Extensive C-terminal deletion in human immunodeficiency virus type 1 Env glycoprotein arising after long-term culture of chronically infected cells


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Human immunodeficiency virus type 1 (HIV-1) chronically infected (CI) cell lines were established from HIV-1,~B/LA~-infected MT-4 cells that survived acute infection. The HIV env gene expressed in the two long-term cultured cell lines differed from that of the lines cultured for shorter periods, by coding for a glycoprotein gpl60 that had the C terminus deleted. One long-term cultured cell line, CI-17, was studied in detail. An insertion of a premature stop codon in the env gene caused about 90% of gpl60 molecules to be truncated (gpl60x), lacking both cytoplasmic and transmembrane domains; these species were secreted into the cell medium, and could form oligomers with other truncated gpl60 molecules as well as with their normal counterparts. CI-17 cells constantly yielded high levels of viral protein and relatively low quantities of infectious virus, without cytopathicity. However, acute infection of fresh MT-4 cells with CI-17-derived virus led to cytopathicity, the rate of which as well as the Env glycoprotein pattern depended on multiplicity: (i) using an infection dose of $10^{-4}$ ID<sub>50</sub>/cell, cells died 7 to 8 days post-infection with normal gp160 synthesis predominating; (ii) with $10^{-2}$ ID<sub>50</sub>, gp160x was produced as early as 48 h post-infection and cell death was delayed. Predominant gp160x formation occurred again when new CI cell lines were obtained with CI-17-derived virus. Thus, two human immunodeficiency virus variants, a normal and a defective one, are persistently expressed in CI-17 cells. The other long-term cultured CI cell line also expressed gp160 with a similar (albeit slightly longer) deletion of a C-terminal region in most molecules, but the cell lines that were cultured for shorter periods did not. These results suggest that the emergence of HIV variants with a C-terminal deletion in the Env glycoprotein, which coexist with normal virus, may play a role in maintaining the long-term growth capacity and viability of CI cells.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection usually results in c.p.e. (Klatzmann et al., 1984), and this may be one mechanism by which CD4<sup>+</sup> lymphocytes are depleted in HIV-infected individuals. Syncytium formation is a prominent feature of HIV-induced c.p.e. in vitro (Lifson et al., 1986), but it does not appear to be required for cell death (Somasundaran & Robinson, 1987; Stevenson et al., 1990; Dedera & Ratner, 1991; Garry, 1989). Different viral gene products may be responsible for c.p.e.: as has been demonstrated for regions of both the glycoprotein gp120 and gp41 subunits (Stevenson et al., 1990; Kowalski et al., 1991), and other viral genes also have been found to play a role (Sakai et al., 1991; Cheng-Mayer et al., 1992).

There are prominent differences in lymphoid cell reaction to HIV in vitro. Most often infection leads to rapid c.p.e. with or without syncytium formation and cell death. In other cases, c.p.e. is moderate, with recurrent cyopathic/non-cyopathic periods, or a rather stable equilibrium between uninfected and infected cells exists (Gallo et al., 1984; Cloyd & Moore, 1990). Some infected cells can retain their morphological integrity and growth potential despite usual viral production. These latter characteristics may result in the establishment of chronically infected (CI) cell lines.

A means for studying the mechanisms of HIV infectivity and c.p.e. is to analyse models in which these processes are hindered. Beside mutagenesis, another approach is to compare acute cyopathic and chronic attenuated HIV infection in vitro, and to analyse the difference in viral protein patterns (Stevenson et al., 1990; Kaplan & Swanstrom, 1991). Previously, two
forms of gp160 were observed in the long-term cultured CI-17 cell line established after acute infection of MT-4 cells with the IIIB/LAI strain: normal gp160, and a truncated form (gp160x) that accumulated in high excess in the cells. Truncated gp160x lacked the Env protein cytoplasmic domain (Veselkovskaya et al., 1993). Here, we have studied the cause of the truncation and its possible biological significance. Our findings suggest that such truncation is apparently an evident result of the long-term culture of CI cells.

Methods

Cell culture and virus. H9 cells continuously producing HIV-1<sub>IIIB/LAI</sub> (Popovic et al., 1984; Wain-Hobson et al., 1991), or the original HIV-1<sub>IIIB</sub>, passed in CEM cells, were used for establishing CI MT-4 cell lines (Miyoshi et al., 1982). CI cell-derived virus was used also to initiate other CI cell lines. Infectious virus titres were determined by end-point titration on MT-4 cells and scored by the Reed and Muench equation, virus activity being monitored by its specific c.p.e. (Harada et al., 1985).

Cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub> in RPMI 1640 medium (Institute of Viral Preparations, Moscow, or GIBCO). 10% fetal calf serum (FCS; Gamaleya Institute of Microbiology and Immunology, Moscow, or GIBCO). Cells were diluted twice a week from 1 to 2 x 10<sup>6</sup> to 0.3-0.6 x 10<sup>6</sup>/ml with fresh medium.

To establish CI cell lines after acute infection and the death of most cells, the surviving fraction was recultivated with a mixture of fresh medium and medium from outgrown MT-4 cells. After several weeks, the c.p.e. declined until cell morphology, ability to cluster, and initial cell growth rate were restored.

Different CI cell lines were analysed. The CI-17 cell line, the first sample of which was frozen only after > 100 passages, was obtained from HIV-1<sub>IIIB/LAI</sub>-infected MT-4 cells. Other cell lines were established with CI-17-derived virus. In one case, infectious virus was first purified by cloning; after acute infection with CI-17-derived virus, virus was titrated by end-point dilution, and infectious virus harvested from the last dilution was used to infect fresh MT-4 cells and establish the new CI-37 line, which was passed for 15 cycles (CI-37-15) before analysis. The CI-789 line was initiated with the HIV-1<sub>IIIB</sub> prototype. It was cultured for a short time after stabilization, after which a fraction of the cells was frozen while the remainder were continuously cultured. After thawing, both the short-term (CI-789-12) and the long-term (CI-789-76) cultured sublines were simultaneously analysed.

For examining the effects of acute infection MT-4 cells were infected with cell-free fluid, usually from CI-17 cells. The standard m.o.i. was 0.1-0.2 (5U; Cetus) was added immediately before amplification. PCR conditions were as follows. First cycle: 5 min at 93 °C, 1 min at 68 °C, 2 rain at 72 °C; second to 35th cycle: 1 min at 93 °C, 1 rain at 68 °C, 10 min at 72 °C; 36th cycle: 1 min at 93 °C, 1 min at 68 °C, 10 min at 72 °C.

Radioimmunoprecipitation assay (RIPA). The fractions were thawed in RIPA buffer: 0.15 m-NaCl, 0.01 m-Tris-HCl pH 7.5, 0.5% sodium deoxycholate, 1% Triton X-100 (TX-100), 1% sodium phospholipid, 0.1% SDS and 10% Iodoacetamide. After centrifugation at 12000 g for 20 min, supernatants were incubated overnight at 4 °C with antibodies. Staphylococcus aureus was added for 2 h with the temperature maintained at 4 °C, and then washed in RIPA buffer. Pellets were suspended in electrophoresis buffer (0.01 m-Tris-HCl pH 8.0, 2% SDS, 5% 2-mercaptoethanol (ME), 25ng/ml bromo-phenol blue, 10% glycerol), boiled for 3 min, centrifuged at 12000 g for 2 min, and then run on a 8–13% SDS–PAGE gradient after which autoradiography was performed.

DNA sequencing. One µg of total chronically or acutely infected cell DNA was diluted in 0.25 mm-dATP, 0.25 mm-dCTP, 0.25 mm-dGTP, 0.25 mm-dTTP, 10 mm-Tris–HCl pH 8.3, 50 mm-KCl, 1.5 mm-MgCl<sub>2</sub> and 0.01% (w/v) gelatin. Two primers (5' TCCGAGATCTTCAGAC-ATAGCA 3'), which respectively correspond to nucleotides 7193 to 7218 and 8364 to 8393 of IIIB DNA plus and minus strands (Ratner et al., 1991). Fractions (1 ml) were collected from the bottom of the tubes and a sample of 15 µl of h<sup>+</sup> or 41to serum/fraction was used for RIPA. Sedimentation markers were ovalbumin (37S) and BSA (515) (Maas & Atkinson, 1990).

Immunostaining of cells. In order to detect Env glycoprotein or CD4 by indirect immunofluorescence, antibodies (1:50) were added for 30 min at 4 °C to 1 x 10<sup>6</sup> cells in 100 µl of culture medium containing a pool of anti-HIV-positive sera from five patients (h<sup>+</sup>), and murine anti-CD4 MAb OKT4 (Ortho Diagnostics).

Protein labelling. One day after chronic infection cell reculture, or 5 to 6 days after acute infection, cells (1 x 10<sup>6</sup>/ml) were incubated for 20 min in methionine-depleted medium without FCS. [35S]Met (specific activity; 5 Ci/mmol, 100 to 300 µCi/ml, Tashkent Institute of Nuclear Physics; or > 1000 Ci/mmol, Amersham) was added. Cells were then washed and frozen.

