A recombinant retrovirus carrying a non-producer human immunodeficiency virus (HIV) type 1 variant induces resistance to superinfecting HIV

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A human immunodeficiency virus (HIV) type 1-infected Hut-78 cell clone (F12) shows a peculiar phenotype: it exhibits an altered viral protein pattern, is a non-producer and is resistant to homologous superinfection. To determine whether this phenotype is dependent upon the expression of the HIV-1 genome integrated therein, the Ssr/I/SsrI F12 provirus [deprived of HIV long terminal repeats (LTRs)] was cloned and inserted in the pLj retroviral vector bearing the neomycin (neo) and Geneticin resistance gene. CD4+ HIV-susceptible CEMss cells (a CEM clone able to form large syncytia 2 to 3 days post-HIV infection) were infected with the recombinant retroviruses rescued from the F12/HIV-pLj-transfected (in either sense or antisense orientation) amphotropic packaging cells PA 317. Neo sense resistant gene clones showed approximately 10 copies of viral DNA/cell (without detectable major deletions) only in episomal form, low viral RNA expression and a viral protein pattern characterized by an uncleaved gpl60, no gp41 and little, if any, p55 gag precursor (as in F12 cells). Superinfection of these F12/HIV DNA-engineered clones with HIV-1 resulted in a significant reduction in the yield of superinfecting HIV. This effect (more pronounced when the clones were maintained under neo selective pressure) was observed in all five retrovirus-infected clones exhibiting the presence and expression of sense episomal F12/HIV DNA but not in two clones bearing an antisense F12/HIV DNA or in one clone bearing only the pLj vector. These results indicate that bio-engineered human CD4+ cells expressing the F12/HIV genome exhibit a significant resistance to HIV superinfection.

Introduction

The resistance of a virus-infected cell to superinfection by the same or by a different virus is known as viral interference. In the life cycle of vesicular stomatitis virus, homologous viral interference (i.e. resistance against the same virus) is mediated by defective interfering particles (Mirakhur & Peluso, 1988; Peluso & Moyer, 1988; Stamminger & Lazzarini, 1989). An intracellular immunization against human immunodeficiency virus (HIV) infection via a homologous interference phenomenon was first outlined by Trono et al. (1989), who demonstrated a reduction in the release of superinfecting HIV-1 from cells harbouring a gag-mutant defective HIV-1 provirus. The failure of any HIV-1 isolate to replicate in HIV-1 chronically infected cells also has been demonstrated by immunological methods (Hart & Cloyd, 1990). Le Guern & Levy (1992) have observed that both HIV-1 and HIV-2 isolates can superinfect CD4+ HIV-2-infected cells, whereas in clones infected by either of the HIV types and exhibiting the usual CD4 downregulation, superinfection could not be demonstrated. The underlying mechanism(s) of the viral interference was not elucidated since the resistance to HIV-1 superinfection was correlated with the downregulation of CD4 receptor sites mediated by their intracellular binding with the viral gp160 glycoprotein precursor. No attempts, however, were made to find out whether superinfecting HIVs had or had not entered CD4+ downregulated cell clones, as shown by Taddeo et al. (1993). Conversely, Stevenson et al. (1989) demonstrated only a partial resistance to HIV-1 superinfection occurring in CEM cells transfected with a retrovirus construct expressing high levels of HIV-1 gp120/gp41, suggesting the presence of other viral and/or cellular factors involved in the interference phenomenon.

We have characterized an HIV-1-infected, non-producer Hut-78 cell clone (F12) harbouring an HIV-1 variant without major genomic changes and exhibiting the usual CD4 downregulation (Federico et al., 1989). F12 cells are fully resistant to HIV-1 or HIV-2 superinfection, even at high m.o.i. This homologous viral
interference appears to be mediated by a block in the retrotranscription of the first DNA strand of superinfecting HIVs (Taddeo et al., 1993). The possible role of the F12/H1V provirus in the F12 complex phenotype as well as in the homologous viral interference was studied in HIV-1-susceptible CD4+ human cells infected with a recombinant retrovirus harbouring the whole non-producer F12/HIV genome. The SstI/SstI F12/HIV provirus [deprived of both HIV long terminal repeats (LTRs)] was cloned and then subcloned in the pLj (pDOL) retroviral vector (Korman et al., 1987) carrying the neomycin (neo) and Geneticin resistance gene. Amphotropic packaging PA 317 cells (Miller & Buttimore, 1986) were transfected with the F12/HIV-pLj construct and neo-resistant clones were obtained. The supernatants from PA 317 clones were a good source of recombinant retroviruses (carrying the entire F12/HIV genome in either sense or antisense orientation) available for a subsequent abortive cellular infection. F12/HIV-pLj retrovirus-infected CEMss clones (CEM clones able to form large syncytia 2 to 3 days post-HIV infection), carrying approximately 10 copies/cell of episomal viral DNA without detectable major deletions, exhibited a 104- to 105-fold reduction in superinfecting HIV-1 yield as compared to CEMss clones carrying the F12/HIV genome in the antisense orientation. Preliminary data suggest that retrotranscription of superinfecting HIV-1 is quantitatively impared in sense retrovirus-infected CEMss clones.

