case, following the acute encephalitis caused by primary infection of the grey matter, the virus reaches the white matter of the spinal cord and induces demyelination. During this second phase, the persistent virus is found primarily in macrophages and oligodendrocytes [41, 42]. Specific capsid determinants have been shown to be crucial for the persistence of TMEV [43, 44].

Among human picornaviruses, coxsackievirus B (CVB) establishes persistent infections in animal models in heart and pancreatic tissue, depending on the tropism of the virus strain: cardio- or pancreatotropic, respectively [45, 46]. A spatial and temporal correlation between viral replication and tissue damage has been observed in the heart of chronically infected mice [47], suggesting that CVB could be reponsible for chronic pathologies in humans, and this is supported by epidemiological data [5]. Moreover, CVB viruses also establish persistent infections in vitro and ex vivo in human cells, particularly endothelial cells and mesangial cells of the kidney [48, 49].

In the case of PV, viral multiplication was first studied in a very small number of human cell lines as well as primary cultures of monkey kidney cells, using mainly single-cycle growth experiments, which revealed the highly lytic nature of PV [50]. However, in more recent years, when lymphoid, erythroblastoid or neuronal cells were used, persistent infections were readily established in vitro [51–53]. In human erythroblastoid cells, persistence seems to depend on the degree of differentiation of these cells, as well as on the absence of shutoff of host cell syntheses [52, 54]. This appears to be due to host cell factors rather than specific viral determinants. Similarly, in a human neuroblastoma cell model developed in our laboratory [53], all the PV strains that we have tested are capable of persisting in these cells (although with highly variable efficiencies), suggesting that the cell plays the predominant role in the establishment phase. However, in our second model, established in nonneural HEp-2 cells [55], we found that capsid determinants are of crucial importance during the establishment of persistence, as will be developed in greater detail in the following paragraphs. Taken together, these results suggest that, for the same virus, the importance of viral determinants in the phenotype of persistence varies with the cell line.

In vivo, PV persists in immunodeficient and immunocompetent mice [56, 57]. In the latter, it has been shown that the virus persists for months in the brain of unparalyzed mice inoculated with the type 2 Lansing strain, and in the spinal cord of mice paralyzed after inoculation with PV mutants adapted to the murine CNS [58]. These results demonstrate that PV can persist in the CNS of an immunocompetent animal.

Does poliovirus persist in humans?

In its natural host, that is humans, PV is the aetiological agent of acute paralytic poliomyelitis (reviewed in ref. 59). The transmission occurs through the oral route, and the virus first replicates in the oropharynx, particularly in the tonsils. PV crosses the acid barrier of the stomach because of its resistance to low pH and then multiplies in the small intestine, in which it is found at the level of Peyer's patches. PV is excreted and can be found at high titres in faeces. From the primary sites of multiplication in the digestive tract, PV spreads to the cervical and mesenteric lymph nodes. A viraemic phase follows and in most infections, that is 99% of cases, the infection stops at this stage. In the remaining 1% of infections, the virus reaches the CNS and multiplies preferentially in the motor neurons of the spinal cord and brain stem. The lysis of these motor neurons is responsible for the flaccid paralysis which is characteristic of poliomyelitis.

Worldwide eradication of poliomyelitis is currently underway, based on the use of two efficient vaccines: the Salk vaccine, which consists of inactivated strains, and the Sabin vaccine, which is composed of live attenuated strains [60, 61]. Both vaccines confer a strong general immunity, but the attenuated one has the advantage of also conferring good intestinal immunity, following virus multiplication in the gut. However, during virus multiplication, the rapid reversion of the live Sabin strains towards a much less attenuated phenotype than that of the original vaccine strains can cause a very rare iatrogenic disease, vaccine-associated paralytic poliomyelitis [62, 63].

Particularly in North America, 10–50% of survivors of poliomyelitis develop the postpoliomyelitis syndrome (PPS), a variety of new neuromuscular symptoms which arise after decades of clinical stability [14]. These include new muscular atrophies affecting muscles originally affected or, conversely, muscles which seemed to have been spared during the acute disease. Three nonexclusive hypotheses have been proposed to explain this syndrome: the early senescence of originally spared neurons which have been oversolicited in compensation for the loss of neurons during the acute disease, an immunopathologi-

cal mechanism and persistent PV infection [15]. In agreement with the third mechanism, one study has revealed the presence of anti-PV immunoglobulin (Ig)M antibodies in the cerebrospinal fluid (CSF) of about 60% of PPS patients [64], and in three laboratories, PV or enteroviral sequences have been detected in the CSF with varying efficiencies by reverse transcription and polymerase chain reaction (PCR) [65–67]. These results have still to be confirmed, and nothing is known about the types of cells which could eventually permit PV to persist in the human CNS, although our ex vivo studies indicate that neuronal cells can survive PV infection in persistently infected primary cultures of human foetal brain cells [68].

The poliovirus

The PV capsid has an icosahedral symmetry and is composed of 60 copies of each of the four structural proteins VP1–VP4 [69]. The proteins VP1–VP3 have a common β-barrel structure, with eight β strands and two α helices interconnected by loops. The carboxy termini and many of the interconnecting loops are exposed at the capsid surface, whereas the amino termini of these proteins are located inside the capsid in association with protein VP4, which is exclusively internal [70, 71]. A large depression surrounding the fivefold axis of symmetry, called the canyon, contains the site of PV attachment to its cellular receptor for all three PV serotypes [70, 72, 73]. The PV genome is a single-stranded RNA molecule of positive polarity, of approximately 7.5 kb [74]. The viral genome and the corresponding complementary DNA (cDNA) are infectious after transfection in cell culture, allowing the easy construction of point mutants and recombinant viruses (reviewed in ref. 75). The 5′ end of the genome is covalently attached to a small protein, VPg, while the 3′ end is polyadenylated. A unique large coding region is surrounded by two noncoding (NC) regions which are rich in secondary structure [62, 69]. The 5′ NC region is about 740 nucleotides long and contains important signals for the initiation of plus-strand RNA replication and viral protein synthesis, including an internal ribosomal entry site (IRES). The 3′ NC region is about 70 nucleotides long and plays a role in the initiation of minus-strand RNA synthesis. The large open reading frame encodes a precursor protein of 247 kD, which undergoes successive cleavages by viral proteases, to first generate the P1 precursor of the capsid proteins and the P2 and P3 precursors of the nonstructural proteins, and then all of the viral proteins. The nonstructural proteins include the viral proteases, the small VPg polypeptide, the RNA polymerase and the other viral proteins necessary for PV replication. Several of the precursors also have a functional role in PV multiplication [69].

The cycle of PV multiplication, which is entirely cytoplasmic, and lasts about 8 h, is illustrated in figure 1. PV first adsorbs onto a cellular receptor, CD155, which is a protein belonging to the Ig superfamily, the physiological function of which is unknown. This PV receptor (PVR) has three extracellular domains, a transmembrane domain and an intracytoplasmic domain [76–78]. There are four isoforms of the PVR formed by alternative splicing, although only the two integral membrane-bound forms can serve as functional receptors for PV. When fully processed and glycosylated, the two functional isoforms have a molecular weight of about 80 kD [79], but their glycosylation is not required for PV entry. In the PV cycle, PVR is responsible not only for binding the virus but also for inducing conformational modifications of the capsid that are required for uncoating. The first extracellular domain binds the virus, probably within a 30-amino acid sequence composed of β strands C′, C″ and D, whereas the second and/or the third domain optimize virus entry [80]. It has been postulated that cytopathic effects could result from specific PV-PVR interactions [81].