For cell surface protein analysis, labelled cells were incubated with the sera in 0.5 ml of medium at 20 °C for 2 h and washed. Cell extracts were prepared and immunoprecipitated. To analyse the particulate fraction proteins, cell-free medium was centrifuged at 30000 g for 1 h, and pellets were recovered. The non-sedimentable material is referred to as soluble proteins of cell-free medium.

Antibodies. A pool of anti-HIV-positive sera from five patients (h<sup>+</sup>), sero-negative human serum (h<sup>-</sup>), or the following antibodies were used: sheep antibody D7323 (Aalto) (41to) raised against gp41 C terminus [amino acids (aa) 845 to 860]; monoclonal antibodies (MAb) ADP328 (328) (a gift from F. Trinacrid, Hybridolab, Paris, France) raised against gp41 immuno-dominant extracellular epitope (aa 605 to 609); MAb 9305 (DuPont de Nemours, Dreieich, Germany) raised against the gp120 V3 loop (aa 318 to 328); and murine anti-CD4 MAb OKT4 (Ortho Diagnostics).
analysed on day 5 PI; at that time, 44% of cells were dead.

Table 1. **HIV biological activity in different CI cell lines**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Infectious virus titre (ID₅₀/ml)</th>
<th>RT activity (c.p.m./ml)</th>
<th>HIV antigen-positive cells (%)*</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CI-17</td>
<td>$1 \times 10^4$</td>
<td>$1.8 \times 10^5$</td>
<td>approx. 100</td>
</tr>
<tr>
<td>CI-37-15</td>
<td>$3 \times 10^6$</td>
<td>$5.6 \times 10^5$</td>
<td>approx. 100</td>
</tr>
<tr>
<td>CI-789-12</td>
<td>$3 \times 10^8$</td>
<td>$1.2 \times 10^6$</td>
<td>approx. 100</td>
</tr>
<tr>
<td>CI-789-76</td>
<td>$1 \times 10^8$</td>
<td>$1.5 \times 10^5$</td>
<td>approx. 100</td>
</tr>
<tr>
<td>Control MT-4</td>
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<td>$3.5 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI-17</td>
<td>$5 \times 10^8$</td>
<td>$3.7 \times 10^5$</td>
<td>approx. 100</td>
</tr>
<tr>
<td>Al of MT-4†</td>
<td>$4 \times 10^6$</td>
<td>$1.5 \times 10^4$</td>
<td>approx. 100</td>
</tr>
</tbody>
</table>

* As determined by FACS analysis.
† MT-4 cells, acutely infected with CI-17-derived virus, were analysed on day 5 PI; at that time, 44% of cells were dead.

2% FCS. For intracellular labelling, cells were first treated with 1% saponin, and then washed in 0.1% saponin. Phycocerythrin-conjugated goat anti-mouse or rabbit anti-sheep antibodies (1:100) (Biotech) were then added. Cells were washed and analysed with a FACScan (Becton Dickinson) in PBS and 1% paraformaldehyde.

**Results**

**Characterization of the CI cell lines**

All cells of the CI cell lines continuously produced viral proteins (see below) and released cytopathic infectious virus that demonstrated RT activity in the supernatants. As shown in Table 1, the amounts of infectious virus in cell-free medium of long-term cultured CI-17 and CI-789-76 cell lines were about 100-fold lower than levels in the short-term cultured CI-37-15 and CI-789-12 cell lines, or of MT-4 cells acutely infected with CI-17-derived virus analysed 5 days post-infection (PI), but RT activities were only three- to eightfold lower. These data suggest that these long-term cultured cell lines produced excessively more non-infectious than infectious virions.

**Expression of HIV env gene products in the different cell lines**

In CI-17 cells, gp160 appeared mostly as truncated gp160x, which lacked the cytoplasmic tail but retained most of its extra-cellular domains, whereas only a minority of species (approx. 10%) presented as normal gp160 (Veselovskaya et al., 1993). We compared Env glycoprotein patterns in the different HIV-infected MT-4 cell cultures (Fig. 1). As expected, gp160x of CI-17 cells was not precipitated by 328 and 4110 antibodies, which recognize epitopes of gp160 cytoplasmic tail (Fig. 1a compare lanes 3 and 4; Fig. 1b compare lanes 1 and 5). On the other hand, only (or mostly) normal gp160 accumulated in MT-4 cells that were acutely infected with CI-17-derived virus (Fig. 1a, lanes 5 and 6) as well as in the two short-term cultured CI cell lines, CI-37-15 established with cloned CI-17-derived infectious virus, and CI-789-12 established with the LAI prototype (Fig. 1b, lanes 2 and 6, 3 and 7, respectively). However cells of the latter cell line that had been cultured for a longer period (CI-789-76) predominantly expressed a shorter gp160 (Fig. 1b, lanes 4 and 8). This truncated gp160 migrated slightly faster than gp160x (it was more evident when both extracts were run close together; data not shown), which allowed us to rule out the trivial explanation that it resulted from contamination with CI-17 cells during long-term culture (see below). As for gp160x (Veselovskaya et al., 1993), CI-789-76 shorter Env glycoprotein resulted from protein truncation rather than abnormal glycosylation, as determined by SDS-PAGE of the corresponding deglycosylated products (data not shown), and it was precipitated by 41a9, an antibody recognizing the gp41 external immunodominant epitope (data not shown). Thus, the anatomy of these two truncated glycoproteins is quite similar.

