Cells surviving infection by human immunodeficiency virus type 1: vif or vpu mutants produce non-infectious or markedly less cytopathic viruses

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Under conditions in which a clonal cell line (M10) isolated from a human T cell lymphotrophic virus type I-transformed MT-4 cell line was completely killed by infection with wild-type human immunodeficiency virus type 1 (HIV-1), equivalent M10 cells survived infection with HIV-1 vif, vpr or vpu mutant virus after transient cytopathic effects. Several cell clones, which were isolated from the proliferating M10 cells after infection with vif and vpu mutant viruses (M10/vif- and M10/vpu-), had heterogeneous HIV-1 phenotypes in terms of HIV-1 antigen expression, their syncytium forming capacity, reverse transcriptase activity and the infectivity of HIV-1 particles produced. When the replication kinetics of the HIV-1 particles produced were assayed in M10 cells, the clones could be classified into three types, i.e. type I producing non-infectious HIV-1 with low replicative ability and type III producing infectious HIV-1 with a replicative ability similar to that of wild-type HIV-1. HIV-1 major viral cell proteins and virus particle fractions were almost typical in types II and III but not in type I. Electron microscopic examination of particles released by I, II and III clones revealed rare defective, predominantly defective and essentially normal virions, respectively. Northern and Southern blot analyses revealed no apparent deletion in the proviral DNA and mRNA prepared from these clones, except in the case of type I and II clones isolated from M10/vpu- which contained large deletions in the mRNAs for gag and gag-pol proteins. Thus, M10 cells surviving infection with HIV-1 vif or vpu mutants are heterogeneous, persistently expressing HIV-1 antigens and producing non-infectious or less cytopathic virus.

Introduction

Genetic variability is a characteristic feature of human immunodeficiency virus type 1 (HIV-1), the aetiological agent of AIDS (Benn et al., 1985; Alizon et al., 1986; Spire et al., 1989). The time course of the disease is slow and involves the persistence of virus infection. At primary infection, there is an initial viraemia, which is followed rapidly by antibody response and viral clearance (Allain et al., 1986; Goudsmit et al., 1986; Gaines et al., 1987). However, protective immunity is not established, and signs of active infection reappear after an average of 9-8 years (Bacchetti & Moss, 1989). Progression to AIDS correlates with the reappearance of circulating viral proteins (Goudsmit et al., 1986; Paul et al., 1987). In addition, the isolation of infectious HIV-1 is more difficult from asymptomatic carriers (AC) than from individuals in later stages of the diseases (Äsjö et al., 1986; Evans et al., 1987; McElrath et al., 1989). Nevertheless, the presence of HIV-1 infection has been demonstrated in all consecutive HIV-1-seropositive cases, regardless of their clinical stage, by the polymerase chain reaction (PCR) which does not distinguish active from latent infection or defective from replication-competent virus (Jackson et al., 1990). Thus, the characteristic feature of HIV-1 infection is the variability of the persistence period in AC between seroconversion and the development of AIDS. In particular, it is not known whether there are cells, persistently infected and harbouring the HIV-1 genome, which produce defective virus and/or little or no infectious virus. Such cells could contribute to the evolution of infection if specific events in the AC could lead to activation of the persistent state into a productive one (Ho et al., 1987; Fauci, 1988).

We have reported an abnormal HIV-1 life cycle in persistently HIV-1-infected cells obtained after infection of human T cell lymphotrophic virus type I (HTLV-I)-transformed MT-4 cells or its clonal derivative M10 with HIV-1 (Ikuta et al., 1988; Yunoki et al., 1991). The infected MT-4 and M10 cells were drastically reduced in number by single-cell killing rather than by the induction of syncytia. However, the few surviving cells had growth
rates similar to those of uninfected M10 cells and also expressed HIV-1 antigens. About 85% of cell clones isolated from these persistently HIV-1-infected MT-4 or M10 cells (MT-4/HIV-1 or M10/HIV-1, respectively) did not produce infectious HIV-1 particles (Yunoki et al., 1991). Thin-section electron microscopy revealed many abnormal morphologies among the HIV-1 particles released from these cell clones (Ikuta et al., 1988; Goto et al., 1990; Morita et al., 1990). In addition, structural analysis of HIV-1 DNA by Southern hybridization revealed various kinds of proviral deletions in the cell clones (Imai et al., 1991). Recently, we have shown that infection with HIV-1 vif, vpu or vpr mutants produced surviving and HIV-1 antigen-expressing M10 cells (M10/vif', M10/vpu' or M10/vpr' respectively) after transient cell lysis by the infection, whereas infection with nef mutants or wild-type HIV-1 completely killed the cells (Nishino et al., 1991). Here we describe the isolation and characterization of several cell clones isolated from M10/vif' and M10/vpu'.

