Persistent infection of MT-4 cells by human immunodeficiency virus type 1 becomes increasingly likely with in vitro serial passage of wild-type but not nef mutant virus

Yoshii Nishino, Takaaki Nakaya, Koh Fujinaga, Masahiko Kishi, Ichiro Azuma and Kazuyoshi Ikuta*

Institute of Immunological Science, Hokkaido University, Kita-ku, Sapporo 060, Japan

Our previous studies have shown that human immunodeficiency virus type 1 (HIV-1), with mutations in accessory genes such as vif, vpr or vpu, can generate persistent infection of MT-4 cells, whereas infection by wild-type or nef mutant HIV-1 causes extensive cell death. The possibility of generating a naturally attenuated form of HIV-1 with reduced cytopathogenicity in MT-4 cells was examined by in vitro serial passage of the wild-type and a nef mutant form of HIV-1, each derived from the infectious molecular clone pNL432. The ability to cause persistent infection was observed after four passages of wild-type HIV-1 with the frequency of persistence becoming progressively higher with serial passage. In contrast, persistent infection was not observed even after 50 passages of the nef mutant virus. Sequence analysis of the accessory gene loci in genomes recovered from the persistent infections caused by passaged virus revealed mutations in vif and vpr, but not in vpu. The processing of the Env precursor to mature forms was not modified in any of the passages of either wild-type or nef mutant HIV-1. However, when compared with acute infections caused by similarly passaged virus of both wild-type and nef mutant HIV-1, persistent infections by passaged wild-type HIV-1 showed a significant decrease in the cell surface expression and function of Env. Cell surface CD4 was only partially down-regulated on cells acutely infected with the passaged viruses, whereas on cells persistently infected with passaged wild-type HIV-1 it was completely down-regulated. These results suggest that, during serial passage of HIV-1, mutations accumulate at least in the accessory genes vif and vpr in parallel with a lesser interaction between cell surface Env and CD4 molecules, and lead to the generation of less cytopathogenic viruses capable of persistent infection. Our results also suggest an important role for the nef gene product in the generation of HIV-1 strains that are less cytopathogenic.

Introduction

Human immunodeficiency virus type 1 (HIV-1), a member of the lentivirus subfamily of retroviruses, is lymphotropic and highly cytopathogenic (Popovic et al., 1984). Upon primary infection there is an initial viraemia, which is followed rapidly by an immune response and apparent viral clearance (Allain et al., 1986). Nevertheless, the continued presence of HIV-1 has been demonstrated in all HIV-1-seropositive individuals by PCR (Simmonds et al., 1990). Recently, an extraordinarily large number of latently infected cells was observed throughout the lymphoid system from early to late stages of infection (Pantaleo et al., 1993; Embretson et al., 1993). Thus, HIV-1 can remain clinically silent for nearly 10 years (Bacchetti & Moss, 1989), in a state of persistent infection, before the development of AIDS. A characteristic feature of the HIV-1 isolates that arise during this persistent period is their heterogeneity in terms of cytopathogenicity (Fenyo et al., 1988; Tersmette et al., 1988). The mechanisms that act after HIV-1 primary infection to regulate either cell death or latency and/or persistence in the infected host cells are, therefore, essential for our understanding of the pathogenesis of the disease.

We have previously reported an abnormal HIV-1 life cycle in persistently HIV-1-infected cells obtained from the human T cell lymphotropic virus type I-transformed MT-4 cell line or from its clonal derivative, M10, after infection with HIV-1 (Ikuta et al., 1988; Yunoki et al., 1991). In this system, infected cells were drastically reduced in number by the killing of single cells rather than by the induction of syncytia, a mechanism similar to that observed in normal peripheral blood mononuclear cells. However, a few surviving cells continued to grow at a significant rate and were found to have become persistently infected with HIV-1 (Ikuta et al., 1988). Surprisingly, the clones derived from these surviving cells
included both non-producers and producers of defective particles (Goto et al., 1990; Morita et al., 1990; Yunoki et al., 1991; Imai et al., 1991). These observations led us to use this novel cell system to study the mechanism of persistent HIV-1 infection associated with little or no cytopathogenicity. With this system, we have shown that infection by HIV-1 with mutations in accessory genes, such as vif, vpr or vpu, also generates similar persistent infection after transient cytolysis, whereas infection with a nef mutant (nef-1) or with wild-type HIV-1 causes complete killing of M10 cells (Nishino et al., 1991; Kishi et al., 1992, 1993).