The native virion has a sedimentation coefficient in sucrose gradients of 160S, which remains unchanged as long as virus binding to PVR is performed on ice. Once the incubation temperature of infected cells is raised to 37 °C, conformational transitions are induced, and the sedimentation coefficient of the particule is decreased to 135S [82–85]. This altered 135S particle, also named the A-particle, is much more hydrophobic than the virion, has altered antigenic properties and is protease-sensitive [86, 87]. In addition, the 135S particles have lost the internal protein VP4 and expose the amino terminus of VP1 at their surface, this sequence normally being located within the interior of the capsid in native virions [84]. Some of the 135S particles are released in the cell supernatant by way of a process known as elution [87]; the remainder are internalized. The ability of the 135S particles to attach to lipid membranes suggests that they could create a pore in the cellular membrane, thereby releasing the viral genome into the cytoplasm [88, 89]. These particles are infectious, even in the absence of the PVR, but with a very low efficiency [90].

As soon as the viral RNA reaches the cytoplasm, the VPg protein is cleaved from the viral genome by a cellular protease, allowing translation by the cellular machinery. In fact, the PV genome is uncapped, and the initiation of translation therefore depends on the IRES [91, 92]. PV nonstructural proteins are responsible for the shutoff of host cell transcription and translation, for the processing of viral proteins and for viral replication, which occurs in virus-induced membrane complexes [93, 94]. Viral replication starts with the synthesis of the minus strand, generating the double-stranded RNA, that is the replicative form, which subsequently becomes

a replicative intermediate when many viral genomes are replicated simultaneously. The capsid proteins associate in protomers, five protomers form a pentamer and 12 pentamers form a procapsid [95]. It is not yet known whether the viral genome enters a preformed procapsid, or if in fact the capsid proteins condense around the viral genome to give the provirion. A final proteolytic cleavage of the precursor VP0 separates capsid proteins VP2 and VP4, generating the mature virion. Although the process of virus release is not fully understood, massive virus liberation generally occurs through cell lysis. However, it has also been reported that some virus could be liberated via the bursting of vacuoles at the cell surface [96]. In agreement with the possibility of virus liberation in the absence of cell lysis, we and others have obtained high PV titres in the supernatants of

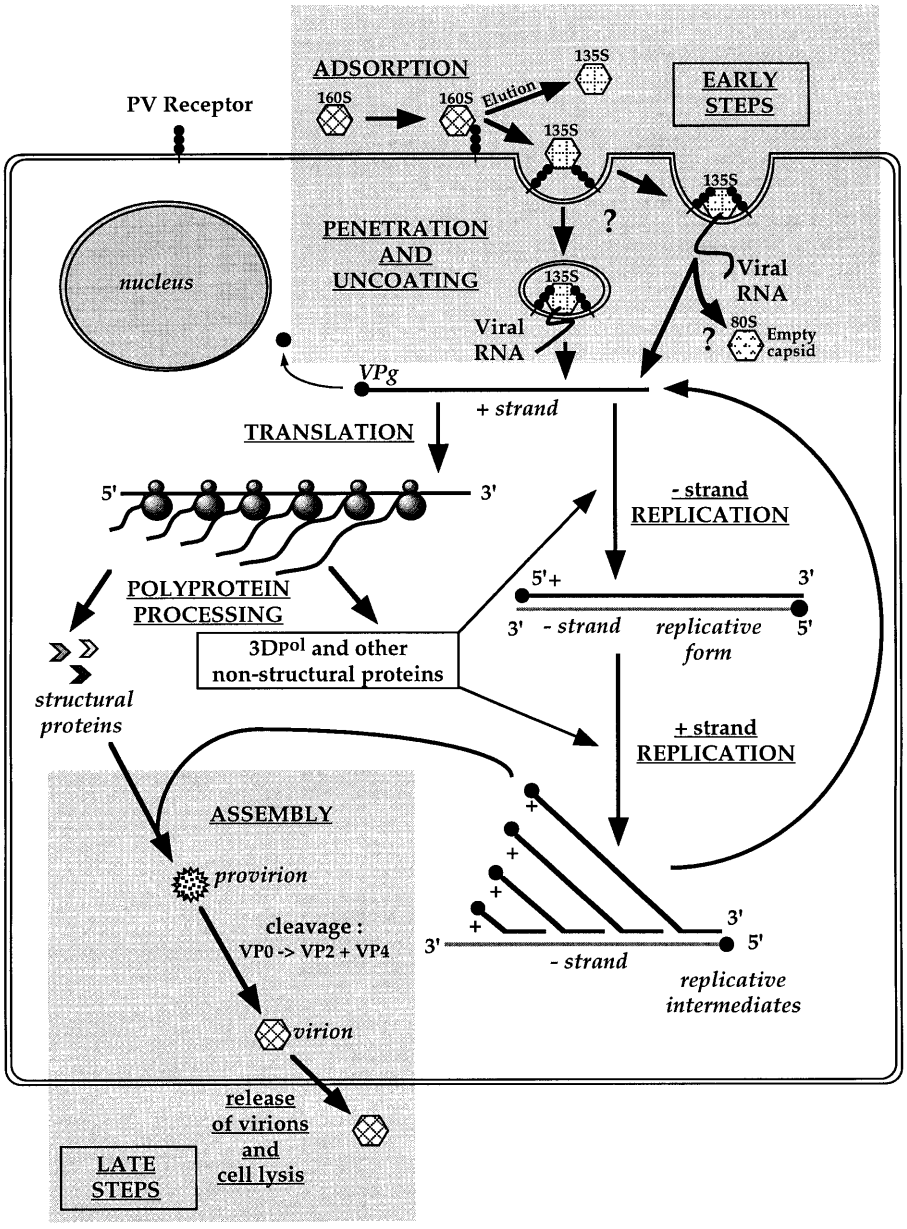

Figure 1. Main steps of the cycle of PV multiplication. The early steps include virus adsorption onto the PVR, conformational modifications of the capsid, decapsidation and RNA release into the cytoplasm (adapted from ref. 80) The following steps of viral protein and RNA syntheses, which are exclusively cytoplasmic, are briefly described in the text. The late steps include capsid assembly and encapsidation of the viral genome, a final proteolytic maturation cleavage and the liberation of mature virions.

some cell lines in the absence, or preceding the appearance, of cytopathic effects [81, 97].

Isolation of poliovirus mutants, PVpi, with a modified cell tropism

In the laboratory, we have established persistent PV infections in human neuroblastoma cells and, more recently, in primary cultures of human foetal brain cells [53, 68, 98]. The former are neuronal cells, and among the latter, cells surviving PV infection were found to belong to the neuronal lineage. After several months of persistent infection of neuroblastoma cells, mutant viruses, called PVpi, were selected [55]. These viruses are both phenotypically and genotypically modified. Indeed, PVpi differ from their parental counterparts in terms of virus antigenicity, thermosensitivity, host range and cell tropism. In addition, they contain numerous mutations in their genomes, and in particular, in the region encoding the capsid proteins [99]. Remarkably, identical substitutions to those present in PVpi were selected during the persistent infection of human foetal brain cells [68].