In contrast to gp120, gp41 was not usually detected, presumably because of the high cell- and virus-specified (p40gag) protein background in the corresponding gel zone, but it was regularly noted after immunoblotting or glucosamine labelling of acutely infected or short-term-cultured CI cells (data not shown).

Among the different cells there were other differences in the HIV protein patterns. As previously reported (Kaplan & Swanstrom, 1991), more efficient p55gag processing that resulted in more p24 was regularly noted in acutely infected relative to chronically infected cells independently of the virus used for acute infection, prototype HIV-1-LAI or virus from any of the chronically infected cell lines. Also, CI-37-15 cell p55gag had a slightly lower electrophoretic mobility, which may be due to selection of a HIV variant arising during clonal purification of the virus used for establishing the CI-37 cell line (Sanchez-Palomino et al., 1993).

In many experiments there was a 200K protein in the precipitates, which also occurred in uninfected cell extracts (data not shown), the apparent amount of which was roughly equivalent to that of the recovered radioactivity in the lane.

Thus, long-term culture of HIV-1-LAI CI MT-4 cells resulted for both cases in the deletion of the cytoplasmic tail of most gp160 species. Apart from the co-expression of normal gp160 in both CI-17 and CI-789-76 cell lines, these findings accord with those found using simian immunodeficiency virus (SIV) systems since it has been shown that prolonged passage of SIV in human cells leads to extensive deletions in the Env glycoprotein cytoplasmic tail whereas minimally passaged virus
expresses full-length glycoprotein (Chakrabarti et al., 1989; Hirsch et al., 1989; Luciw et al., 1992; Marthas et al., 1989; Ritter et al., 1993). This led us to characterize more precisely our truncated Env products using the CI-17 line as a model.

Genetic analysis of gp160x of CI-17 cells

The reason for gp160x truncation was examined by proviral DNA sequencing of the env gene. Six independent 250-nucleotide sequences corresponding to the region of aa residues 640 to 722 were generated from CI-17 cells and three similar sequences were derived from MT-4 cells acutely infected with CI-17-derived virus. Alignment showed greater than 90% nucleotide and protein identity both among the clones and with HIV-IIB/LAI DNA (Ratner et al., 1985). Sequences from CI-17 cells, but not those from acutely infected cells, had a point mutation at nucleotide position 2016 leading to translational stop and generation of truncated gp160 (642 aa in its mature form) that is 12 residues proximal to the transmembrane part (Fig. 2). In two cases where longer sequences were obtained from CI-17 cells tat and rev second exons were unchanged (data not shown). These data indicate that gp160x results from env mRNA premature termination. Accordingly, the predominant formation of gp160x relative to gp160 in CI-17 cells is likely to be related to the increased abundance of the mutant proviral DNA.

The simultaneous expression of the mutant and normal proviruses may provide a selective advantage for cell growth during long-term CI cell culture, which led us to examine the conditions under which their products interact and whether mutant DNA expression leads to the formation of defective virions.

gp160x secretion into CI-17 cell-free medium

At least most of the gp160 transmembrane domain is needed for anchoring at the cell membrane (Owens et al., 1994), and thus we determined the distribution of truncated and normal Env glycoprotein in CI-17 cell cytoplasm, on the external membrane, and in cell-free particulate and soluble fractions, after 6-h labelling (Fig. 3).
The glycoprotein patterns in the cytoplasm and on the cell membrane were similar, with lower amounts of gp160 and gp160x on the latter (Fig. 3a, lanes 1 and 2). The predominance of p24 over p55 in the particulate fraction is indicative of the presence of viral particles. Gp120, but also gp160x and some gp160 (as shown by selective immunoprecipitation with 41to antibodies) were regularly found, although total recovery of viral proteins was rather poor (Fig. 3a, lane 4; Fig. 3b, lanes 1 and 2). In contrast, massive recovery of particle-free soluble gp160x and gp120 was noted in the medium (Fig. 3a, lane 5). In spite of its lower mobility in the gel, the identity of soluble gp160x was indicated by the absence of the 41to epitope (Fig. 3b, lanes 3 and 4). In the soluble fraction we also observed an approx. 30K component (indicated as env in Fig. 3b, lane 3) in which covalently linked glucosamine was present (data not shown). This component should most probably be the C-terminal product of gp160x cleavage at the normal site.