Methods

Cloning of F12/HIV and insertion in pLj retroviral vector. Total DNA extracted from F12 cells was digested with the SstI enzyme. A fraction of approximately 9 kb was isolated through a 5 to 25% NaCl gradient, extensively dialysed, concentrated and then ligated with an SstI-digested pUC19 plasmid. The screening of transformed JM109 ampicillin-resistant bacterial cells was performed with a 32P-labelled (α-dCTP, NEN-DuPont, sp. act. 3000 Ci/mmole) SstI/SstI probe excised from the psp 64/BH10 plasmid.

From a large-scale preparation of F12/HIV-pUC plasmid, the SstI insert was excised and the 3' cohesive ends were converted into blunt ends by the T4 polymerase enzyme (Boehringer Mannheim, BBR). At the same time, the 5' cohesive ends of the BamHI-cut pLj retroviral vector were converted into blunt ends through the ‘filling-in’ reaction led by the Klenow fragment of DNA polymerase I (BBR), and then dephosphorylated. The transformation of JM109 bacteria with the product of ligation between the pLj vector and the F12/HIV genome gave rise to several kanamycin-resistant colonies, which were screened for sense or antisense orientation of the F12/HIV insert afterwards. An accurate restriction analysis was performed in order to verify the absence of evident deletions or rearrangements in the F12/HIV insert.

Cell cultures, cloning and transfection. CEMss cells, infected or transfected CEMss clones, F12 and B. E. 5 cells (Folks et al., 1986) were grown in RPMI-1640 medium (Flow Laboratories) with 10% fetal calf serum (FCS), and were then split biweekly. NIH 3T3 and PA 317 cells were cultured in Dulbecco’s modified MEM (Flow) with 10% FCS. All neo-resistant CEMss clones were maintained in 0.8 mg/ml Genentin (also designated G418, Sigma) which was about 50% active; while 0.5 mg/ml Genitin was sufficient to maintain both NIH 3T3 and PA 317 clones.

Transfection experiments were performed using the calcium phosphate co-precipitation method (Wigler et al., 1979). Cultures were exposed to Geneticin (0.5 mg/ml for both NIH 3T3 and PA 317 cells, 1 mg/ml for CEMss cells) 48 h after transfection. After 15 to 20 days, NIH 3T3 or PA 317 resistant colonies were picked up with vacuum grease-treated cloning cylinders. Conversely, the neo-resistant CEMss cells were cloned by the limiting dilution method in 96-well plates in the presence of 1 mg/ml Geneticin.

Recombinant retrovirus detection and infection of HIV-susceptible cells. Supernatants from selected PA 317 clones were harvested, clarified at low speed and ultracentrifuged for 8 h at 50000 g. Retrovirus titres were estimated by infecting polybrene-pretreated (4 mg/ml for 6 h) NIH 3T3 cells with serial dilutions of the concentrated preparations. Resistant colonies were scored after 20 days of culture in the presence of neo. Retrovirus titres were recorded as neo-resistant c.f.u. Polybrene-treated CEMss cells were infected twice with approximately 1 c.f.u./cell and 24 h later were cultivated in the presence of 1 mg/ml Geneticin.

HIV superinfection and detection. Clarified supernatants from H9/HTLV-IIIb cells were ultracentrifuged and then used at different concentrations to superinfect CEMss clones. Supernatants of superinfected CEMss clones were collected, clarified and centrifuged in a TL100 Beckman ultracentrifuge at 100000 r.p.m. for 15 min at 4°C. Reverse transcriptase (RT) assay was performed as previously described (Rossi et al., 1988). HIV titres (expressed in syncytiuhorming units, s.f.u.) were determined by infecting poly-l-lysine-pretreated CEMss cells with serial dilutions of the viral preparations, and by scoring the number of syncytiuh 4 to 5 days post-infection (p.i.). Electron microscopic analysis was performed as described (Federico et al., 1989).

Fluorescence-activated cell sorting (FACS) analysis. CD4 receptors on plasma membrane were detected by indirect immunofluorescence analysed by a cytofluorimeter. Briefly, 106 cells were incubated with the appropriate concentration of an anti-CD4 monoclonal antibody (Ortho Diagnostics) in 100 μl PBS containing 2.5% FCS. After an incubation of 60 min in ice, samples were washed twice in PBS, and further incubated for 60 min in ice with 100 μl of 1:20 goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Ortho Diagnostics). After two washes with PBS, samples were fixed with 3% (v/v) of formaldehyde and analysed in a cytofluorimeter (FAC-scan, Becton Dickinson).

