Identification of a human cytomegalovirus mutant in the pp150 matrix phosphoprotein gene with a growth-defective phenotype

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Following amplification by PCR of a portion of the matrix phosphoprotein pp150 gene, electrophoretic analysis revealed the simultaneous presence of two viral variants of human cytomegalovirus in the blood of a heart transplant recipient. Repeated denaturation-anealing cycles during the amplification reaction led to the formation of heteroduplex molecules with altered electrophoretic mobility. Sequence analysis of the amplification products showed the presence of a viral variant carrying an in-frame three nucleotide deletion, which caused the absence of an aspartic acid in the corresponding protein. Attempts to plaque-purify the deletion mutant were unsuccessful, suggesting that the variant was growth-defective.

Human cytomegalovirus (HCMV), a β-herpesvirus, is an important opportunistic pathogen capable of producing severe disseminated infections and organ syndromes in immunocompromised individuals, such as AIDS patients or transplant recipients (Gorensek et al., 1988; Saltzmann et al., 1988). Although serological methods capable of differentiating and classifying field isolates of HCMV are still lacking, viral variants can be differentiated on the basis of genomic variability. Use of techniques capable of revealing viral DNA polymorphisms has made it possible to demonstrate the simultaneous infection of single immunocompromised (AIDS) patients by different HCMV strains. DNA restriction analysis revealed differences in HCMV strains isolated from different organs in the same patient (Drew et al., 1984; Spector et al., 1984) or in multiple strains simultaneously isolated from the same patient’s blood (Gerna et al., 1992).

During a study conducted to verify whether a pair of PCR primers relevant to an internal portion of the HCMV gene for the matrix phosphoprotein pp150 could be used for amplification of a number of isolates (Zipeto et al., 1990, 1992; Gerna et al., 1991), we detected the simultaneous presence of two viral variants in an isolate (VR3010) from peripheral blood leukocytes of a heart transplant recipient. The two variants were revealed by the presence of two bands in PAGE after PCR amplification. One of the bands corresponded to the size (257 bp) expected from the sequence of the reference strain AD169 (Jahn et al., 1987a), whereas the other corresponded to a larger (350 to 380 bp) segment (Fig. 1a). Both amplification products were specifically recognized by an internal pp150 oligonucleotide probe in a Southern blot hybridization experiment. The presence of both the standard (O) and the slower (H) band was confirmed in other sequential isolates from the same patient. In addition, the two bands (with approximately constant relative intensity) could be detected after PCR amplification in a series of sequential samples of polymorphonuclear leukocytes from the same patient (data not shown).

Restriction analysis of the two amplified segments separated on polyacrylamide gel seemed to indicate the presence in the H segment of a 100 to 120 bp insertion between the TaqI and BstNI sites (Fig. 2). The putative insertion was suggested by the slower migration of the TaqI–BstNI restriction fragment from the purified H segment, with respect to the corresponding fragment from the O segment. When the PCR amplification products were analysed by electrophoresis on agarose gel, instead of polyacrylamide gel, the two bands migrated very close to each other (Fig. 1b). Taken together, these results suggested that the sequence difference between the H and O segments induced a conformational difference, rather than a difference in length. This resulted in a different electrophoretic migration velocity which was best revealed by PAGE rather than agarose gel electrophoresis.

When O and H segments separated by PAGE were individually cloned in Bluescribe, excised and analysed again by PAGE, only the O band was detected. The sequence of the cloned segments was then determined by the dideoxynucleotide chain termination method, with the Sequenase kit (version 2.2, U.S. Biochemical
Fig. 1. Polyacrylamide (a) and agarose gel (b) electrophoresis of the amplification product of a segment of the ppi50 gene from reference strain AD169 (lanes 1), polymorphonuclear leukocytes of the immunosuppressed patient (lanes 2) and a DNA-negative control (lanes 3). Two bands of different mobility (O, standard and H, slower) are clearly visible in (a) lane 2, whereas in (b) lane 2 the two bands practically comigrate. M, Size markers (pBR322-HaeIII).

Corporation) using [α-35S]dCTP, with both forward and reverse primers. Sequence analysis showed the presence in both H and O clones of two different sequences, one perfectly corresponding to the published sequence of the reference strain AD169 (Jahn et al., 1987a), the other with a three nucleotide deletion. The deletion was in phase with the original open reading frame and caused the absence of an aspartic acid in position 719 of the coded protein (Fig. 2). Sequencing data were therefore in agreement with the conformational hypothesis. Two viral populations were simultaneously present in the patient under study, one with a normal copy of the gene coding for ppi50, and the other containing a small deletion. This was confirmed by cloning and sequencing the amplification products obtained from two other viral isolates from the same patient, one recovered 1 week prior to, and the other 2 weeks after VR3010 isolation. Repeated cycles of denaturation, annealing and extension during PCR amplification gave rise to the formation of normal homoduplexes (N/N), deleted homoduplexes (D/D) and heteroduplexes (N/D). It is known that extra bases stacked into the double helix produce a wedge that kinks the DNA (Hsieh & Griffith, 1989; Rice & Crothers, 1989; Wang & Griffith, 1991). Thomas et al. (1986) reported that a three base deletion in one strand of a 268 base pair DNA fragment produced a marked retardation in its mobility, suggesting that the three base loop produced a kinked molecule. It is plausible then to conclude that the small three nucleotide loop occurring in heteroduplexes introduced a curvature in the DNA molecule that considerably reduced its migration velocity in PAGE.