An interesting property of some PVpi is that they have an extended host range. Most PV strains, neurovirulent in primates, are unable to multiply in the murine CNS and are therefore not paralytogenic in mice upon intracerebral inoculation. In contrast, PVpi strains derived from the neurovirulent Mahoney strain were found to be adapted to the murine CNS and paralytogenic in mice [100]. Indeed, mutations corresponding to mouse adaptation determinants are regularly selected in PVpi derived from both attenuated and neurovirulent strains. These determinants affect the early steps of the PV cycle, that is adsorption, penetration and uncoating.

PVpi also have a modified tropism at the level of the host cell. They are generally more lytic for neuroblastoma cells than the parental PV strains, and conversely they are less lytic in nonneural HEp-2 cells, as estimated by the ratio of titers on neuroblastoma vs. HEp-2 cells, and the diameter of plaques on the latter cell type [55].

PVpi establish persistent infections in HEp-2 cells

In correlation with modified cell tropism, PVpi can establish persistent infections in HEp-2 cells, which are derived from an epidermoid carcinoma of the larynx [55]. However, the persistent infection of HEp-2 cells is quite different from that observed for the neuroblastoma cell model, from several points of view. First, it is clear that in the case of HEp-2 cells, the viral strain is of crucial importance for the establishment of the persistent infection, as only the PVpi are capable of persisting in these cells; all of the laboratory PV strains tested are fully lytic in HEp-2 cells. Moreover, we have shown by coinfection experiments that the lytic phenotype is dominant during this establishment phase [32]. The fact that both the lytic and the persistent phenotypes are observable in HEp-2 cells make this the model of choice for studying the mechanisms of PV persistence. Second, the multiplicity of infection has also proven to be extremely important in the case of HEp-2 cells. In fact, persistence is only established at low multiplicities, for example 10^{-4} virions per cell for point mutants containing a very limited number of mutations. Third, cytopathic effects are always observable in infected HEp-2 cell cultures, even during the maintenance phase. Finally, the spontaneous cure of cell cultures or, in contrast, the lysis of the entire culture are sometimes observed in the case of the HEp-2 cell model, something which has never been observed with the neuroblastoma model of persistent PV infection. The characterization of persistently infected HEp-2 cell clones led us to propose that the maintenance of infection in these cells depends on the expression of a host factor involved in PV multiplication, the stability of which may be responsible for an equilibrium between an abortive and a lytic virus cycle (fig. 2) [32]. Virus-cell coevolution is observed to occur during persistent infection of HEp-2 cell cultures, and the study of a certain number of cell clones has helped to shed light on the mechanisms involved in the maintenance phase, and in particular, on the role of the cell in the persistent infection of HEp-2 cells by the PV [97].

Mechanisms of maintenance of persistent PV infection in HEp-2 cells

The maintenance of persistent PV infection has been studied in cells producing virus and in cells cured either spontaneously or by growth at supraoptimal temperature (39 °C). One of the prominent features of the maintenance phase is that cells selected during persistent infection display various phenotypes, suggesting that cells may survive infection by way of different mechanisms [97]. Cells producing 1–100 virions per cell are generally resistant to superinfection, and harbor an average of 3000 to 40,000 PV genome equivalents per cell [32]. Viral genomes are sometimes localized in specific regions of the cytoplasm. Cells which were cured may be highly susceptible or in contrast resistant to superinfection, or they may have an intermediate phenotype [97]. Some cell clones display a phenotype which is stable over several years, while others are phenotypically very unstable. These differences can in part be explained by the level of expression of the PVR, which, depending on the cell clone, varies from the background level of receptor-negative murine cells to the level of expression found for the fully permissive parental HEp-

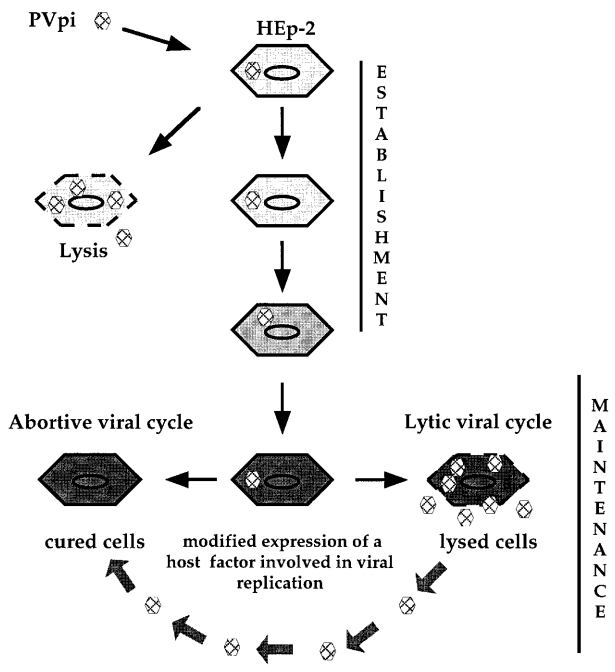

Figure 2. Schematic representation of a persistent PV infection in an HEp-2 cell culture. Only PVpi or PV mutants harbouring specific mutations can establish persistent infections. In the first weeks post-infection many cells are lysed, but some of them survive infection. During the maintenance phase of persistent PV infection, only a minority of cells demonstrate cytopathic effects and are lysed; the majority survive infection and divide. The spontaneous cure of the culture is sometimes observed, suggesting that the PV cycle is abortive in part of the culture. In some persistently infected HEp-2 cultures, the equilibrium between the lytic cycle and the abortive cycle can be displaced in one sense or the other, according to the culture conditions (cell density, incubation temperature etc.) [32].

2 cells. However, some of the cured cell clones display selective permissivity, since they are susceptible to the wild-type Mahoney strain and partially resistant to the attenuated Sabin 1 (S1) strain [97]. The selective permissivity therefore depends on viral determinants, and some of them have been localized to regions of the viral capsid which are involved in the early steps of the virus cycle, including the internal amino terminus of VP1, and the well-exposed B-C loop of the same protein which is located at the fivefold axis of symmetry. Interestingly, the cellular determinants involved in the selective permissivity of certain HEp-2 clones also implicate the early steps of infection by modifying the level of PVR expression, and the efficiency of uncoating transitions which occur subsequent to adsorption.