Considerable genetic and phenotypic diversity in the HIV-1 strains isolated after 15 serial passages of a clonal virus population in MT-4 cell cultures was recently reported (Sanchez-Palomino et al., 1993). In this study, we sought to make use of such heterogeneity for the isolation of less cytopathogenic forms of HIV-1 following in vitro serial passages of a cytopathogenic wild-type and nef-1 HIV-1 in M10 cells. Our results showed that the rate of occurrence of persistent infection by wild-type HIV-1 increased with serial passage, whereas the HIV-1 nef-1 virus failed to initiate a persistent infection even after a large number of passages. The mechanism of persistent infection by passage of wild-type HIV-1 seems to be due to a lower interaction of HIV-1 Env and CD4 on the cell surface which, in turn, may be due to mutations in at least the accessory genes vpr and vif.

**Methods**

**Cells and viruses.** The M10 clonal cell line (Yunoki et al., 1991), which was derived from the MT-4 cell line (Miyoshi et al., 1982) was cultured in complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum) at 37 °C in a CO2 incubator. The inocula for infection by passage of wild-type HIV-1 were harvested, washed with PBS and lysed in 20 mM-Tris-HCl pH 8-3). The cell lysate was incubated with 100 μg/ml of proteinase K for 1 h at 55 °C and then further heated for 10 min at 100 °C. PCR amplifications of the viral sequences were carried out for 30 cycles, each consisting of 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C, using a PCR kit (Perkin Elmer Cetus) with primers vif1 (5' TCTTGCTCTCCTCTGTCGAGTA 3'; nucleotides 5518 to 5539) and env3 (5' CCCCATAATAGACTGTGACCCACA 3'; nucleotides 5806 to 5828) as a probe.

**PCR amplification and nucleotide sequencing.** Acutely or persistently HIV-1-infected cells were harvested, washed with PBS and lysed in DNA extraction buffer (0.5% Tween 20, 50 mM-KCl, 2.5 mM-MgCl2, 20 mM-Tris- HCl pH 8-3). The cell lysate was incubated with 100 μg/ml of proteinase K for 1 h at 55 °C and further heated for 10 min at 100 °C. PCR amplifications of the viral sequences were carried out for 30 cycles, each consisting of 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C, using a PCR kit (Perkin Elmer Cetus) with primers vif1 (5' GACATAAAAGTGATGCGCAAGA 3'; nucleotides 4995 to 5017), vpr1 (5' TCTTGCTCTCCTCTGTCGAGTA 3'; nucleotides 5828 to 5806), vif1 (5' AAGCCACCTTTGCCTAGTGTTA 3'; nucleotides 5551 to 5539) and env3 (5' CCCCCATAAAGACCTGCGCAACCACA 3'; nucleotides 6344 to 6320) as described previously (Saiki et al., 1988).

**Syncytium formation assay.** Syncytium formation was assayed by incubation of the human T cell line MOLT-4 (clone number 8; Kikukawa et al., 1986) with infected cells at a ratio of 10:1 as described previously (Kishi et al., 1992). After incubation for 24 h at 37 °C, the formation of syncytia was observed. Relative percentages of syncytia were calculated from the average of triplicate assays.

**Southern blot analyses.** These were carried out as described previously (Kishi et al., 1993). Total cellular DNA samples were digested with HindIII and then subjected to Southern blot hybridization using 32P-labelled HindIII fragments of the HIV-1 DNA insert of pNL432 (nucleotides 531 to 9600) as a probe.

**Immunoprecipitation and SDS–PAGE.** Acutely or persistently HIV-1-infected cells were labelled for 16 h with 100 μCi/ml of L-[35S]Protein Labelling Mix containing 77% methionine and 18% cysteine (1180 Ci/mmole; Du Pont NEN) in RPMI-1640 medium containing 10% of the normal concentrations of methionine and cysteine. A cell-free fraction was prepared by low-speed centrifugation, followed by centrifugation of the supernatant at 70000 r.p.m. for 30 min at 4 °C in a TLA-100.3 rotor (Beckman) to pellet virus particles and then ligated into a Bluescript II SK(-) vector (Stratagene). Nucleotide sequencing was carried out by the dideoxynucleotide chain termination method using the Sequenase version 2.0 kit (USB).

**Western blot analysis.** Western blotting was carried out as described previously (Goto et al., 1990). The cell lysates in 0.5% Nonidet P40 included both non-producers and producers of defective particles (Goto et al., 1990; Morita et al., 1990; Yunoki et al., 1991; Imai et al., 1991). These observations led us to use this novel cell system to study the mechanism of persistent HIV-1 infection associated with little or no cytopathogenicity. With this system, we have shown that infection by HIV-1 with mutations in accessory genes, such as vif, vpr or vpu, also generates similar persistent infection after transient cytolysis, whereas infection with a nef mutant (nef-1) or with wild-type HIV-1 causes complete killing of M10 cells (Nishino et al., 1991; Kishi et al., 1992, 1993).