Methods

Cells and viruses. A clonal cell line, M10 (Yunoki et al., 1991) isolated from an HTLV-I-transformed MT-4 cell line (Miyoshi et al., 1982), was cultured in complete medium (RPMI 1640 medium supplemented with 10% foetal bovine serum) at 37 °C in a CO₂ incubator. HIV-1 mutants in vif (pNL-Nd) and vpu (pNL-Ss) were prepared as described previously (Nishino et al., 1991) from the pNL4.3 plasmid containing full-length HIV-1 DNA (Adachi et al., 1986). Plasmids pNL-Nd and pNL-Ss were constructed by inserting frameshift mutations at the NdeI and Sspl restriction sites. Vif and vpu proteins in pNL-Nd and pNL-Ss consisted of only 28 and 32 N-proximal amino acids. The conditioned media from SW480 cells transfected with these plasmids as described previously (Adachi et al., 1987) were used as HIV-1 inocula after filtration through a 0.22 pm membrane filter. HIV-1-infected M10/vif' and M10/vpu' cells, which had survived infection with the conditioned media from SW480 cells (Nishino et al., 1991), were cultured in complete medium. M10/vpr' cells were also isolated. These had survived infection with conditioned media from SW480 cells transfected with pNL-AF2 (Nishino et al., 1991). This plasmid was constructed from pNL4.3 by inserting a frameshift mutation at the AfllI restriction site (Ogawa et al., 1988).

MOLT-4/HTLV-IIB and MOLT-4/LAV-I cells, obtained by serial passage of the acute lymphocytic leukaemia-derived cell line MOLT-4 (Minowada et al., 1972) after infection with both the HTLV-IIB (Popovic et al., 1984) and LAV-1 (Barré-Sinoussi et al., 1983) strains of HIV-1, were also cultured in complete medium and used as controls.

Isolation of cell clones. Cell clones were isolated from M10/vif', M10/vpu' and M10/vpr' cells by limiting dilution in a 96-well microplate (flat-bottom; Corning) which contained a single cell/well in RPMI 1640 supplemented with 20% foetal bovine serum and cultured for 2 to 3 weeks at 37 °C in a CO₂ incubator.

Immunofluorescence (IF) test. Indirect IF tests of cell smears fixed with cold acetone were carried out as described previously (Ikuta et al., 1989) using either 500-fold serum dilutions from an HIV-1-seropositive subject (IF titre 1:4096) or culture fluid from a hybridoma clone producing mouse monoclonal antibodies (MAbs) to HIV-1 gag p24 (V107) or p18 (V17) (Ikuta et al., 1989). The membrane IF (MIF) test with OKT4 (Ortho-mune) for cell surface CD4 expression was performed as described previously (Ikuta et al., 1989).

HIV-1 infectivity titration. HIV-1 infectivity in the conditioned medium was titrated in M10 cells in a 96-well microplate (flat-bottom; Corning) as described previously (Ikuta et al., 1987). Briefly, M10 cells infected with serial 10-fold dilutions of conditioned media were cultured for 5 days at 37 °C in a CO₂ incubator. HIV-1-specific antigens were identified by an IF test using serum from the HIV-1-seropositive subject, and titres were expressed as TCID₅₀/ml.

Reverse transcriptase (RT) activity assay. RT activity in the conditioned medium was assayed as described previously (Ogawa et al., 1989). Briefly, the medium was mixed with a reaction mixture containing poly(rA).oligo(dT) and [α-³²P]TTP (800 Ci/mm; New England Nuclear). After incubation for 3 h at 37 °C, the mixture was dotted onto DEAE filter paper (Whatman DE81). RT activity was visualized on X-ray film.