Mechanisms of establishment of persistent PV infection in HEp-2 cells: the critical role of determinants located in the viral capsid

As mentioned earlier, the virus plays a crucial role during the establishment phase of persistence in the HEp-2 model. The importance of the viral capsid, in particular, was first revealed by studying a type 1 PVpi, S11, derived from the attenuated S1 strain, after 6 months of persistent infection in neuroblastoma cells. PVpi S11 is persistent in HEp-2 cells, whereas the S1 strain is totally lytic in these same cells, allowing us to identify the genomic region responsible for the persistent phenotype by constructing recombinant mutants between the two viruses [101]. In fact, only a recombinant virus carrying the majority of the capsid protein-encoding region from S11 persists in HEp-2 cell cultures, when cells are infected at a multiplicity of 10^{-2} virions per cell (fig. 3). Genome sequencing revealed that for this recombinant mutant, all of the missense mutations are localized in the VP1 and VP2 capsid protein genes, although silent mutations are spread over the whole region from the *VP2* to the 2A protease gene. The fact that recombinant viruses carrying only either the *VP1* or the *VP2* gene from S11 do not persist under the same conditions suggests that important determinants are localized in both proteins and that they cooperate to produce the persistent phenotype. A more recent study conducted with the type 3 wild-type Leon strain, and a PVpi, L2-2, deriving from it, after 2.5 months of persistent infection in neuroblastoma cells, confirmed that the determinants involved in the phenotype of persistence in HEp-2 cells are also present in the capsid protein-encoding region for this serotype [102].

Two strategies were used successfully to identify the determinants of persistence present in the capsid-encoding region. The first one, used for type 1 PV (S1 strain), was based on the observation that during persistent infection of HEp-2 cells the PVpi become progressively more lytic for HEp-2 cells. This suggested that reversions might occur at positions involved in the persistent phenotype, a fact that was confirmed by sequencing the genomes of mutant viruses isolated after several weeks of persistent infection in HEp-2 cell cultures [103]. The second strategy, used for type 3 PV (Leon and Sabin 3 strains), was based on the assumption that determinants important for PV persistence would be consistently selected, even in the genomes of PVpi derived from different PV strains of the same serotype. Indeed, this was proven to be true when the genomic sequences were compared for two type 3 PVpi [102]. A series of point mutants carrying decreasing numbers of PVpi mutations were constructed for both types 1 and 3, and their phenotypes

of persistence were determined. A cumulative effect was generally observed in function of the number of determinants present in the viral capsid. Further, the study of these point mutants allowed us, in the case of both serotypes, to identify two determinants of persistence, present in the capsid proteins VP1 and VP2 (fig. 4) [102, 103]. However, there are several important differences between the two serotypes. In fact, each type 1 determinant appears to be sufficient, by itself, to confer the persistent phenotype to the S1 strain, while both type 3 determinants are required to confer the capacity to establish persistent infections efficiently to the type 3 Leon strain. Even more important, the localization of the determinants on the three-dimensional structure of the capsid is quite different for the two serotypes, since both type 1 determinants are on the capsid surface, whereas only one of the type 3 determinants is on the capsid surface, the other one being inside the capsid. The fact that type 3 mutants carrying only one determinant do not persist efficiently in HEp-2 cells may be related to the wild-type nature of the Leon strain, which may replicate more efficiently than the S1 attenuated strain in cell culture, and therefore be naturally more lytic, requiring a greater number of determinants in order to persist.

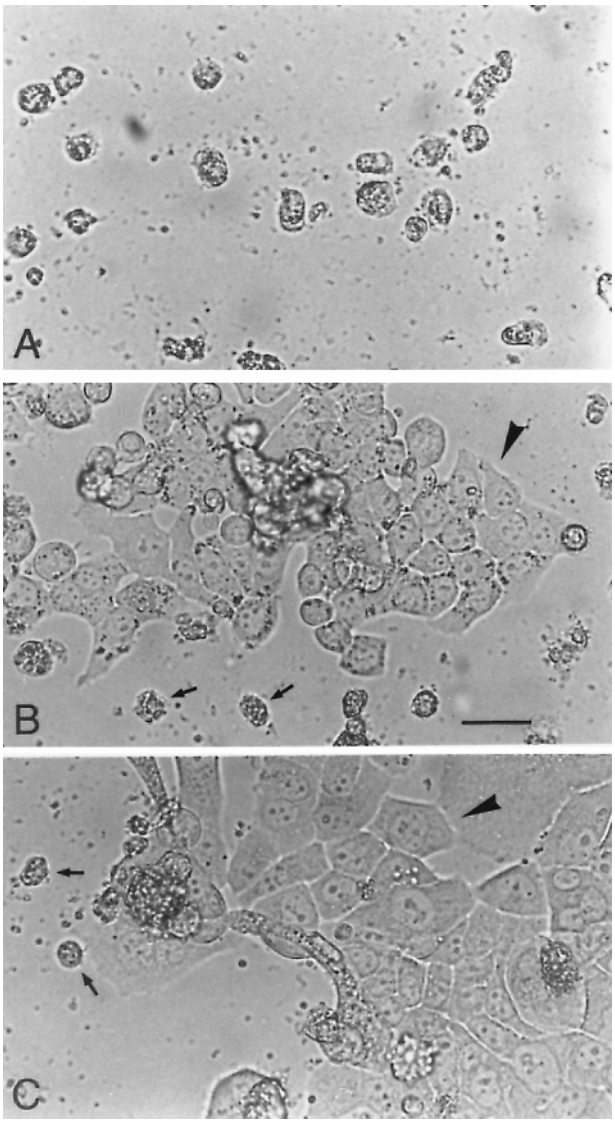

Figure 3. Light micrographs of HEp-2 cell cultures, 3 months after infection with PV strains S1 (*A*); a recombinant between S1 and PVpi S11, carrying the capsid protein genes *VP2*, *VP3* and *VP1* of the PVpi S11 (*B*); and the PVpi S11 (*C*) All S1-infected cells died after infection. The PVpi S11 and the recombinant PV established persistent infections [101]. In cultures of persistently infected cells, patches of growing cells are shown with arrowheads and cells with cytopathic effects are shown with small arrows. The magnification is the same for all three micrographs: bar, 40 μm.

When the multiplication of PV mutants was studied by titrating the virus produced after infection of cells at low multiplicity, that is under the same conditions as those used to establish persistent infections, a delay was observed during the first few days for the persistent mutants (fig. 5) [102, 103]. This was confirmed by looking for viral antigen in infected cells: both the proportion of infected cells and the average amount of viral antigen are lower in cells infected by the type 3 persistent double mutant than in cells infected by the parental lytic strain (fig. 6).

In order to better understand this delay in infection, we chose to study the virus cycle at the molecular level, using the persistent mutants of both serotypes which contain only one or two determinants. The type 1 determinants (table 1) correspond to a His → Tyr substitution at amino acid position 142 of VP2, and a Val → Ile substitution at amino acid position 160 of VP1 [103]. The first one is localized in the canyon and could therefore interact directly with the PVR. The second residue is at the interface between protomers, above a hydrophobic pocket in the canyon floor [73]. It could alter the plasticity and the stability of the capsid, which may be important for liberation of the viral genome during PV entry into the cell. Therefore, both of these determinants may affect the early steps of the PV cycle. The type 3 determinants (table 1) correspond to a Val → Leu substitution at amino acid position 13 of VP2, inside the capsid, and to an Asp → Asn substitution at amino acid position 290 of VP1, in antigenic site 3a, at the capsid surface [102]. The first residue is located near a β-sheet structure, located at the interface between pentamers, which is formed during capsid assembly, and is involved in the stability of the capsid [104]. An amino acid change at this position could possibly modify the conformational changes induced by the receptor during uncoating, or affect virus assembly