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**Antibodies.** Anti-HIV-1 antibodies, murine monoclonal antibodies (MAbs) to Gag p24 (V107; Ikuta et al., 1989), Env gp120 (0.5; Matsushita et al., 1988) and Nef 3' ORF (SS; Du Pont NEN) and the serum from an HIV-1 seropositive subject were used. OKT4 (Ortho Diagnostic Systems) was used for the recognition of the V3-V4 domain of CD4 (Berger et al., 1988).

**Isolation of serial passages of HIV-1.** The M10 cells were infected for 1 h with wild-type and nef-1 HIV-1 in the conditioned medium from the transfected SW480 cells. After being washed, the infected cells were cultured in complete medium at 37 °C for 2 to 4 days. Similarly, the HIV-1 in the conditioned medium was serially passaged at an m.o.i. of 0.1 to 1, up to 20 times for wild-type HIV-1 and up to 50 times for nef-1 HIV-1. These passages were designated wt-1 to wt-20 and nef-1 to nef-50, respectively.
Cytopathogenicity of HIV-1 in serial passage

Fig. 1. Kinetics of replication and cytopathogenic properties of serial passages of wild-type and nef- HIV-1. M10 cells were mock-infected or infected with wt-1, wt-4, wt-12, wt-20, nef-1, nef-10, nef-20, nef-30, nef-40 or nef-50 at an m.o.i. of 0.01 for 1 h at 37 °C. After being washed, the cells were seeded at 5 × 10^5/ml in complete medium. The media were replaced with fresh complete medium 4 days after infection and thereafter every 3 days. The viable cells were counted 2 and 4 days after infection and thereafter every 3 days by the trypan blue dye exclusion method. Similarly, the percentage of HIV-1 antigen-positive cells in the viable cell population was determined by the IF test with serum from an HIV-1-seropositive subject.

were subjected to SDS-PAGE and the proteins in the gel were electrophoretically blotted onto nitrocellulose sheets.

Flow cytometry. The binding activities of MAbs to the cell surface of infected cells were analysed by flow cytometry as described previously (Ohki et al., 1992). Briefly, cell pellets were suspended in 25 μl of appropriately diluted murine MAb and the mixture was incubated at 4 °C for 30 min. After being washed twice with RPMI-1640 supplemented with 5% fetal bovine serum, the cells were incubated with a 40-fold dilution of FITC-conjugated anti-mouse IgG (Dakopatts). After incubation at 4 °C for 30 min, the cells were washed with the same medium, fixed with 5% formalin in PBS and then analysed with a Becton Dickinson FACScan system.

Results

Generation of persistent infection by serial passage of wild-type HIV-1, but not by nef- HIV-1

The cell killing activities of a sample from each serial passage of wild-type and nef- HIV-1 were determined in M10 cells. M10 cells were infected at the same m.o.i. (0.01) and, after incubation at 37 °C for 1 h, were cultured at a cell density of 5 × 10^5/ml in complete medium. Representative results for the kinetics of killing shown by wild-type HIV-1 (wt-1, -4, -12 and -20) and nef- HIV-1 (nef-1, -10, -20, -30, -40 and -50) are shown in Fig. 1.

The appearance of IF assay-positive cells was observed first at 2 days post-infection for all virus infections. Thereafter, the proportion of IF assay-positive cells rose to almost 100% by 4 days post-infection for all viruses. RT activity in the conditioned media showed kinetics very similar to those of the IF test (Fig. 2). In particular, the RT activity in the conditioned medium of cells infected with nef- viruses was only transient in all serial passages. Nonetheless, all of the HIV-1 viruses examined initially replicated similarly in M10 cells. However, M10 cells increased in number 19, 16 and 13 days post-infection with wt-4, wt-12 and wt-20 passaged virus respectively, although they were completely killed by wt-1 infection. Thereafter, cells derived from these infections showed continuous proliferation, continuous expression of HIV-1 antigens (Fig. 1) and continuous production of particles with RT activities (Fig. 2), indicating that they had
become persistently infected with HIV-1. These cell cultures were termed M10/wt-4, M10/wt-12 and M10/wt-20 respectively.

These results suggested that the survival of cells following infection with wild-type HIV-1 became more likely with the passage number of the infecting viral stock. Moreover, when the HIV-1 particles recovered from persistently infected M10/wt-4 and M10/wt-20 cells were assayed for their abilities to kill M10 cells, it was found that their cytopathogenicity was greatly reduced (Fig. 3), although the original wt-4 and wt-20 viral stocks remained strongly cytopathogenic (Fig. 1).