Nucleic acid analysis. Southern and Northern blot analyses were essentially performed as described (Federico et al., 1989). A 32P-labelled SstI/SstI F12/HIV insert was used as probe at a sp. act. of about 5 x 106 to 8 x 106 c.p.m. /μg. Low M DNA was extracted by the Hirt procedure and digested as indicated in the manufacturer's recommendations.

RNA-PCR analysis was performed on samples treated twice with RNase-free DNase (BBR) followed by heat-inactivation of the DNase and by two phenol-chloroform extractions and ethanol precipitation. Each sample was also checked by DNA-PCR in order to detect the presence of possible DNA contamination. Two-hundred ng of total RNA, extracted by the guanidine isothiocyanate method (Chirgwin et al., 1979), was retrotranscribed for 90 min at 42°C in a 20 μl final volume containing 400 units of RNasin, 250 μM of each of the four deoxynucleoside triphosphates, 150 ng of the ‘reverse’ oligonucleotide probe excised from the pSp 64/BH10 plasmid. The radiolabelled (α-dCTP, NEN-DuPont, sp. act. 3000 Ci/mmole) SstI/SstI probe was used as a radiolabelled SstI/SstI F12/HIV insert was used as probe at a sp. act. of about 5 x 106 to 8 x 106 c.p.m. /μg. Low M DNA was extracted by the Hirt procedure and digested as indicated in the manufacturer's recommendations.

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patients' sera, each serum showing the complete pattern of antibody to HIV proteins in Western blot analysis; (iii) 60 μg/sample of Protein Lysis buffer (25 mM-Tris-HCl, pH 8.0, 3 mM-MgCl₂, 1 mM-EDTA pH 8.0) containing 0.1% SDS and heat inactivated at 95 °C. Total cell lysates resulting from this procedure were used for PCR amplification as described above.

For detection of superinfecting HIV-1, primers able to discriminate between vif sequences of the F12/HIV genome (Carlini et al., 1992) were utilized in the PCR amplification. The vif probe, in the 'forward' orientation, was 5' ATATCAAGCAGGACATAAC. For the mitochondrial DNA, primers previously published were utilized (Wrischnik et al., 1985). The primer sequences are: 'forward' 5' TGTCGATTCATTGTGTGGCTCCC (in the F12/HIV gag region) and 'reverse' 5' GGCAAGCAAGGAGCTAGAAC. The vif sequence is located in the same position in both F12/HIV and the BH10 molecular clone.

**Results**

**Cloning of F12/HIV provirus in pUC19 plasmid and its subcloning in the pLj retroviral vector**

An SstI-digested pUC19 plasmid was ligated with the 9 kb fraction of the SstI-digested F12 cellular DNA in order to clone the F12/HIV genome. The SstI/SstI F12/HIV proviral DNA was inserted in both sense and antisense orientations in the pLj retroviral vector, carrying the neo resistance gene (Fig. 1). As already described for different HIV-1 isolates (Pescador et al., 1985; Ratner et al., 1985; Srinivasan et al., 1989; Wain-Hobson et al., 1985), in the F12/HIV variant the SstI restriction sites map in the R regions of LTRs excluding virtually all the regulatory sequences of the 5' LTR and the poly(A) signal of the 3' LTR, but saving all the HIV open reading frames (ORFs). The complete nucleotide sequence of F12/HIV (Carlini et al., 1992) shows that the SstI site is located in the same position in both F12/HIV provirus and the BH10 molecular clone.

**Molecular and biological characterization of F12/HIV-pLj-transfected PA 317 clones**

The amphotropic packaging cell line PA 317 was transfected with the pLj vector or with the F12/HIV-pLj construct in either sense or antisense orientation. Some neo-resistant clones were molecularly and biologically characterized. The F12/HIV-pLj construct integrated into all PA 317 clones analysed (Fig. 2a) and retained the integrated F12/HIV-pLj construct even after prolonged cultivation without neo selection pressure. The RNA pattern strongly resembles that observed in parental F12.

![Fig. 1. A restriction map of the F12/HIV-pLj construct. The arrows in the F12/HIV genome indicate restriction sites which are absent from the F12/HIV genome. The SstI/SstI integration site is located in the same position in both F12/HIV and the BH10 molecular clone.](image-url)
cells (Federico et al., 1989) (Fig. 2b). Conversely, a single band of approximately 13 kb was detected in PA 317 clones transfected with the antisense construct indicating the absence of splicing donor/acceptor sites in that orientation (not shown).