Syncytium formation assay. Syncytium formation was assayed by incubation of the CD4-positive human T cell line, MOLT-4 clone no. 8 (Kikukawa et al., 1986) with HIV-1-infected cells at a ratio of 10:1 as described previously (Somasundaran & Robinson, 1987). After incubation for 24 h at 37 °C, the formation of giant cells was observed. Relative percentages of syncytia were calculated from the average of triplicate assays.

Immunoprecipitation and SDS-PAGE. Cells were labelled for 16 h with 100 μCi/mg of L-³²P methionine and 18% cysteine (1170 Ci/mm; New England Nuclear) in RPMI 1640 medium containing one-tenth the normal concentrations of methionine and cysteine. A cell-free fraction was prepared by low-speed centrifugation, followed by centrifugation of the supernatant at 35000 r.p.m. for 1 h in an SW50.1 rotor (Beckman) to pellet virus particles and give a supernatant containing the soluble protein fraction. The cell and virus particle lysates and the soluble protein fraction diluted with lysis buffer (0.5% NP40, 0.5% sodium deoxycholate, 0.05 M-Tris-HCl pH 7.2, 0.1 M-NaCl, 1 mM-EDTA, 1 mM-PMSF) were immunoprecipitated with the serum from an HIV-1 seropositive subject or V107 MAb as described previously (Ikuta & Luftig, 1986). Proteins in the immunoprecipitates were analysed by SDS-PAGE (separation gel, 10 to 15% linear gradient polyacrylamide gel; spacer gel, 4% gel) as described previously (Ikuta et al., 1989).

DNA and mRNA structural analyses. DNA and poly(A)+ RNA fractions were prepared from cells as described previously (Chomczynski & Sacchi, 1987; Imai et al., 1991). Southern and Northern blot hybridizations were carried out as described previously (Imai et al., 1991). A ³²P-labelled HindIII DNA fragment of HIV-1 DNA encompassing almost the entire HIV-1 genome (nucleotides 531 to 9606) of pNL4.3 was used as a probe. The CD4 probe was a ³²P-labelled BamHI DNA fragment encoding the soluble extracellular 368 amino acids of CD4 (Morikawa et al., 1990). The β-actin probe was a ³²P-labelled HindIII DNA fragment (about 400 bp) of the human β-actin gene (Wako Pure Chemical Industries). For Northern blot hybridizations, the same filter which blotted with poly(A)+ RNA was used after each probe was removed. PCR was carried out for 30 cycles, each consisting of 2 min of denaturation at 94 °C, 1.5 min of annealing at 55 °C, and 2.5 min of extension at 72 °C using a PCR kit (Perkin Elmer Cetus) with two sets of primers (nucleotides 3267 to 3288/6320 to 6344 and 4368 to 4390/6320 to 6344) as described previously (Saiki et al., 1988). PCR was carried out with a third set of primers (nucleotides 7617 to 7641/7799 to 7820) under the same conditions with the exception that extension was for 1 min at 72 °C. The DNA fragments amplified by the latter primers were directly sequenced by the method of Engels et al. (1988) using a ³²P-labelled synthetic oligonucleotide (representing bases 7691 to 7712) as a primer.
Electron microscopy: Ultrathin sections of cells were examined using a Hitachi H-800 electron microscope as previously described (Goto et al., 1990).

Results

Cell clones isolated from M10/vif− and M10/vpu− cells

Clones of M10/vif−, M10/vpu− and M10/vpr− cells, obtained as survivors after infection of M10 cells with vif, vpu and vpr mutants (Nishino et al., 1991), were generated by limiting dilution in 96-well microplates. HIV-1 antigen, detected using the IF test with serum from an HIV-1-seropositive subject, was expressed in all clones obtained from M10/vif− and M10/vpu− cells, but not in any clone from M10/vpr− cells. Seven clones were isolated from M10/vif− and M10/vpu− cells and designated Vif-1 to Vif-7 and Vpu-1 to Vpu-7. Data on the characterization of these clones are summarized in Table 1.