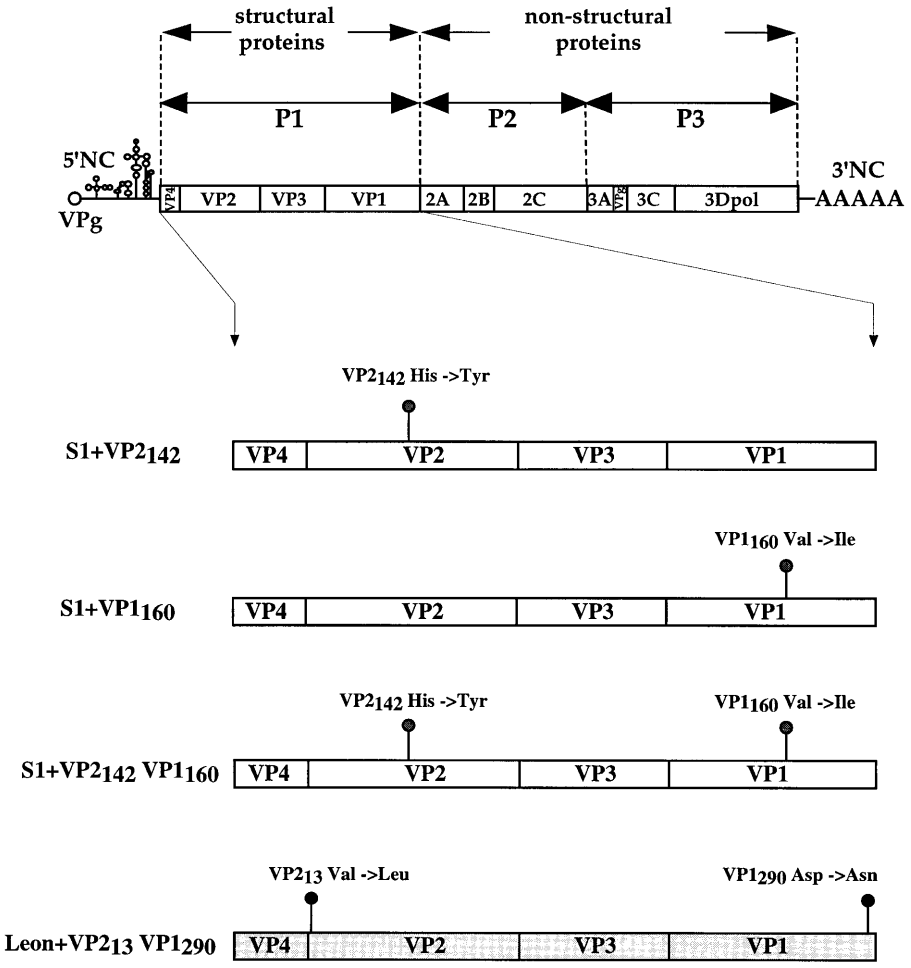

Figure 4. Persistent PV point mutants used to elucidate the molecular mechanisms of PV persistence. The organization of the PV genome is shown in the upper part of the figure. The 5′ and 3′ NC regions flank the single large open reading frame encoding the precursor polyprotein, which is shown as an elongated rectangle. The protein precursors P1, P2 and P3 and the mature proteins are indicated. The small VPg polypeptide, covalently attached to the 5′ end of the genome, the main secondary structures of the 5′ NC region and the poly(A) sequence at the 3′ end of the genome are represented schematically. The capsid protein-encoding region is shown in the lower part of the figure for four persistent PV mutants, in white for the mutants of serotype 1 and in grey for the mutant of serotype 3. The names of the mutants are given on the left-hand side of the figure. The missense mutations differentiating the persistent point mutants from their respective parental strains are indicated by spheres above the VP1 and VP2 capsid protein-encoding genes [102, 103]. NC = noncoding.

late in the cycle. The second residue may be involved in PV-receptor interactions, as it has been shown that such interactions involve not only the residues lining the canyon but also amino acids located in the highly variable, exposed antigenic sites on the capsid surface [105].

Persistent phenotype is associated with modifications of the early steps of the PV cycle

In the case of the type 1 determinants, studies conducted by other groups with these same residues in the wild-type Mahoney strain have clearly shown that they are involved in the early steps of the virus cycle [73, 82]. In the case of type 3 determinants, transfection of infectious RNA, allowing the bypass of the early steps of the PV cycle, indicated that the post-entry steps for the persistent mutants carrying only one or two mutations were comparable to those of the wild-type Leon strain [102]. Single-step growth curve experiments revealed a delay in viral production in the first hours following infection for the persistent viruses, and altogether these results suggested that the early steps are affected for type 3 mutants. We therefore chose to focus on the early steps of the PV

cycle in the hope of elucidating the molecular mechanisms of PV persistence in our HEp-2 cell model.

The early steps of PV infection begin by virus binding to the PVR. It was surprising to find that all type 1 and type 3 persistent point mutants bind more efficiently than the parental viruses, and that each of the determinants contributes independently to this enhanced adsorption, as this is not, a priori, in favour of persistence. Further, when elution of the 135S particles at 37 °C, which is generally considered to be an indicator of PV uncoating, was compared, the efficiency varied depending on the mutant and the serotype. In fact, the type 1 point mutants carrying Ile at position 160 of VP1 (fig. 4) elute much more efficiently than either of the other persistent or lytic viruses. This could certainly contribute to the persistent phenotype by lowering the virus density at the cell membrane. Similarly, the type 1 point mutant carrying Tyr at position 142 of VP2 elutes slightly more efficiently than the lytic S1 strain. In contrast, the type 3 persistent double mutant, as well as each of the corresponding single mutants, elutes less efficiently from the infected cells than the lytic parental virus, and this, like the enhanced adsorption, must be responsible for an increased number of particles at the cell surface. Thus in the case of the type 3 mutants the elution process does not seem to be directly linked to the persistent phenotype [102, 103].

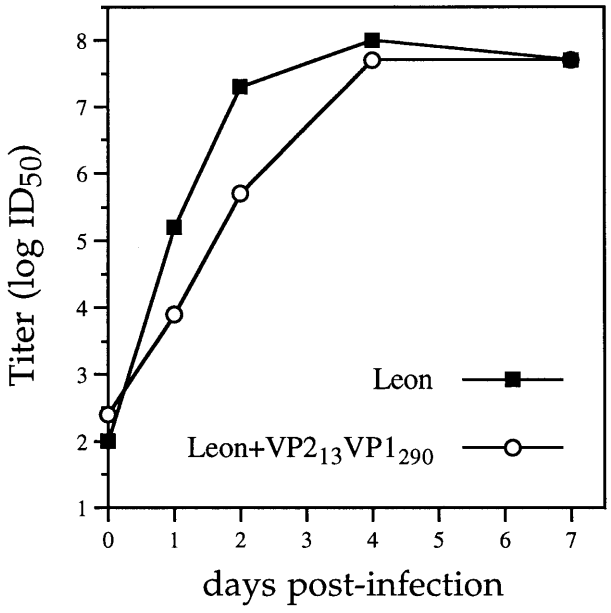

Figure 5. Virus production under the same conditions as those required to establish persistence. Cells were infected with the lytic parental Leon strain and the persistent point mutant Leon + $VP2_{13}VP1_{290}$, at a multiplicity of 10^{-4} infectious doses 50 (ID_{50}) per cell. At the times indicated, intra- and extracellular viruses were pooled and titered. A delay in the production of the persistent mutant is clearly visible in the first 2 days post-infection.