The ability of several neo-resistant PA 317 clones to release recombinant retroviral particles was analysed by infecting NIH 3T3 cells with their supernatants and scoring the neo-resistant colonies. As shown in Table 1, the retroviral titres of supernatants of F12/HIV-pLj-transfected clones were generally lower (10- to 100-fold) than those of the pLj-transfected ones, possibly because of the large viral genome inserted in the vector. A non-specific inhibitory effect of the F12 genome in either orientation could also not be excluded. In any case, supernatants from PA 317 clones, clarified and concentrated by ultracentrifugation, were able to infect polybrene-pretreated CEMss cells.

Characterization of CEMss clones infected by the recombinant retrovirus

CEMss cells infected by recombinant retrovirus preparations (in either sense or antisense orientation) (see Methods) were selected in 0.8 mg/ml Geneticin for about 20 days. The neo-resistant cell population was then cloned by the limiting dilution method and more than 100 colonies were recovered.

Southern blot analysis of high Mr HindIII-restricted CEMss DNA identified CEMss clones A11, C6, B4, D6 and E4 infected with sense F12/HIV-pLj, and clones D7 and H9 infected with antisense F12/HIV-pLj constructs. As for G7 clone and F12 cells, the former is the CEMss clone transfected with pLj only, whereas the latter are our internal positive control.

Table 1. **Titration of supernatants of neo-resistant PA 317 clones**

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>RT (c.p.m./ml)</th>
<th>Titre (c.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLj 9-1</td>
<td>6 × 10⁴</td>
<td>8 × 10⁴</td>
</tr>
<tr>
<td>27-1</td>
<td>1.6 × 10⁴</td>
<td>2 × 10⁴</td>
</tr>
<tr>
<td>F12/HIV-pLj</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense 13-1</td>
<td>10⁴</td>
<td>5 × 10²</td>
</tr>
<tr>
<td>13-2</td>
<td>9 × 10⁴</td>
<td>10²</td>
</tr>
<tr>
<td>29-5</td>
<td>10⁴</td>
<td>5 × 10²</td>
</tr>
<tr>
<td>Antisense 16-1</td>
<td>1.2 × 10⁴</td>
<td>9 × 10²</td>
</tr>
<tr>
<td>16-3</td>
<td>9 × 10⁴</td>
<td>3 × 10²</td>
</tr>
<tr>
<td>32-1</td>
<td>2 × 10⁴</td>
<td>9 × 10²</td>
</tr>
</tbody>
</table>

* RT activity and infectious titres of the supernatants from pLj- and F12/HIV-pLj- (both sense and antisense) transfected PA 317 clones. Retrovirus titres are reported as neo-resistant c.f.u./ml, measured on NIH 3T3 fibroblasts.
Interfering non-producer HIV variant

Fig. 4. DNA- (a to c) and RNA-PCR (d) analysis of retrovirus-infected CEMss clones. (a) DNA extracted by the Hirt method was run in a 0.8% agarose gel for 36 h at 1 V/cm, blotted and hybridized as described (see legend of Fig. 2). Undigested extrachromosomal DNAs from F12/HIV-pLj-infected antisense (D7, lane D), sense (A11, C6, D6, B4, lanes A to C and E) CEMss clones are shown. (b) Hirt-extracted undigested DNAs from the sense C6 clone cells, obtained in 1989 (lane A) and in 1991 (lane B), are shown. (c) Hirt-extracted DNA was EcoRI- (lanes A to D) or PvuI- (lanes E to H) digested, run and hybridized as described in the legend to Fig. 2 and in (a) above. In lanes A to D and E to H are sense clones A11, C6, D6 and E4. As a positive control (C+) HIV-1-(pNL4-3)-infected CEMss cells were used. (d) 200 ng of total RNA was extracted from F12/HIV-pLj retrovirus-infected sense (A11, D6, C6, E4, B4) or antisense (H9, D7) CEMss clones, retrotranscribed and amplified by PCR as described in Methods. HIV gag-specific primers were used. As a positive control (C+), 200 ng of F12 total RNA was processed, and the same amount of uninfected CEMss cellular RNA was the negative control (C−). On the left side, the length of the amplified products is shown.

Fig. 5. DNA-PCR analysis, performed with F12/HIV gag primers, of serially diluted whole cell lysates from 8E.5 cells (a), F12/HIV-pLj antisense D7 (b) and F12/HIV-pLj sense A11 and C6 (c and d) CEMss clones. On the right side, the fragment sizes of HaeIII-digested φX174 DNA are indicated. On the left side, the length of the amplified products is shown.

but were found only in an unintegrated state even after 20 days of culture with or without selective pressure. This was also verified in Hirt-restricted DNA. The enzymes used were EcoRI, which cuts once in the vpr region of the F12/HIV genome (Carlini et al., 1992) and at the 3′ end of the neo resistance gene (see Fig. 1), and PvuI, which does not cut in the F12/HIV genome but linearizes the retroviral vector by cutting in the psi region (Fig. 4c). It does appear that the abortive infection induced in CEMss cells was due to a recombinant retrovirus not showing major genomic deletions. Similar results were obtained after the recombinant retroviral infection of human or murine cell lines, such as HeLa or NIH 3T3 (data not shown). This suggests that unknown mutation(s) in the integrase sequences and/or function, which occurred in either the F12/HIV genome or in the strain of pLj retroviral vector used (Donehower & Varmus, 1984; Schwartzberg et al., 1984), may account for its inability to integrate into the host genome. This episomal viral DNA showed a remarkable stability over a > 2 year time interval (Fig. 4b).