The synthesis of HIV-1 gag antigens was detected using an IF test employing MAbs to p24 (V107) and p18 (V17) in all of these clones, with the exception of Vif-1 which failed to react with either MAb and Vif-2 which reacted only with V107. In contrast, cell surface CD4 antigen expression using the MIF test with OKT4 was detectable in about 80% of Vif-1, but not in the other clones. However, Northern blot analysis with 32P-labelled CD4 cDNA as a probe revealed that representative clones expressed CD4 mRNA (Fig. 1a). A similar rate of expression of β-actin mRNA was observed in the same filter as for CD4 mRNA (Fig. 1b). The syncytium forming capacity of the clones isolated from M10/vif− cells was generally lower than those isolated from M10/vpu− cells. Conditioned media from clones seeded at 5 x 10⁵ cells/ml were harvested after being cultured for 3 days, and examined for both RT activity and HIV-1 infectivity. Strong RT activity was detected in the conditioned media from Vif-5, Vif-6 and Vpu-6. In the case of Vif-4, Vif-7 and Vpu-7, RT activity was lower but detectable, whereas that from the other clones was at the level given by conditioned medium of uninfected M10 cells. In contrast, significant infectivity titres were detected by the regular assay method in the conditioned media of Vif-5, Vpu-6 and Vpu-7, but not in the media from the other clones. HIV-1 replication was next examined in these clones over 11 days in culture. For this, M10 cells were infected for 1 h with conditioned media from these clones. After washing with complete medium, infected cells were seeded at 5 x 10⁵ cells/ml in complete medium and the cell density was adjusted to 5 x 10⁵ cells/ml every 4 days thereafter. RT activity in the conditioned media from these infected cells was measured daily for the first 8 days in culture and again at 11 days after infection (Fig. 2). The results revealed substantial differences in the rate of appearance of RT

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>IF with*</th>
<th>MIF with OKT4</th>
<th>Syncytium formation</th>
<th>RT activity†</th>
<th>Infectivity (TCID₅₀/ml)†</th>
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<tr>
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<tr>
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<td>+</td>
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<tr>
<td>MO/LAV‡</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>10⁶</td>
<td>&lt;10⁰⁵</td>
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* IF test with the serum of an HIV-1-seropositive subject (Pa.) and MAbs to HIV-1 gag p24 (V107) and p18 (V17).
† The conditioned media from cells seeded at 5 x 10⁵ cells/ml and harvested after culture for 3 days were used for RT activity and HIV-1 infectivity.
‡ MOLT-4/HTLV-IIIB.
§ MOLT-4/LAV-1.
Fig. 1. Expression of CD4 mRNAs in cell clones isolated from M10/vif- and M10/vpu- cells. The poly(A)^\prime^ RNAs extracted from Vif-1, Vif-7, Vif-5, Vpu-4, Vpu-5 and Vpu-6 cells (lanes 1 to 6) were subjected to electrophoresis on a 1% agarose gel. A filter blotted with these mRNAs was hybridized with 32P-labelled DNA probes for (a), CD4 and (b) human \(\beta\)-actin. Poly(A)^\prime^ RNA extracted from MOLT-4/LAV-1 cells (lanes 7) was used as a control.

Attenuation of HIV-1 particles produced by clones isolated from M10/vif^- and M10/vpu^- cells

The correlation between the replicative and cell killing activities of the HIV-1 particles produced by representa-
tive clones isolated from M10/vif^- and M10/vpu^- cells was examined in M10 cells (Fig. 3). Clones Vif-1 and Vpu-4 as type I, Vif-7 and Vpu-5 as type II and Vif-5 and Vpu-6 as type III were selected as representative of producers of non-infectious HIV-1, infectious HIV-1 with low replicative ability, and infectious HIV-1 with rapid replicative ability, respectively. M10 cells were infected with the conditioned media from these cell clones at 37°C for 1 h. Conditioned medium from SW480 cells which had been mock-transfected or transfected with either pNL432, pNL-Nd or pNL-Ss were used as controls for mock infection or wild-type HIV-1, vif mutant and vpu mutant infection, respec-