Thus, massive gp160x secretion confirms that it lacks a transmembrane domain. 'Spontaneous' secretion of gp160 by a chronically infected cell line (Kalyanaraman et al., 1988, 1990), and more recently secretion of a similarly truncated gp160 (Hallenberger et al., 1993; Owens et al., 1994), have also been reported. In addition, unprocessed gp160 may also be present on virions, especially when the cytoplasmic tail is shortened due to insertion of a translational stop in the env gene (Dubay et al., 1992).

**Hetero-oligomerization of gp160**

In some experiments in which CI-17 cell extracts were precipitated with antibody 41to, a minor band at the same position as gp160x was noted. We investigated whether this band might result from gp160x coprecipitation with normal gp160 through gp160x-gp160 hetero-oligomer formation.

Indeed, env glycoproteins are oligomerized into dimers and higher-ordered structures (Pinter et al., 1989; Schawaller et al., 1989; Doms et al., 1990; Earl et al., 1990; Weiss et al., 1990). Oligomerization precedes gp160 cleavage and possibly plays a role in its folding, but since cleavage probably destabilizes intermolecular
connections within oligomers, gp120 and gp41 present mostly as monomers in sucrose gradient (Earl et al., 1990). Oligomerization is assumed to involve mainly the extracellular part of the transmembrane glycoproteins of retroviruses (Einfeld & Hunter, 1988, 1994). Thus, we examined using sucrose gradient sedimentation whether truncated gp160x may be inserted into homo- and/or hetero-oligomers. The gp160 from either acutely infected or CI-17 cells and gp160x from CI-17 cell extracts mostly appeared in the 11S (fractions 4 to 6) but also in the 7S zone (fractions 8 to 9), which included most of gp120 (Fig. 4a, b), and this indicates that, as previously proposed (Berman et al., 1989; Earl et al., 1992; Hallenberger et al., 1993) gp160x oligomerization does not require gp160 cytoplasmic and transmembrane moieties (Einfeld & Hunter, 1988).

To test whether 41to antibody-precipitable gp160x originated from coprecipitation of hetero-oligomers with gp160, part of CI-17 cell extracts were heated in 1% SDS–1% ME to dissociate oligomers and, after centrifugation, samples of sucrose-gradient fractions were precipitated by either h+ serum or 41to antibodies. 41to antibody-precipitable gp160x in the untreated samples was mostly found in the dimeric (fractions 5 to 7) and bottom fractions (Fig. 4c), whereas oligomers (Fig. 4d) and almost all 41to antibody-precipitable gp160x (Fig. 4e) disappeared from the denatured extracts. Altogether, these findings are consistent with the formation of gp160–gp160x hetero-oligomers.

**Distribution of normal HIV provirus among CI-17 cells**

The presence of hetero-oligomers suggested that both gp160 and gp160x synthesis occurred within the same cells. However, it was still possible that CI-17 cells were a mixture of gp160- and gp160x-expressing cells, only a subset of which produced both glycoproteins. Two approaches were used to examine this further.

We determined the proportion of 41to antibody-positive cells by fluorescence-activating cell sorting.
Fig. 4. Sedimentation analysis of HIV glycoproteins in CI-17 and acutely infected cells. Cytoplasmic extracts from 6-h labelled acutely infected (a) or CI-17 cells (b to e) were fractionated in sucrose density gradient under native conditions (a, b, c) or after heating for 1 min in 1% SDS, 1% ME (d, e). Fractions from the bottom of each tube were collected and proteins were analysed by RIPA with h+ (a, b, d) or 41to sera (c, e). Arrows designate the positions of marker proteins in the gradients. HIV proteins precipitated from whole CI-17 cell extract (lanes indicated as E) were used as electrophoretic markers in (a) and (b).

(FACS) analysis (Fig. 5), using MT-4 cells acutely infected with CI-17-derived virus or uninfected cells as positive and negative controls, respectively. As detected by an anti-V3 MAb (9305), there was substantial Env accumulation in the CI-17 as in the acutely infected cells (day 5 PI), and surface CD4 was strikingly reduced in both. Intracellular immunostaining with 41to antibody showed homogeneous labelling, indicating that gp41
Fig. 5. HIV Env glycoprotein and CD4 expression of A1 and CI-17 cells. MT-4 cells were stained either with MAb OKT4, or with anti-V3 MAb 9305 or antibody 41-to (after saponin permeabilization), before or after (day 5) acute infection by CI-17 cell-derived virus (10⁴ ID₅₀/cell). Mock-infected CI-17 cells were similarly examined. Results are presented as relative cell numbers versus fluorescence intensity. ---, Negative control fluorescence.