To assess how many copies of episomal viral DNA/cell were present in sense and antisense retrovirus-infected CEMss clones, a DNA-PCR experiment was carried out on sequentially diluted whole cell lysates of sense (C6, A11) and antisense (D7) clones as compared with a similarly diluted whole cell lysate of 8E.5 cells (Folks et al., 1986) used as a control since they contain only one copy/cell of HIV-1 proviral DNA. Data in Fig. 5 indicate that both sense and antisense CEMss clones contain approximately 10 copies/cell of proviral DNA.
Fig. 6. RIPA of HIV-specific proteins in either 'sense' (A11, D6, C6) or 'antisense' (AS) retrovirus-infected CEMss clones immunoprecipitated with either HIV-negative (−) or -positive (+) human sera. Cell lysates from both F12 and D10 clones were used as positive controls, the latter being a producer HIV-1 chronically infected Hut-78 clone (Federico et al., 1989). 14C-labelled size markers (Amersham) and HIV proteins detectable in RIPA are also marked.

In the retrovirus-infected CEMss cells, both the F12/HIV-pLj (Fig. 4d) and the neo resistance gene (data not shown) mRNAs were only detectable by RNA-PCR analysis, suggesting a very low transcriptional activity of both Moloney leukaemia virus (MLV)LTRs and simian virus 40 (SV40) promoters present in the unintegrated retroviral vector. Data are shown for gag primers only. When env primers are used, two PCR product bands are visible (data not shown). This finding may be accounted for by a preferential overamplification of a single DNA strand which runs with a slight delay with respect to the amplified dsDNA in a non-denaturing gel (J. J. Sninsky, Cetus Corporation, personal communication). At any rate, the specificity of the PCR products is confirmed by the hybridization with the specific oligoprobes.

All sense retrovirus-infected clones showed, as in F12 parental cells, the presence of uncleaved env glycoprotein precursor (three representative clones are shown in Fig. 6), whereas the uncleaved p55 gag protein, present in low amounts in F12 cells (Federico et al., 1989), was not easily detectable in any of the infected CEMss clones. At best, very faint bands were visible on the actual autoradiogram. For the sake of clarity, therefore, all retrovirus-infected clones are scored (−) in Table 3 with respect to the uncleaved F12 p55. No F12/HIV-specific proteins were detected in CEMss clones infected with the supernatants of PA 317 clones transfected with the F12/HIV-pLj construct in the antisense orientation.

Fig. 7. CD4 FACS analysis of retrovirus-infected CEMss clones carrying only pLj (c, G7 99.72% CD4+), sense (e, A11 94.30%, f, C6 96.69%) or antisense (d, AS 99.95%) F12/HIV sequences. HeLa (a) and CEMss (b) cells are, respectively, the negative (< 1%) and positive (99.81%) controls.
Table 2. RT activity of supernatants from F12/HIV-pLj retrovirus-infected CEMss clones after HIV-1 superinfection*

<table>
<thead>
<tr>
<th>Time (days) after infection with 4 × 10^3 s.f.u./10^6 cells</th>
<th>6</th>
<th>10</th>
<th>14</th>
<th>16</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CEMss clones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- neo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEMss</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Antisense D7</td>
<td>30652</td>
<td>228200</td>
<td>201800</td>
<td>Cell death</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>35600</td>
<td>422100</td>
<td>166600</td>
<td>Cell death</td>
<td></td>
</tr>
<tr>
<td>+ neo</td>
<td></td>
<td></td>
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<td></td>
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<td>Antisense D7</td>
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<td>45408</td>
<td>34231</td>
<td>31218</td>
</tr>
<tr>
<td>Sense</td>
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<td>0</td>
<td>1580 (3-4)</td>
<td>5845 (17-0)</td>
<td>9375 (30-0)</td>
</tr>
<tr>
<td>A11</td>
<td>0</td>
<td>8400 (80-8)</td>
<td>10500 (23-0)</td>
<td>21800 (63-6)</td>
<td>5600 (17-9)</td>
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<tr>
<td>B6</td>
<td>0</td>
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<td>1300 (4-1)</td>
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<td>9490 (20-8)</td>
<td>6150 (17-9)</td>
<td>6967 (22-5)</td>
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<td>10035 (22-0)</td>
<td>13700 (40-0)</td>
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<td>30691 (67-5)</td>
<td>34771 (101)</td>
<td>21981 (70-4)</td>
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<tr>
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<td>0</td>
<td>1580 (3.4)</td>
<td>5845 (17.0)</td>
<td>9375 (30.0)</td>
</tr>
</tbody>
</table>