Fig. 2. Kinetics of the appearance of RT activity in the culture medium of M10 cells infected with HIV-1 particles produced by cell clones isolated from M10/vif- and M10/vpu- cells. M10 cells (2.5 × 10^6 cells) were infected for 1 h at 37°C with culture media (1.5 ml) from cell clones Vif-1 to Vif-7 and Vpu-1 to Vpu-7. As a control, M10 cells were infected with the culture medium from MOLT-4/LAV-1 (MO/LAV). After being washed with complete medium, the M10 cells were seeded at 5 × 10^5 cells/ml in complete medium. The cell numbers were again adjusted to 5 × 10^5 cells/ml in fresh complete medium 4 and 8 days after infection. The conditioned medium of the infected M10 cells was harvested daily for 8 days and again at 11 days after infection. The RT activity was then examined. Days post-infection are indicated at the top of the figure.
Less cytopathic HIV-1

Fig. 3. Kinetics of the replicating and cytopathic abilities of HIV-1 particles produced by cell clones isolated from M10/vif- and M10/vpu- cells. M10 cells (2.5 × 10⁶ cells) were infected for 1 h at 37 °C with culture media (1.5 ml) from Vif-1, Vif-7, Vif-5, Vpu-4, Vpu-5 and Vpu-6. Similarly, M10 cells were mock-infected with conditioned media from untransfected SW480, or infected with conditioned media from SW480 transfected with pNL432, pNL-Nd, pNL-Ss as wild-type (wt) HIV-1, vif mutants and vpu mutants, all of which contained HIV-1 titres of about 10².5 TCID₅₀/ml. After being washed with complete medium, the cells were seeded at 5 × 10⁵ cells/ml in fresh complete medium every 4 days for first 8 days and thereafter every 3 days. The viable cells were counted daily by trypan blue exclusion for the first 8 days and thereafter every 3 days after infection. Simultaneously, the percentage of HIV-1 antigen-positive cells in the viable cells was determined by the IF test with serum from an HIV-1-seropositive subject.

Characterization of HIV-1 proteins in the representative cell clones

The representative clones shown in Fig. 3 were also analysed by immunoprecipitation followed by SDS-PAGE for HIV-1 proteins. The cells were labelled with L-³⁵S protein labelling mix for 16 h. The cell, virus particle and soluble protein fractions were then immunoprecipitated with serum from the HIV-1-seropositive subject (Fig. 4) and MAb V107 (Fig. 5). HIV-1 env but not gag proteins were found in all fractions of the type I Vif-1 clone. Both env and gag proteins were detected in the cell fraction, but only env appeared in the virus particles produced from type III clones had markedly reduced cytopathogenic activity against M10 cells compared with that of the original vif and vpu mutant viruses.

When at different times after infection, the viable cell numbers were examined, it was found that even the HIV-1 particles produced from type III clones had markedly reduced cytopathogenic activity against M10 cells compared with that of the original vif and vpu mutant viruses.

Characterization of HIV-1 proteins in the representative cell clones

The representative clones shown in Fig. 3 were also analysed by immunoprecipitation followed by SDS-PAGE for HIV-1 proteins. The cells were labelled with L-³⁵S protein labelling mix for 16 h. The cell, virus particle and soluble protein fractions were then immunoprecipitated with serum from the HIV-1-seropositive subject (Fig. 4) and MAb V107 (Fig. 5). HIV-1 env but not gag proteins were found in all fractions of the type I Vif-1 clone. Both env and gag proteins were detected in the cell fraction, but only env appeared in the virus particles produced from type III clones had markedly reduced cytopathogenic activity against M10 cells compared with that of the original vif and vpu mutant viruses.
Fig. 4. Immunoprecipitation of HIV-1 proteins in cell clones isolated from M10/vif- and M10/vpu- cells. Uninfected MOLT-4, MOLT-4/HTLV-IIIB, MOLT-4/LAV-1, uninfected M10, Vif-1, Vif-7, Vif-5, Vpu-4, Vpu-5 and Vpu-6 (lanes 1 to 10) were similarly labelled with [35S]methionine and [35S]cysteine for 16 h at 37 °C. (a) Cell, (b) virus particle and (c) soluble protein fractions were immunoprecipitated with the serum from the HIV-1-seropositive subject used in the IF test. HIV-1 proteins in the immunoprecipitates were separated by SDS-PAGE. The $M_r$ values of the proteins were calculated by comparing their mobilities with those of marker proteins using a calibration kit (Pharmacia Fine Chemicals).