Table 2. CI-17 cell-free and cell-associated virus infectivity

<table>
<thead>
<tr>
<th></th>
<th>Infectious titre (ID₅₀/sample) in:</th>
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<tbody>
<tr>
<td></td>
<td>Cell-free</td>
</tr>
<tr>
<td>Number of cells per sample*</td>
<td>10⁶</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>5.6 × 10⁶</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>2.7 × 10⁶</td>
</tr>
</tbody>
</table>

* Ten ml of cell suspension were pelleted, the cells were washed with 40 ml of fresh medium, and then counted. Virus infectivity of the original cell-free fluid and from the recovered cells was evaluated by end-point titration, using eight replicates for each dilution. Cultures were monitored for up to 10 days, and infectious titres were assessed by the resulting c.p.e.

C terminus was expressed in most (if not all) CI-17 cells. Similar results were noted with other anti-Env or -Gag antibodies (data not shown). Such immunostaining pattern as this indicates that all CI-17 cells express normal gp160.

Presuming the necessity of normal env gene expression for effecting HIV infectivity, cell-associated infectivity was then estimated by end-point titration of washed CI-17 cells. ID₅₀ was 3 and 0.5 viable cells in two independent experiments, respectively (Table 2), which indicates the capacity of most cells to produce infectious virus. In both experiments infectivity of cell-free medium was much lower than that of the corresponding washed cells, which allowed us to discount any significant role of carried-over virions in cell-associated infectivity. Thus both approaches strongly suggest the activity of the normal virus in most CI-17 cells.

In spite of the lack of a specific marker for gp160x, other data suggest its widespread distribution among CI-17 cells: (i) the occurrence of gp160/gp160x hetero-oligomers; (ii) the apparent excess of mutated over normal proviral DNA, as of gp160x over gp160; (iii) the fact that most env gene products that complexed and coimmunoprecipitated with CD4 were gp160x, given that intracellular CD4 is present in all cells (data not shown).

Detection of defective virus activity in CI-17 cell supernatants

In several experiments a minor gp160x-like component was noted in extracts of MT-4 cells that were acutely infected with CI-17-derived virus (see Fig. 1 a lane 6). This suggested that gp160x-producing activity might be transmitted from CI-17 to other cells through the generation of defective virions with limited replication capacity.

To examine this and since the replication rate of the defective virus should depend on the input m.o.i., we infected fresh MT-4 cells with CI-17-derived virus at three multiplicities: standard (10⁻⁴), 10⁻⁶ and 10⁻² ID₅₀/cell. Cell viability and viral protein synthesis were then monitored (Fig. 6a). Independently of the m.o.i., most infected cells were eventually killed by the virus, but the kinetics of c.p.e. and the pattern of Env expression depended on the m.o.i. Infection at the standard m.o.i. yielded the expected results: synthesis of viral proteins on day 4 PI, and almost total cell death by day 7. Infection at low m.o.i. led to considerable delay in both HIV protein synthesis and cell death. Infection with the high m.o.i. resulted in delayed cell death as well as the early gp160x appearance. In particular, on day 7 PI, whereas almost all cells infected with the standard m.o.i. were dead, 38% of cells infected with the high m.o.i. were still alive and 22% were still viable on day 10. Production of gp160x was noted as early as 2 days PI, a timing compatible with a single virus replication cycle (Kim et al., 1989). The lack of Gag protein synthesis at that time might then be explained by delayed accumulation of full-sized HIV genomic RNA relative to partially spliced mRNA used for translation into Env (Felber et al., 1990; Schwartz et al., 1990).

Several new chronically infected lines were also established with CI-17-derived bulk virus. Under these conditions, stabilization of the new line was always accompanied by the transition of Env glycoprotein expression from dominant gp160 to gp160x production.
(a) Multiplicity of acute infection (ID$_{50}$/cell)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>$10^{-5}$</th>
<th>$10^{-4}$</th>
<th>$10^{-2}$</th>
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<tr>
<td>Cell viability (%)</td>
<td>83</td>
<td>71</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>70</td>
<td>70</td>
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<td></td>
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<td></td>
<td>22</td>
<td>83</td>
<td>0</td>
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</table>