* RT values are expressed in c.p.m./ml after background subtraction. The percentage values relative to the control antisense (+ neo) CEMss D7 clone are shown in parenthesis.

\[5 \times 10^3\] (a) F12/HIV-pLj retrovirus-infected either sense (C6) or antisense (D7) clones were infected with two different m.o.i.s (a, 4 × 10^3; b, 2 × 10^3 s.f.u./ml) of HIV-1, both in the presence of neo. At different days post-infection, cell supernatants were collected and tested for RT activity. RT values are given as c.p.m./ml of culture supernatant. (b) On day 10 post-infection, duplicate neo-treated cell cultures (4 × 10^3 s.f.u./ml) were deprived of the antibiotic and reseeded to monitor HIV-1 yields in the following days. (●) C6 or (▲) D7 clones in the presence of neo; (○) C6 or (△) D7 clones deprived of neo. Each experimental point is the average of duplicate samples.

As expected, no RT activity was detectable even in 100-fold concentrated supernatants of F12/HIV-pLj retrovirus-infected CEMss clones, since all retrovirus-infected clones were fully non-producer as were F12 cells. Accordingly, RIPA, performed on 10-fold concentrated supernatants of the [35S]methionine–cysteine-labelled clones, failed to show HIV-specific proteins (not shown). Finally, membrane CD4 receptor sites were equally detectable by the FACS analysis in > 94% retrovirus-infected CEMss cells as in parental CEMss cells. The lower signal shown in Fig. 7 indicates that all retrovirus-infected CEMss cells expose fewer CD4 molecules/cell than the parental uninfected CEMss cells.

**HIV-1 challenge of CEMss clones infected with the F12/HIV-pLj recombinant retrovirus**

Selected clones from the recombinant sense or antisense retrovirus-infected CEMss cells were superinfected with either 2 × 10^3 or 4 × 10^3 s.f.u./10^6 cells. In the absence of the neo selective pressure, HIV-1 yields from CEMss clones carrying the retrovirus-infected antisense F12/HIV-pLj sequences were comparable to those from HIV-1-infected parental CEMss cells (Table 2), thus representing the most suitable control for these experiments. In fact, in the F12/HIV-pLj sense retrovirus-infected C6 clone the yields of superinfecting HIV-1 were over 10-fold lower than in the antisense clone D7 (Fig. 8 a). The magnitude of this effect was higher if the cells were continuously kept under neo selective pressure. Exposure to the antibiotic delayed and reduced HIV-1 production per se in clones infected with the recombinant retrovirus carrying the F12 genome in antisense orientation (Table 2, compare lines 3 and 2). This may reflect a neo-induced subtle toxicity that was not detected by thymidine, uridine and methionine pulsing of any of three recombinant retrovirus-infected sense (All, C6, D6) and one antisense (D7) CEMss clones tested (data not shown).

In a parallel experiment, duplicate cultures of C6 and D7 clones exposed to 4 × 10^3 s.f.u./10^6 cells had the antibiotic washed out on day 10 p.i. and were reseeded to monitor virus yields. Removal of Geneticin resulted in markedly increased yields of the superinfecting HIV-1 (as measured by syncytium formation and RT activity) in cultures of the antisense retrovirus-infected D7 clone, as compared to the unwashed cultures of the same clone. Conversely, the low HIV-1 yields from cultures of clone...
Table 3. Characterization of retrovirus-infected CEMss cell clones

<table>
<thead>
<tr>
<th>CEMss clone</th>
<th>Expression of CD4 sites</th>
<th>F12-HIV uncleaved gp160</th>
<th>Resistance to HIV superinfection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>F12 sense retrovirus-infected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A11§</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>B6§</td>
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<td>C6§</td>
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<td>E4§</td>
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<tr>
<td>B4§</td>
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<tr>
<td>F12 cells</td>
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</table>

* -, No inhibition; +, < 10-fold inhibition; + +, > 10-fold inhibition of the yield of superinfecting HIV-1 (m.o.i. 4 x 10<sup>3</sup> s.f.u./10<sup>6</sup> cells); + + + +, full inhibition of the yield of superinfecting HIVs (Taddeo et al., 1993) even at the m.o.i. 5 x 10<sup>4</sup> s.f.u./10<sup>6</sup> cells.
† pLj-infected CEMss clone.
§ Antisense F12/HIV-pLj-infected CEMss clones.
§ Sense F12/HIV-pLj-infected CEMss clones.