As the modified elution of the mutants indicated that uncoating transitions were modified for both the type 1 and the type 3 PV, we studied the conformational changes in the virus induced by the PVR. In the case of the type 1 persistent mutants, both determinants appear to be responsible for accelerated uncoating in comparison with the lytic S1 strain, as they are each associated with an increased ratio of 135S to 160S particles, as early as 4 min after decapsidation was initiated by a temperature shift from 0 °C to 37 °C. However, it must be noted that the effect is much stronger with the determinant at position VP1-160. Therefore, although the greatly accelerated 160S to 135S transition may be involved in the persistence of the type 1 mutant carrying an Ile at position VP1-160, it appears that a second molecular mechanism must be responsible for the comparable persistence observed with the mutant carrying a Tyr at position VP2-142 [103].

The results obtained with the type 3 persistent double mutant are particularly remarkable, since this virus seems to undergo novel capsid transitions following adsorption, generating a form which sediments with a coefficient of about 147S [102]. Moreover, these capsid transitions occur at 0 °C, whereas the 160S to 135S transitions normally observed require a temperature greater than 32 °C [88]. In fact, the 147S forms are detected as early as 15 min after infection at 0 °C, and we have demonstrated, using PVR-negative and PVR-positive murine cells, that these forms appear specifically upon interaction with the PVR. Neither membrane-bound intact virions of 160S nor cell-associated 135S particles are detected at any time post-infection with the persistent mutant, although empty capsids are found both associated with the cells and in the extracellular medium. Interestingly, the 147S form is found exclusively in association with the cells, indicating that this form is not eluted either. Altogether, these results suggest that the persistence of the type 3 mutant carrying both determinants at positions VP2-13 and VP1-290 is linked to the generation of a novel form of PV, which remains tightly associated with the infected cells [102]. It may be that the typical 160S to 135S capsid transitions do not – or only rarely – occur with this virus.

Our results show that in the case of both serotypes, the viral determinants affect the early steps of the PV cycle (table 1). In particular, it seems that modified PV-PVR interactions are responsible for the persistent phenotype of the mutants, although the enhanced elution and uncoating of the type 1 mutant carrying the determinant at position VP2-142 are most probably not the

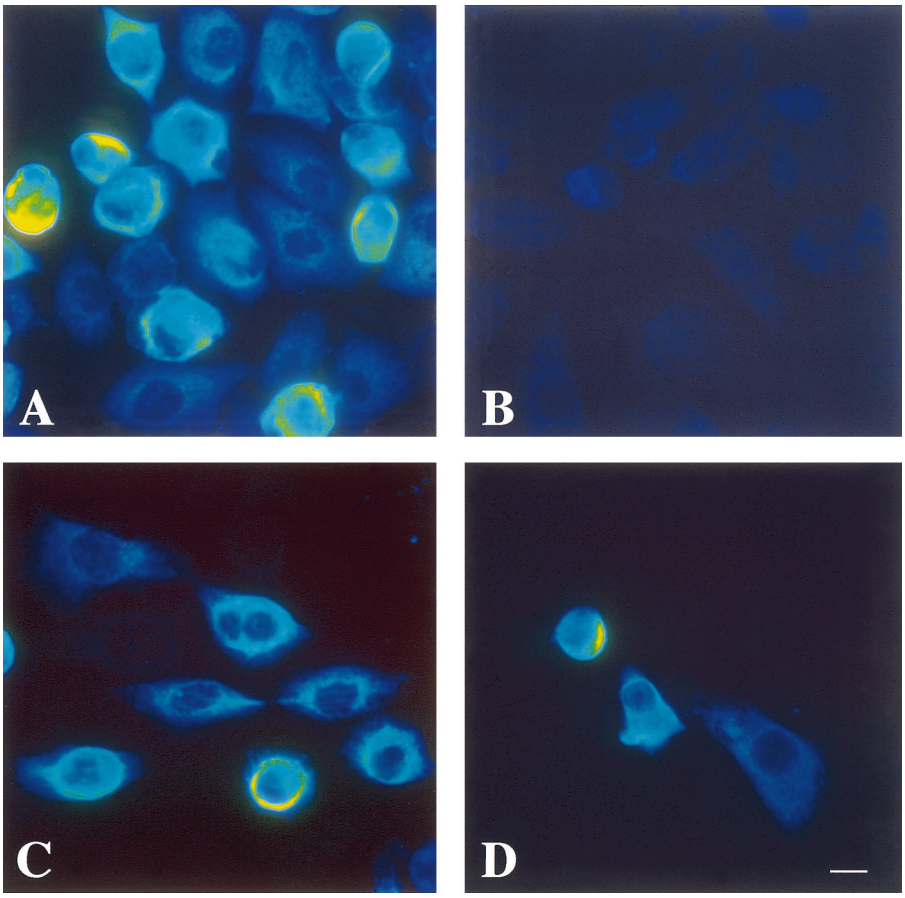

Figure 6. Detection of viral antigen in PV-infected HEp-2 cells by indirect immunofluorescence and confocal microscopy (Image: R. Hellio) HEp-2 cells were infected at a multiplicity of 1 ID_{50} per cell either by Leon (*A* and *B*), by the persistent point mutant Leon + $VP2_{13}VP1_{290}$ (*C*) or by a Leon-derived recombinant PV carrying the capsid protein-encoding region of a type 3 PVpi (*D*) Seventeen hours after infection the subconfluent cells were fixed, and viral antigen was stained with an anti-PV type 3 rabbit serum (*A*, *C* and *D*), using the preimmune rabbit serum as a negative control (*B*) and anti-rabbit fluorescein isothiocyanate (FITC)-labelled goat IgGs. Fluorescence was measured and represented with colours from black (negative) to red (extremely positive). The blue, green and yellow represent intermediate increasing values of fluorescence. The white bar indicates 10 μm. There are more cells positive for viral antigen in HEp-2 cultures infected by the lytic Leon strain (*A*) than in cultures infected with each of the persistent mutants (*C* and *D*).

only mechanisms of viral persistence. In this case it cannot be excluded that late steps, such as capsid assembly, may also be involved. In addition, when the results concerning the two serotypes are considered together, it seems that the generation of the 147S form is not absolutely required for the persistent phenotype, since the two type 1 mutants carrying one or the other of the determinants persist in the absence of any detectable 147S production [103]. Finally, as we have previously described [103], both of the type 1 single point mutants persist less efficiently than the type 1 double mutant carrying the two determinants together. It will therefore be interesting to investigate the molecular mechanisms responsible for this increased persistence. Possibly, a 147S form is also generated upon interaction of the type 1 double mutant with the PVR, as was observed in the case of type 3 PV.