Fig. 5. Immunoprecipitation of HIV-1 gag proteins in cell clones isolated from M10/vif- and M10/vpu- cells. The same (a) cell and (b) virus particle fractions as used for Fig. 4 were immunoprecipitated with V107 MAb to HIV-1 gag p24.

particle and soluble protein fractions of the type I Vpu-4 clone (Fig. 4 and 5). More HIV-1 proteins were detected in the type III Vif-5 clone than in the type II Vif-7 clone, especially in the virus particle fraction (Fig. 4 and 5). The HIV-1 gag p24 protein levels in the virus particle fractions of the type II Vpu-5 and type III Vpu-6 clones were low compared with those in the corresponding cell fractions (Fig. 4 and 5).
Characterization of HIV-1 provirus DNA and mRNA in the representative cell clones

DNA was extracted from the representative clones, M10 and MOLT-4/LAV-1 cells, the latter two being used as controls. After digestion of these DNAs with \textit{XbaI}, Southern blot hybridization analyses were carried out using \(32\text{P}\)-labelled nearly full-length HIV-1 DNA as a probe. There is no \textit{XbaI} site within HIV-1 DNA, and consequently all clones of types I to III gave single bands varying in length (dependent on the \textit{XbaI} sites in the flanking regions) and a heterogeneous DNA band in uncloned MOLT-4/LAV-1 cells (Fig. 6a). These results indicated the presence of one copy of HIV-1 provirus DNA per diploid genome in these clones.

Digestion of MOLT-4/LAV-1 DNA with \textit{HindIII} gave bands of 4.32, 2.1, 1.48, 0.63 and 0.56 kbp that hybridized with a full-length HIV-1 DNA probe (Fig. 6b). The plasmid pNL432 gave 4.32, 2.1, 1.48 and 1.18 kbp bands that hybridized with the same probe (data not shown). DNAs from the representative clones showed hybridizing bands similar to those of MOLT-4/LAV-1 except for type I Vpu-4 and type II Vpu-5 (Fig. 6b).

Vpu-4 lacked the 4.32 kbp \textit{HindIII} fragment but retained the 2.1, 1.48 and 1.18 kbp fragments (Fig. 6b). The 4.32 kbp fragment was replaced by a 2.1 kbp fragment which also hybridized with a 4.32 kbp \textit{HindIII} fragment probe (Fig. 6c). The intensity of the 2.1 kbp hybridizing signal was almost twice those of the 1.48 and 1.18 kbp fragments (by densitometric analysis and correction for \(M_r\) values of DNA fragments) (Fig. 6b). Therefore, it was likely that, in Vpu-4 DNA, a sequence of nearly 2.2 kbp had been deleted from the 4.32 kbp

\begin{figure}
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\includegraphics[width=\textwidth]{Fig6}
\caption{Southern blot analyses of proviral DNA in cell clones isolated from M10/\textit{vif} and M10/\textit{vpu} cells. Genomic DNAs (10 \(\mu\)g) from uninfected M10, MOLT-4/LAV-1, Vif-1, Vif-7, Vif-5, Vpu-4, Vpu-5 and Vpu-6 cells (lanes 1 to 8) were digested with (a) \textit{XbaI} and (b) \textit{HindIII}, then subjected to electrophoresis on 0.45% and 0.6% agarose gels, respectively. The filters blotted with these DNAs were hybridized with a \(32\text{P}\)-labelled \textit{HindIII} fragment containing a nearly full-length HIV-1 DNA. The same filter used for (b) was hybridized with a \(32\text{P}\)-labelled 4.32 kbp \textit{HindIII} fragment as a probe after removal of the probe (c).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig7}
\caption{Northern blot analysis of HIV-1 mRNA in cell clones isolated from M10/\textit{vif} and M10/\textit{vpu} cells. After removal of the probes used for Fig. 1, the same filter blotted with poly(A)\textsuperscript{+} RNAs extracted from Vif-1, Vif-7, Vif-5, Vpu-4, Vpu-5, Vpu-6 and MOLT-4/LAV-1 (lanes 1 to 7) was hybridized with a \(32\text{P}\)-labelled \textit{HindIII} fragment containing nearly full-length HIV-1 DNA as a probe. Exposure time for autoradiography was 2 h for (a) and 30 min for (b).}
\end{figure}

\textit{HindIII} fragment or that some form of duplication event had occurred in addition to the deletion. PCR analysis supported the former possibility. An approximately 1.1 kbp DNA fragment was amplified with a pair of primers (nucleotide 3267 to 3288 in the gene coding for \textit{pol} RT,
and 6320 to 6344 in the gene coding for env gp120). However, no fragment was amplified with a set of primers (nucleotides 4368 to 4390 in the gene coding for pol endonuclease and 6320 to 6344) (data not shown). This result suggested that the deletion site of HIV-1 DNA in Vpu-4 might reside between the 3' terminus of the pol gene and the 5' terminus of the env gene.