C6 (bearing the sense F12/HIV-pLj retrovirus) did not change as a consequence of the neo removal, again as compared to the unwashed culture of the same clone (Fig. 8b).

Table 2 shows the RT values recorded when the experiment described in Fig. 7(a) was repeated and extended to four other clones (A11, B6, D6 and E4) exhibiting the phenomenon of interference and also to the B4 clone, which was shown not to express F12/HIV RNA molecules (see RNA-PCR assay, Fig. 4d).

These data were confirmed by electron microscopic observation (data not shown). In the HIV-1-superinfected pLj-infected CEMss G7 clone, a large increase in HIV-1 virions released after neo removal was clearly detected. The absence of intracytoplasmic viral particles in these cells maintained under neo selective pressure rules out the possibility that the increased HIV yields after neo removal were due to a massive release of intracellularly accumulated virions. Instead, very few retroviral particles were released from the HIV-1-superinfected F12/HIV-pLj-infected C6 clone with or without selective pressure.

All RT-positive supernatants obtained from retrovirus-infected CEMss clones after HIV superinfection infected fresh CEMss cells as efficiently as H9/HTLV-III<sub>B</sub> supernatants (as indicated by syncytium formation, data not shown), thus proving that superinfected CEMss clones were not releasing defective HIV particles.

In conclusion, HIV protein expression and resistance to HIV superinfection have been detected in all five CD4<sup>+</sup> retrovirus-infected clones (A11, B6, C6, D6, E4, exhibiting the presence and the expression of sense episomal F12/HIV DNA), but not in CD4<sup>+</sup> sense retrovirus-infected clone B4, where the expression of sense episomal F12/HIV DNA was not demonstrated. Controls, consisting of one retrovirus-infected CD4<sup>+</sup> clone bearing only the pLj vector (G7), and of two retrovirus-infected CD4<sup>+</sup> clones (D7, H9) bearing an antisense episomal F12/HIV DNA, did not show any of the observed phenomena (Table 3). Both qualitatively and quantitatively, the patterns of F12 proviral expression and resistance to HIV-1 superinfection are similar but not superimposable in the original F12 clone and in the cells carrying the sense F12/HIV-pLj construct. In fact, (i) the former is CD4 downregulated and the latter are CD4<sup>+</sup>, and (ii) the pattern of F12 proviral expression in F12 cells is both quantitatively (more gp160) and qualitatively (small but detectable amounts of p55) different from that of the sense clones studied. Further, (iii) the levels of F12 resistance to HIV superinfection are several-fold higher than in sense bioengineered CEMss clones (Table 3).

**PCR analysis of retrotranscription of cDNA in HIV-1 superinfection**

In F12 cells, the life cycle of superinfecting HIV-1 and HIV-2 is blocked late during retrotranscription (Taddeo et al., 1993). To determine whether a similar mechanism was operative in superinfected retrovirus-infected sense CEMss clones, lysates from equal numbers of HIV-1-superinfected sense A11 and C6 and antisense D7 CEMss clones were tested by PCR. Taking advantage of some nucleotide mutations in the F12 vif gene (Carlini et al., 1992), a pair of primers able to discriminate between the endogenous F12/HIV and the superinfecting HIV-1 HXB2 viral genomes was utilized.

Smaller amounts of the retrotranscription products in HIV superinfected clones (sense versus antisense orien-
Interfering non-producer HIV variant

Fig. 9. DNA-PCR analysis of cell lysates from HIV-1-superinfected cultures of sense (C6, A11) or antisense (D7) retrovirus-infected CEMs clones. Samples at different dilutions (from 1 : 2 to 1 : 256) were amplified using H9/HTLV-III, specific vif primers (a) or primers recognizing sequences from mitochondrial DNA (b). The length of the amplified products is shown on the left.

tation) were obtained by serially diluting all cell lysates. As shown in Fig. 9, the vif signal disappears at the 1 : 32 to 1 : 64 dilutions in the superinfected sense CEMs clones, whereas the same signal disappears in the superinfected antisense CEMs clone only at the 1 : 256 dilution. Conversely, in all dilutions of both sense and antisense cell lysates, the mitochondrial DNA could still be amplified. These data correlate well with the difference in superinfecting virus release between sense and antisense CEMs clones, suggesting that the inhibition may occur at a step after virus adsorption but before proviral integration.

Discussion

CD4 downregulation, as a consequence of its intracellular binding to the viral glycoprotein precursor gp160, may account for the homologous viral interference occurring in HIV-1-infected cells. The existence of CD4-independent modes of HIV cell entry has been outlined by different authors (Ikeuchi et al., 1990; Li et al., 1990), who described HIV-1 susceptibility of cells not expressing detectable amounts of CD4 mRNA or protein. Further, the failure of HIV to enter animal cells highly expressing the human CD4 ligand strongly suggests the necessity of other still unknown cellular factors (Chesebro et al., 1990; Maddon et al., 1986).