Several questions arise from the results obtained with the type 3 double mutant, concerning the 147S form. First, the role of the 147S form in PV persistence remains to be defined. Second, the question of the precise nature of the 147S form must be addressed. Is it a PV uncoating intermediate which is normally too furtive to be seen but which would be more stable and thus detectable in the case of the persistent mutant? Is it an abortive end product specifically formed by an atypical interaction between the PVR and the mutant virus? Or is it merely a poorly efficient alternative to the 135S

particle? The corresponding hypotheses are illustrated in figure 7 [102]. It is also possible to imagine that if important signals transduced upon PV-PVR interactions are indeed responsible for cell lysis, as has previously been suggested [81], then these signals could be modified when PVR interacts with the persistent viruses, or even with the 147S form, permitting virus production with little or no cell lysis. Experiments are currently underway to distinguish between these possibilities in order to better our understanding of the molecular mechanisms of PV persistence in HEp-2 cells, and perhaps eventually that of PV uncoating.

Importance of the multiplicity of infection

Our results indicate that PVs carrying the viral determinants of persistence, that modify the early steps of the PV cycle, are capable of establishing a persistent infection in HEp-2 cell cultures, although only when infections are initiated at very low multiplicities. Several factors could be responsible for this requirement. First, viral RNA genomes, even after cloning, are always mixed populations of mutants because of the high mutation rate caused by viral RNA polymerases and the lack of an appropriate correction mechanism [106]. The virus dilution required to infect cells at a low multiplicity could allow the elimination of emerging lytic mutants. This is important, since our previous results indicate that the lytic phenotype is dominant over the persistent phenotype during the first days after infection [32]. Second, it cannot be excluded that noninfectious viruses, which may exceed the number of virions by several orders of magnitude in all viral stocks (and thus differ from the DI), contribute to cell lysis. Indeed, the number of particles detectable by electron microscopy always exceeds the number of virions. These defective viruses could also be eliminated by dilution. Finally, we propose that when HEp-2 cell cultures are infected at a high multiplicity by persistent viruses carrying only one or two mutations, the large number of viruses per cell could compensate for their inefficiency during the early steps of the viral cycle, bringing about the lysis of the entire culture in the first hours or days post-infection, as is observed with a lytic virus (fig. 8A). However, when HEp-2 cells are infected by these same persistent viruses at very low multiplicities, that is with one virion per 10,000 cells, the inefficiency of the early steps could eventually be responsible for modified transduction signals as described in the preceding paragraph, or a delayed viral cycle in each infected cell. This could in turn leave the infected cell the time to produce certain factors necessary for its survival, allowing it to become resistant to superinfection by PV. A certain number of these cells could then grow slowly, even in the presence of virus, producing colonies that are visible after several weeks of persistent infection (fig. 8B).

Conclusion

We have identified viral determinants present in the PV capsid which are involved in the establishment of persistent infections in HEp-2 cells in vitro. Moreover, these determinants profoundly affect the early steps of

Table 1. Determinants of PV persistence present in the viral capsid.

Virus serotype	Number of viral determinants necessary for PV persistence	Viral determinants identified (amino acid residue and viral protein)	Location on the three-dimensional structure of the capsid	Step(s) of the viral cycle affected
1*	1	Tyr-142 in VP2 or Ile-160 in VP1	E-F loop of VP2 in the canyon, on the capsid surface β strand E, in the interface between protomers on the capsid surface	receptor-binding and receptor-mediated conformational changes (see text for full details)
3†	2	Leu-13 in VP2 and Asn-290 in VP1	amino-terminal region of VP2, on the inside of the capsid, at the interface between adjacent pentamers carboxy-terminal region of VP1, exposed at the surface in neutralization antigenic site 3a	

*The type 1 viral determinants were identified in the genome of a PVpi, S11, derived from the S1 strain. †The type 3 viral determinants were identified in the genome of a PVpi, L2-2, derived from the Leon strain.