Fig. 6. Induction of gp160x under different experimental conditions. (a) HIV protein patterns in acute infection cells using different m.o.i. of CI-17-derived virus. Virus from CI-17 cell-free medium with an initial infectious titre of $1.5 \times 10^3$ ID$_{50}$/ml was concentrated 100-fold, followed by appropriate dilution. Two ml of each dilution were incubated with $3 \times 10^7$ MT-4 cells for 2 h at 37 °C, and cell concentrations were then adjusted. Thus, different m.o.i. were used: $10^{-5}$, $10^{-4}$ and $10^{-2}$ ID$_{50}$/cell. At different times after infection (as indicated), cell viability was monitored by trypan blue exclusion, and HIV protein pattern was examined after RIPA with h$^+$ serum of 2-h labelled cell extracts. (b) Truncated Env expansion during transition from acute to chronic infection cell line status. CI-17-derived virus was used at $3 \times 10^{-5} ID_{50}$/cell. Cell viability and Env glycoprotein patterns were monitored at the indicated times PI; h$^+$ serum was used for RIPA. (c) Truncated Env expansion during coculture of gp160x-producing and normal Env-producing CI cell lines. CI-17 cells ($5 \times 10^6$) were mixed with $1 \times 10^7$ of short-term-cultivated CI-789 cells (CI-789-15) and incubated under standard conditions. At the indicated times, viral glycoproteins were analysed by RIPA with h$^+$ serum. The results obtained with individually cultured CI-789 (CI-789-21) and CI-17 lines are indicated by * and **, respectively.

(Fig. 6b). Though the initial gp160:gp160x ratio depended on the input m.o.i. of acutely infected cells (a low m.o.i. led to delay of gp160x appearance and of the time when the new chronically infected cell line stabilized), the final ratio was very close to that of original CI-17 cells. Also, when CI-17 cells were co-cultured with short-term cultured CI-789 cells (CI-789-15), which do not produce gp160x, visible expansion of gp160x synthesis was again
detected in spite of the initial CI-789 cell excess and lack of cytopathicity in the co-culture (Fig. 6c). However, when infectious virus from CI-17 underwent preliminary purification by limited dilution, the resulting chronically infected line did not display gp160x production, at least during a short-term culture period (Fig. 1, line CI-37).

Discussion

We established several chronically infected lines from MT-4 infected cells with different sources of HIV-1_HXB/LAI-containing culture fluid or derivatives thereof. All the cells of these lines expressed HIV antigens, were surface CD4-negative, and continuously produced cytopathic infectious virus. But, in contrast to results with acute infection, no c.p.e. was noted in chronically infected cultures and cells apparently grew normally. A prominent difference between the different chronically infected cell lines was the emergence of C-terminal truncated gp160, which occurred in both cases where cells were initially infected with bulk HIV-1_HXB/LAI and the lines had been cultured for long periods. Monitoring the CI-789 line directly showed the transition from normal to truncated gp160 expression occurred as a function of culture length. Both long term chronically infected cell lines expressed normal gp160 as a minor product.

This led us to consider that gp160 truncation may be a natural phenomenon occurring in long-term chronic infection and we then examined the reason for truncation and its possible biological consequences using the CI-17 line as a model. Massive secretion of gp160x, gp120 and of an apparent gp41 N-terminal product of gp160x cleavage at the normal site, previous results from gp160x epitope mapping (Veselovskaya et al., 1993), and genetic analysis all indicated that the reason for truncation was a stop codon mutation in the env gene occurring just before the membrane spanning domain of gp160. Although the truncated gp160 of the CI-789-76 line was not analysed as thoroughly, its electrophoretic mobility, lack of 41to and presence of 41a9 antibody epitopes and apparently normal glycosylation pattern allow us to speculate that a similar mechanism applies.

Viral protein synthesis and RT levels in the CI-17 and CI-789-72 cell lines were comparable to those of the other chronic or acutely infected cells, but infectious virus yields were about 100-fold lower, indicating that a higher release of non-infectious virions occurs. Detection of gp160x–gp160 hetero-oligomers in CI-17 cell extracts, homogeneous intracellular immunostaining with antibodies to gp120 and gp41 C terminus, and the high capacity of individual CI-17 cells to produce infectious virus, indicate that both gp160 species are present in most (if not all) CI-17 cells.

Our findings indicate that two types of proviral DNA, a defective one (gp160x-inducing) and the normal (gp160-inducing), are coexpressed in CI-17 cells. They also strongly suggest that the two corresponding HIV variants, one defective and one infectious, replicate there. The product of the mutated env gene, gp160x, lacks both ‘anchoring’ domains but it conserves most of the gp160 ectodomains, and it presents characteristics of normal gp160: proteolytic cleavage with formation of gp120 (Veselovskaya et al., 1993), and oligomerization. In contrast to gp160, gp160x is extensively secreted when not integrated into hetero-oligomers. Persistence of the normal virus in CI-17 cells may be assumed from the continuous synthesis of untruncated gp160 and from the infectivity and c.p.e.-inducing capacity of CI-17 cell-free medium virus in fresh MT4 cells.