In Vpu-5, two HindIII fragments of about 4.0 and 2.2 kbp hybridized with the 4.32 kbp HindIII fragment probe (Fig. 6c), suggesting the presence of two copies of an HIV-1 provirus genome per diploid genome. If this were the case, Vpu-5 may contain two defective HIV-1 proviruses, with deletions of approximately 0.3 and 2.0 kbp. However, as the result of Southern blot hybridization analysis after digestion with XbaI clearly showed a single band (Fig. 6a), the number of copies inserted in the Vpu-5 genome could not be established and further analysis may be required.

Northern blot analysis showed that, with the exception of Vpu-4 and Vpu-5 which had a 7 kb mRNA in place of the 9.2 kb mRNA, all clones contained mRNAs (9-2, 4-3 kb and several species around 2 kb) similar to those in MOLT-4/HIV-1 cells (Fig. 7).

To date, no deletion has been detected in type III clones which produced HIV-1 particles with replicative abilities comparable to that of wild-type HIV-1, but having markedly reduced cytopathic effects against the cells. Recently, it has been reported that the env region contains the major determinant of HIV-1 cytopathogenicity (Cheng-Mayer et al., 1990), and more recently that the cytopathic effect of HIV-1, not its replicability ability, is attenuated by a mutation affecting the env gp41 amino terminus (Kowalski et al., 1991). Therefore, we amplified the gp41 region by PCR with a combination of primers (nucleotides 7617 to 7641/7799 to 7820) following direct sequencing of the 5' terminal 12 bases in the env gp41 gene in type III Vif-5 and Vpu-6 clones. However, no sequence changes were detected (data not shown).

Morphological differences of HIV-1 particles in cell clones

Clones of types I to III isolated from M10/vif− and M10/vpu− cells were examined by electron microscopy. The morphology of HIV-1 particles produced by the clones is shown in Fig. 8. The results showed rare defective HIV-1 particles in type I clones, the predominance of defective doughnut-shaped HIV-1 particles with a lower percentage of intact particles in type II clones, and predominantly intact HIV-1 particles in type III clones.

Discussion

In our previous report, cytopathicity was compared between four HIV-1 mutants defective in the regulatory genes vif, vpr, vpu and nef (Nishino et al., 1991). The effect of mutations of the other regulatory genes tat and rev was not examined, since such mutants were not infectious (Adachi et al., 1991). Their growth kinetics and cell killing activity were examined under the same conditions using a clonal cell line, M10, isolated from MT-4 cells, since the life cycles of the vif and vpu mutants
varied in different cell lines; these were vif mutant-infected C8166 (Sodroski et al., 1986), A3.01 (Ogawa et al., 1989), H9 or MOLT-3 (Fisher et al., 1987) and vpu mutant-infected Jurkat cells (Terwilliger et al., 1989). In the culture system using M10 cells, the efficiency with which surviving cells, continuously proliferating and expressing HIV-1 antigens, were obtained has been shown to vary using different HIV-1 mutants (Nishino et al., 1991). Persistently HIV-1-infected M10 cells were obtained 4 days after infection with a vif mutant when adjusted to $5 \times 10^5$ cells/ml, with a vpu mutant when adjusted to $1 \times 10^6$ but not $5 \times 10^5$ cells/ml, and with a vpr mutant when adjusted to $2 \times 10^6$ but not $1 \times 10^6$ or $5 \times 10^5$ cells/ml. Consequently, under the conditions used in this study ($5 \times 10^5$ cells/ml), survivor cells were observed only in vif mutant infection but not in vpu mutant infection (Fig. 3). Infection with nef mutant or wild-type virus leads to complete cell death even when the cells are adjusted to $2 \times 10^6$ cells/ml (Nishino et al., 1991). Cell clones expressing detectable levels of HIV-1 antigens were isolated following limiting dilution of M10/vif- and M10/vpu- cells (Table 1); however, while several cell clones isolated from M10/vpr- cells did not synthesize detectable quantities of HIV-1 antigen (data not shown).