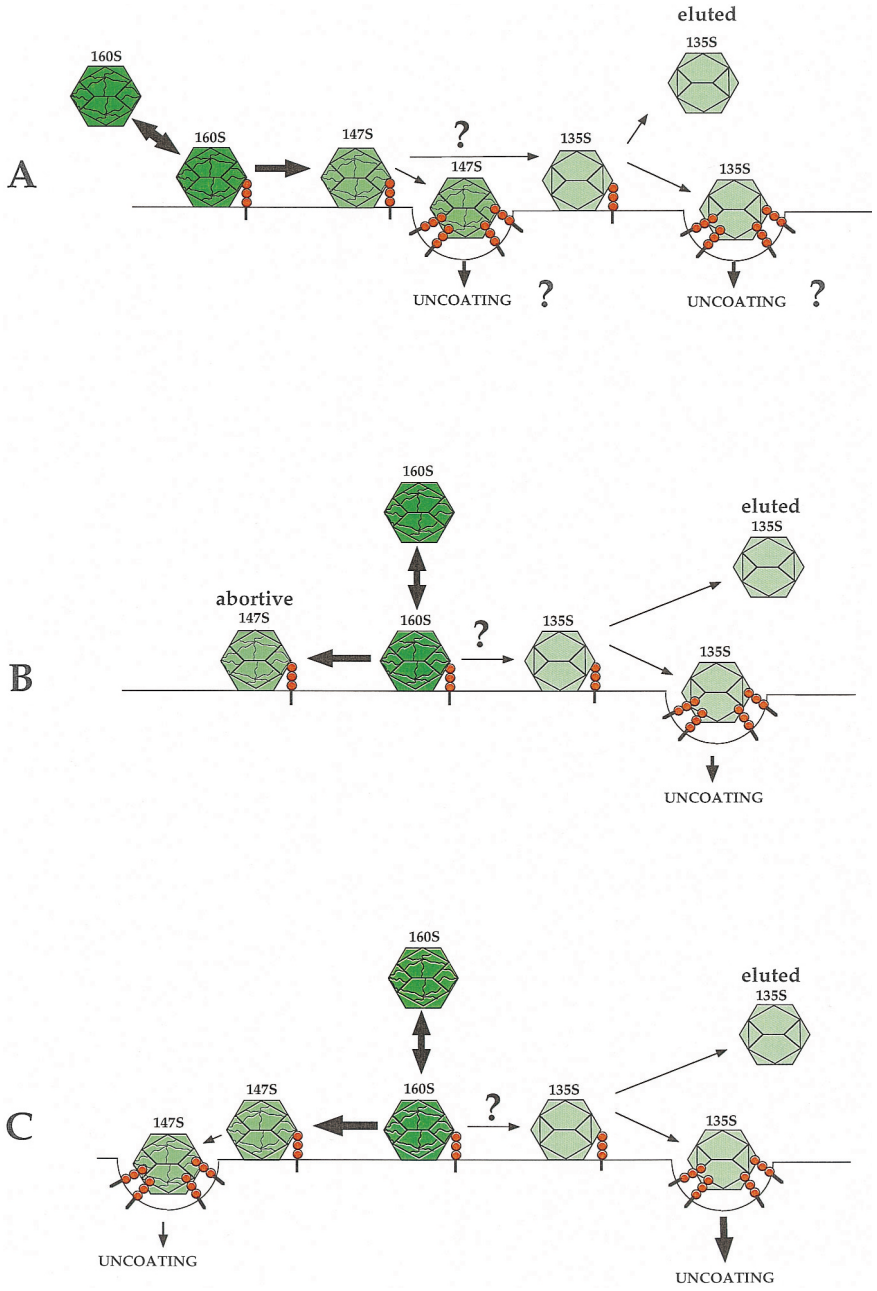

Figure 7. Three possible roles in PV uncoating for the 147S form detected after contact between the persistent mutant Leon + $VP2_{13}VP1_{290}$ (in green) and the PV receptor (in red). The thick and the thin arrows represent the more and the less frequent transitions, respectively. (*A*) The 147S form could be a true viral entry intermediate, which would be much more stable for the persistent mutant than for lytic PV strains and therefore detectable. The 147S form of the persistent mutant would be defective, thereby delaying the penetration and uncoating steps. (*B*) The 147S form could be a nonfunctional conformation of the capsid, specific to the persistent mutant, preferentially generated by PV-PVR interactions. This abortive end product would reduce the efficiency of the normal 160S to 135S uncoating transitions. (*C*) The 147S form could be a poorly efficient alternative to the 135S particle, preferentially produced upon interaction of the persistent mutant with the PV receptor and once again responsible for inefficient viral uncoating. The schematic representation of the 160S to 135S transitions is adapted from ref. 80. These three hypotheses have been discussed in greater detail elsewhere [102].

the PV cycle. This finding is an interesting one, as a link between viral persistence and modification of the early steps of virus multiplication has also been described in the case of reovirus [36]. However, with reovirus, viral determinants affecting the early steps, and in particular viral disassembly, seem to be involved in the maintenance rather than the establishment phase of the persistent infection. Finally, with the murine hepatitis virus, the early steps of the virus cycle are limiting and therefore favourable to the establishment of persistence [107]. However, in this case, the persistence depends on the adaptation of the virus to modified or new receptors.

Perspectives

At the present time, very little is known about persistent PV infections in vivo, and in fact, the link between the PPS and PV persistence remains hypothetical. However, if PV sequences should ever be repeatedly isolated from PPS patients, it would be interesting to determine whether or not mutations present in these sequences correspond to the determinants which we have identified in the PV capsid during persistent infection in vitro. Further, the construction of infectious viruses carrying these sequences could eventually permit us to evaluate the validity of our in vitro models in comparison with what happens during PV persistence in vivo.

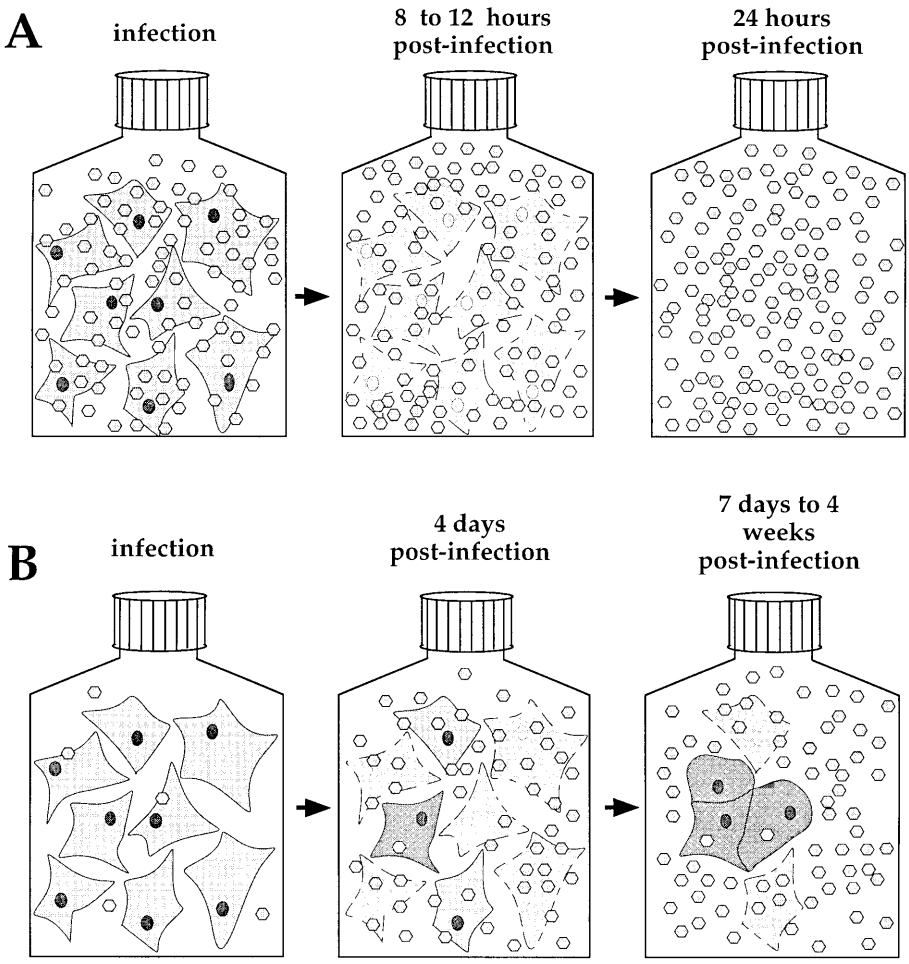

Figure 8. Effect of the multiplicity of infection on the establishment of persistent infections in HEp-2 cell cultures. (*A*) When cells are infected by a persistent point mutant (e.g. Leon + $VP2_{13}VP1_{290}$) at a high multiplicity, the large number of viruses (small hexagons) per cell compensate for their inefficiency during the early steps of the viral cycle, thereby bringing about the lysis of the entire culture within a few days. (*B*) In contrast, when cells are infected by the same persistent virus, but at a very low multiplicity of infection, the modified and inefficient early steps would be responsible for a delayed viral cycle and/or modified transduction signals. The infected cell would produce factors further limiting the viral cycle, allowing cell survival (dark grey). Cells increasingly resistant to PV infection would then arise due to cell selection by the virus.

Taken together, the studies of PV-host cell interactions in vitro have revealed that, for the same virus, the mechanisms of viral persistence may vary considerably with the origin and the differentiation state of the cells (neuronal/nonneuronal), the virus strain (PV/PVpi and PV mutants) and the phase of the persistent infection (establishment/maintenance). Despite this diversity, it appears that very often the early steps of the viral cycle are modified, and this may involve viral as well as cellular determinants, such as the PVR [97]. Indeed, the level of PVR expression is frequently lower in neuroblastoma and HEp-2 cells persistently infected by PV [32, 97]. It is important to note that the viral determinants responsible for the establishment of persistent infections in HEp-2 cells were initially selected in the genome of PVpi, that is during the persistent infection of human cells of neuronal origin [53, 55]. The fact that the same type 1 determinants were selected when PV was grown in murine cells expressing a mutant PVR [73] suggests that the PVR present at the surface of neuroblastoma cells could be slightly modified. This hypothesis is currently being studied in the laboratory. Concerning the viral determinants of persistence, although it appears that there is not a strict correlation between the persistent phenotype of a PV mutant and the newly identified capsid transitions of 160S to 147S, observed upon interaction with the PVR, it remains possible that this transition facilitates the establishment of persistence. It will be particularly interesting in the future to further characterize this particle in order to understand its possible roles in the early steps of the PV cycle and in PV persistence.

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