We tested whether CD4+ HIV-susceptible human cells engineered with the F12/HIV genome would exhibit the same peculiar and complex phenotype as parental CD4- F12 cells and, if they do, whether they were endowed with homologous viral interference. The answers to these questions were unknown since the published data (Federico et al., 1989) did not conclusively show that the F12/HIV genome (and not other unknown cellular factors) was responsible for the F12 phenotype. No viral factor(s) peculiarly encoded by the F12/HIV genome are known, other than those apparently mediating the viral interference occurring in cells productively infected by HIV (Hart & Cloyd, 1990). The total inability of the F12/HIV genome to express the full complement of viral proteins was exploited in order to generate bioengineered CD4+ HIV-susceptible cells resistant to HIV-1 infection like the F12 clone but unable to release infectious virus productively.

CEMss cells were infected by recombinant retroviral particles harbouring the F12/HIV genome (in either sense or antisense orientation) in order to test whether cell clones expressing F12/HIV-related proteins were protected against HIV superinfection. All F12/HIV ORFs have been inserted under MLV LTR control. The F12/HIV genome and the neo resistance gene were inserted in the same construct since the cotransfection of an HIV infectious molecular clone with a pNeo plasmid met with failure (Adachi et al., 1986). Furthermore, we wanted to obtain the correct construct available for a recombinant retrovirus rescue in the absence of regulatory sequences from different promoters (i.e. LTRs from both MLV and HIV). Data shown in Fig. 4 demonstrate that the whole F12/HIV genome (obviously without its LTRs) was present in the retrovirus-infected F12/HIV-pLj CEMs clones as no major genomic deletions were detected following restriction with EcoRI or PvuI.

As the TAR sequences were excised by the SstI digestion, the tat–TAR interaction does not occur in the F12/HIV-pLj construct, which results in the block of any trans-activation (Cullen & Greene, 1989; Dingwall et al., 1989; Gatignol et al., 1989; Sharp & Marciniak, 1989). It is also conceivable that in CD4+ CEMs cells
bearing the F12/HIV-pLj construct several complex interactions among HIV regulatory proteins do not take place. The homologous viral interference detected in F12/HIV-pLj-infected CEMss clones is probably explicable only by the low level expression of F12/HIV-related structural proteins promoted by the construct.

In the recombinant sense and antisense retrovirus-infected CEMss clones, the retrotranscribed DNA remains in an unintegrated state, probably because of infected CEMss clones, the retrotranscribed DNA related structural proteins promoted by the construct. In the recombinant sense and antisense retrovirus-infected CEMss clones, the retrotranscribed DNA remains in an unintegrated state, probably because of infected CEMss clones, the retrotranscribed DNA related structural proteins promoted by the construct. In the recombinant sense and antisense retrovirus-infected CEMss clones, the retrotranscribed DNA remains in an unintegrated state, probably because of infected CEMss clones, the retrotranscribed DNA related structural proteins promoted by the construct. In the recombinant sense and antisense retrovirus-infected CEMss clones, the retrotranscribed DNA remains in an unintegrated state, probably because of infected CEMss clones, the retrotranscribed DNA related structural proteins promoted by the construct.

The resistance to HIV superinfection of CEMSS clones was maximal if kept under neo selective pressure during superinfection. A possible massive loss of the F12/HIV-pLj construct in neo-deprived cultures may be ruled out by the detection of the retroviral DNA even after 15 days of culture without the antibiotic (data not shown). Rather than a direct effect of the antibiotic on F12/HIV-pLj expression (mRNA production is not impaired by this treatment), the large size of the F12/HIV-pLj retroviral construct may account for its putative intrinsic genetic instability, a property already demonstrated by other retroviral genomes (Omer et al., 1983; Shimotohno & Temin, 1982). The possible emergence of a few revertants lacking whole or crucial parts of the construct in neo-deprived cell cultures may be hypothesized but not proven because Southern and Northern blot techniques and even PCR analysis are not useful in identifying such revertants.

Yields of superinfecting HIV in antisense-infected clones grown in the presence of neo are lower than in the same clones not exposed to the antibiotic (Table 2). Following the removal of the antibiotic on day 10 p.i., a sharp increase in RT activity and in syncytium formation was observed. Conversely, in retrovirus-infected CEMss clones, removal of the antibiotic did not result in any increase of the RT activity (Fig. 8b). The intracellular immunization induced by the F12/HIV genome may have already prevented HIV superinfection. In these clones, the emergence of revertants after neo removal could be irrelevant.

These results point to the F12/HIV genome as an interesting non-producer interfering HIV genome. Genomic chimeras containing selected fragments of this genome as well as the corresponding parts of an HIV-1 infectious molecular clone may define the portion(s) of the F12 provirus responsible for the F12 phenotype and for the viral interference phenomenon.

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