Seven cell clones isolated from each of the M10/vif- and M10/vpu- cells, designated Vif-1 to Vif-7 and Vpu-1 to Vpu-7, respectively, were characterized in terms of their replicative ability and the cytopathicity of the HIV-1 particles they produced. The results showed that these cell clones could be classified into at least three types: type I producing non-infectious HIV-1, type II producing infectious HIV-1 with low replicative ability and type III producing infectious HIV-1 with a replicative ability comparable to that of wild-type HIV-1 (Fig. 2 and 3). However, even type III clones produced markedly less cytopathic HIV-1 when compared with either wild-type HIV-1 or with the original vif and vpu mutants (Fig. 3). These results indicate that type III clones contained provirus DNA with additional mutation(s) as well as that of vif or vpu, which affected HIV-1 cytopathicity. However, no apparent HIV-1 mutation site was identified by Southern and Northern blot analyses (Fig. 6 and 7), or by immunoprecipitation (Fig. 4 and 5). Recently, it was reported that the HIV-1 cytopathic effect, but not replicative ability, was attenuated by a mutation affecting the env gp41 amino terminus (Kowalski et al., 1991). However, no such mutation was identified in type III cell clones (data not shown). Therefore, these clones might be useful for understanding another mechanism involved in single-cell killing.

On the other hand, the kinetics of replication and cytopathicity of HIV-1 produced from type II cell clones seems to resemble those of HIV-1 isolates from AC, but not from AIDS patients (Tersmette et al., 1988). In addition, PCR analysis revealed the possibility of the cells being persistently infected with HIV-1, but not producing infectious HIV-1 (Jackson et al., 1990), indicating an HIV-1 life cycle similar to that of the type I clones isolated here. Structural analysis of HIV-1 proviruses revealed large deletions in the vpu-4 and vpu-5 clones (Fig. 6). In vpu-4 DNA, a sequence of nearly 2.2 kbp had been deleted from the 4.32 kbp HindIII fragment (Fig. 6b and c). Northern blot analysis of poly(A)$^+$ RNA from the vpu-4 clone also revealed an approximately 2.2 kb deletion in the 9.2 kb mRNA coding for the gag and gag-pol proteins (Fig. 7). A similar deletion in the provirus DNA was also observed in a cell clone, H2-5, isolated from MT-4 cells which survived infection with HIV-1 in the conditioned medium of MOLT-4/HTLV-IIIB cells as reported previously (Imai et al., 1991). The H2-5 cell clone was found to contain proviral DNA with a deletion of 2558 bp, corresponding to the 3' half of pol gene, the entire vif and vpr genes and the 5' terminal region of the tat gene (Imai et al., 1991). Southern blot analysis suggested the presence of two copies of HIV-1 DNA containing 0-3 and 2-1 kbp deletions per diploid genome of Vpu-5 (Fig. 6c). Although the structure of these HIV-1 DNAs is not clear at present, Northern blot analysis of poly(A)$^+$ RNA from the Vpu5 clone revealed 7 kb instead of 9.2 kb mRNA (Fig. 7), indicating that the 7 kb mRNA was transcribed from an HIV-1 genome containing a 2.1 kbp deletion, but not from one containing a 0-3 kbp deletion. However, both the Vpu-4 and Vpu-5 clones continuously expressed HIV-1 antigens (Table 1), indicating that these antigens expressed by mutant HIV-1 genomes play a major role in the host immunological response in AC. Recently, we reported that several defective HIV-1 producer cell clones isolated from HIV-1-infected MT-4 cells could be superinfected with infectious HIV-1, resulting in the production of infectious HIV-1, and that complementation and/or recombination events in the superinfected cells may account for the production of infectious HIV-1 virions (Yunoki et al., 1991). Studies on superinfection between the clones isolated from M10/vif- and M10/vpu- cells are now in progress in order to understand the difference in the efficiency of isolation of infectious HIV-1 between AC and AIDS patients.

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