Presuming that the defective genome by itself cannot give rise to infectious particles in CI-17 cells, we propose a helper activity for the normal virus. After formation of viral nucleoids by coating of genomic RNAs of both variants with internal proteins from either, the corresponding subviral particles are coated at random by normal and probably by chimeric glycoprotein oligomers. Thus, homo- and hetero-diploid virions, associating or not with defective and normal viral genomes, that are continuously produced by CI-17 cells, have an identical potential to infect new susceptible cells.

Acutely infected cell samples with different m.o.i.s of CI-17-derived virus were designed to verify this point, inasmuch as one could consider that increasing m.o.i. from 100-fold to 10^{-2} ID_{50}/cell should lead to about 1% of cells infected by normal virus, 10% by defective virus, and 0-1% by both (not taking into account heteroploid particles). The findings of a shorter delay of viral protein production observed with this m.o.i., as well as formation of truncated gp160x as early as 48 h after infection, i.e. during the first cycle of HIV replication (Kim et al., 1989), accord with this defective particle model. Alternatively, decreasing the m.o.i. should result in a lower proportion of cells infected by the defective virus. Indeed, no gp160x was found during the acute infection period in MT-4 cells infected by CI-17-derived virus at 10^{6} ID_{50}/cell. However, transition to chronically infected status was accompanied again by the rapid appearance of gp160x, which did not occur if CI-17-derived infectious virus was first purified (CI-37 line). Preliminary propagation of CI-17-derived virus into acutely infected cells was the prerequisite for the lack of rapid gp160x-inducing capacity in new chronically infected cell lines. Thus, CI-17 mutant HIV genome is apparently expressed as virions that accumulate in excess over infectious virus, may infect new cells, and induce at least one abortive round of replication.

It is not yet known whether the env stop codon
mutation in CI-17 is associated with mutation(s) in other viral gene(s), but the activity of the defective virus in acute infections with high m.o.i. of CI-17-derived virus indicates an overall normal viral genome activity. Analysis of individual proviral DNA clones from CI-17 cells is now in progress.

Culture of the CI-789 line initiated with bulk HIV-1(LAI) also led to production of truncated Env, similar but not identical to gp160x, although the production occurred less rapidly than when using CI-17-derived virus. Again this protein accumulated in excess over the normal product. Thus, the predominant synthesis of anchor-less gp160 should provide a selective advantage for long-term chronically-infected cells, the nature of which is not yet clear, but for which the following two major possibilities apply. (i) Attenuation of the still present virus-induced c.p.e. in chronically infected cells could result from the lack of gp160 anchoring sequence. This may be achieved by decreasing, for example, the harmful effect of CD4–Env intracellular complexes (Koga et al., 1990; Crise & Rose, 1992; Lu et al., 1994) or the direct cytotoxicity of gp160 cytoplasmic tail (Miller et al., 1991; Shimizu et al., 1992). Alternatively this may arise from (ii) the direct effect of massive Env protein secretion on cell growth and/or survival. It would then be necessary to suppose that secreted Env proteins alleviate the supposed toxic effects of cell-bound Env or, alternatively, that chronically infected cells become sensitive to some unspecified cell-stimulating effect of secreted Env products. These possibilities are now under consideration.

The biological properties of the HIV defective variants described here resemble in many respects the so-called defective interfering particles of many animal viruses (Holland et al., 1980; Nayak et al., 1989). Defectiveness is generally associated with a more or less long internal deletion in the viral genome, leading to the impossibility of independent replication. In CI-17 cells, however, the defective variant is apparently capable of expressing most if not all of its own genes. This conclusion derives from the early induction of gp160x in MT-4 cells initially infected with CI-17-derived virus at an m.o.i. of 0·01 infectious particle/cell, and from the observation that CI-17 cells synthesize Gag and apparent Gag–Pol fusion proteins in amounts that do not allow us to ascribe their expression solely to the normal virus genome. Thus, in this case the predominant expression of the defective genome in CI-17 cells results most probably from its higher abundance relative to the normal genome.

Irrespective of env glycoprotein phenotype, all chronically infected lines examined here were efficiently depleted of surface CD4. Because HIV-induced c.p.e. may be related to early replication steps–virus attachment and/or entry (Fermin & Garry, 1992)–or to the cytoplasmic accumulation of unintegrated proviral DNA (Weller et al., 1980), blocking reinfection through surface CD4 depletion is a plausible mechanism for lack of virus-induced c.p.e. in chronically infected cultures (Stevenson et al., 1988).

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