In order to confirm this interpretation, the purified O fragment was denatured at 98 °C for 10 min and then renatured at 70 °C for 90 min. PAGE showed that both the slower (H) and the faster (O) bands were present after renaturation. A similar result was obtained when the purified H fragment was treated and analysed in the same way (Fig. 3). These results could be explained by assuming that fragment O contained both N/N and D/D homoduplexes. Denaturation separated the homoduplexes and subsequent annealing created both homo- and heteroduplexes. Fragment H, on the other hand,
contained only heteroduplexes, which were separated by
denaturation and gave rise to formation of both hetero-
and homoduplexes upon annealing. This interpretation
was further confirmed by the separate re-amplification of
purified O and H bands. Each of them gave rise to both
the slower and the faster electrophoretic bands in PAGE.

In order to compare the whole genomes of the wild-
type and of the deleted strain by restriction analysis, we
attempted to isolate the two viral variants present in
isolate VR3010 by plaque purification. DNAs from
individual plaques were then amplified by PCR with the
same primers and run on PAGE, in order to identify pure
clones. Out of 50 plaques analysed in this way, 47
contained the pure clones, whereas three were still mixed,
containing both the wild-type and the variant. The
amplification products from pure clones were then
challenged (by co-denaturation and annealing) with the
amplification product (using the same primers) of the
reference strain AD169. This procedure showed that
whenever pure clones were isolated, they were always of
the wild-type. We never found a pure clone of the variant
strain.

Since a direct comparison between isolated variant
and wild-type genomes could not be performed, we
carried out a comparative restriction analysis of isolate
VR3010 (containing both wild-type and variant) and a
pure wild-type clone. DNA from viral isolates was
extracted and digested with four different restriction
enzymes (EcoRI, HindIII, PstI, BglII). Digested DNAs
were electrophoresed in 0.7% agarose gel and transferred
onto a nylon membrane. Restriction patterns were
revealed by Southern blot hybridization (Chandler &
McDougall, 1986) using four 32P-labelled subgenomic
fragments cloned in cosmids (pCM1015, pCM1049,
pCM1058, pCM1075) and spanning almost the entire UL
fragment of the HCMV genome (Fleckenstein et al.,
1982). The patterns from the wild-type and the VR3010
isolate were overlapping. However, both strains differed
significantly from the reference strain AD169 (data not
shown).

Considering the high genomic variability among
unrelated field isolates of HCMV (Chandler &
McDougall, 1986), it is possible to conclude that the
wild-type and the variant strain in isolate VR3010 are
closely related. It seems plausible to speculate that the
variant originated by mutation from the wild-type.
Furthermore, considering our failure to plaque-purify
the variant strain, it seems reasonable to conclude that
the deleted variant is growth-defective and can only be
propagated in the presence of co-infecting wild-type. It is
interesting in this respect to note that the presence of
the defective genome seems to inhibit the proliferation of
the wild-type itself, since in mixed plaques the c.p.e. occurred
much later than in pure wild-type clones.

Finally, we observed that serial passages of mixed
clones caused a reduction of the mutant virus population
(Fig. 4). This could be explained by the lack of infectivity
of the mutant strain. If this were the case, only cells
infected either by the wild-type virus or simultaneously
by both the wild-type and the mutant virus would have
produced new viral particles. Cells infected only by the
mutant virus would not produce viral progeny, resulting
in a progressive reduction of the mutant fraction in the
viral population. Why in vivo the two viral variants are
present in approximately the same proportion, whereas
in vitro the deleted mutant is unable to propagate
independently to any appreciable extent, remains to be
determined.

Our data do not allow conclusions to be drawn on the
role of the identified mutation of matrix phosphoprotein
pp150 in the defective phenotype; however, our findings
indicate a strong association between pp150 mutation
and the growth-defective viral phenotype observed. The
gene for HCMV matrix phosphoprotein pp150 is located
in the UL segment of the viral genome (Jahn et al., 1987a)
and codes for a 1048 amino acid polypeptide. Computer
analysis indicates that the protein possesses highly
hydrophilic regions, often including β-sheets (Mach
et al., 1989). The protein is strongly basic, contains many
proline residues and is highly phosphorylated. Together
with the phosphoprotein pp65, pp150 is the main con-
stituent of the viral matrix. The corresponding gene is
expressed in the late phase of infection (Jahn et al., 1987b; Scholl et al., 1988). The coding nucleotide sequence does not show significant homologies with open reading frames of other herpesviruses (Jahn et al., 1987a). The deletion described here occurs at a highly hydrophilic region, located on the protein surface. The region is preceded by an α-helix and followed by a β-sheet (analysis performed with Geneworks for Apple Macintosh, Intelligenetics Incorporated). It is possible that the mutation site is relevant to the structure of the corresponding protein, and that an altered pp150 structure can somehow inhibit viral replication. Further studies on this mutant could give useful information about the role of pp150 in the viral life cycle and on the structure-function relationship of this important viral component. The simple procedure described here (amplification followed by PAGE) could be of general use to reveal small deletions that would not be detected by standard restriction analysis. Polymorphisms of this kind could be easily demonstrated in genes of interest in diploid organisms.

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