Fig. 2. Kinetics of appearance of RT activity in culture medium of M10 cells infected with serial passages of wild-type and nef- viruses. The RT activities of the culture media prepared from the same cultures of M10 cells, mock-infected or infected with wt-4, wt-20, nef-10 or nef-50 that were used for the experiments shown in Fig. 1 were determined and visualized on X-ray film.

Fig. 3. Almost complete loss of cell killing ability in HIV-1 particles produced in M10 cells persistently infected with wt-4 and wt-20. M10 cells were mock-infected or infected with HIV-1 from the culture medium of persistently infected cells M10/wt-4 or M10/wt-20 at an m.o.i. of 0.01 at 37 °C for 1 h. The cell numbers were adjusted to 5 × 10⁶/ml in fresh complete medium 4 days after infection and thereafter every 3 days. The viable cell number and the percentage of HIV-1 antigen-positive cells were determined as described in the legend to Fig. 1.

Fig. 4. Western blot analysis of Nef protein in cells acutely or persistently infected with serial passages of wild-type or nef- HIV-1. Lysates of uninfected M10, acutely wt-20-infected M10 [wt-20(A)], M10/wt-20 [wt-20(P)], and acutely nef-50-infected M10 [nef-50(A)] cells were separated by SDS–PAGE. The proteins were blotted onto a nitrocellulose membrane and those which reacted with anti-Nef or anti-Gag p24 murine MAb were detected using a biotin–avidin system. The Mᵣ values of the proteins were calculated by comparing their mobilities with those of marker proteins using a calibration kit for Mᵣ determination (Pharmacia-LKB).
Cytopathogenicity of HIV-1 in serial passage

These results demonstrate that wild-type HIV-1 stocks are mixtures of a cytopathogenic and less cytopathogenic form of the virus, and that the proportion of viruses with lowered cytopathogenicity increases gradually with serial passages and they are amplified in the generation of a persistent infection. In the absence of selection imposed by the generation of persistence, the low cytopathogenicity phenotype remains only a minor component of the population, even after 20 passages, since wt-20 infections could only produce survivor cells after gross cell killing (Fig. 1).

In contrast to the wild-type, none of the passages of the nef virus examined generated survivor cells within 22 days of culture, although the rate of cell death was delayed to some degree with serial passages (Fig. 1).

HIV-1 DNA analysis of cells acutely or persistently infected with HIV-1 serially passaged virus

The kinetics of replication and cytopathogenic activities of the serially passaged samples of wild-type and nef-HIV-1 imply that the nef gene product plays an important role in the generation of the less cytopathogenic HIV-1 variants that lead to persistent infection. Consistent with this, Western blot analysis showed that the level of expression of Nef in survivor M10/wt-20 cells was...
comparable with that in acutely wt-20-infected M10 cells (Fig. 4). However, there was no expression of any Nef-related protein in the acutely nef-50-infected M10 cells, although the same lysate contained a greater level of Gag proteins (Fig. 4). The dramatic difference in the levels of cytopathogenicity exhibited by serial passages of wild-type and nef-1 HIV-1 prompted us to examine the genome structure of virus from representative passages to assess any correlation between mutation(s) in certain HIV-1 gene(s) and the ability to generate persistent infection in M10 cells.

Samples of HIV-1 DNA in M10 cells acutely or persistently infected with serial passages of wild-type HIV-1 were characterized by Southern blot hybridization. The results indicated that, in M10/wt-20 cells, full-length HIV-1 DNA was present and was similar in structure, as far as could be determined, to HIV-1 DNA isolated from the cells acutely infected with wild-type and nef-1 HIV-1, irrespective of the passage number of the virus stock used (data not shown). Thus, mutation(s) that give rise to persistent infection in M10 cells with serially passaged wild-type HIV-1 are not gross and are likely to be small deletions or point mutations.

We previously reported that HIV-1 with mutations in accessory genes, such as vif, vpr or vpu, could generate persistent infections in M10 cells (Nishino et al., 1991; Kishi et al., 1992, 1993). Thus, mutations in these accessory genes might contribute to the generation of persistent infections by serial passage of wild-type HIV-1. To examine this possibility, DNA representatives of the vif, vpr and/or vpu genes present in M10/wt-20 cells were recovered for sequence analysis. DNA clones 1 to 4 and 5 to 9 were cloned from PCR products, amplified with primers vif1a/vpr1 and vif1b/env3 respectively, and their nucleotide sequence was determined (Fig. 5). Substitution of threonine (ACC codon) for isoleucine (AUC codon) in the vtf gene and truncation of the vpr gene by the appearance of stop codon TAG in place of TGG at nucleotides 5670 to 5672 were observed in clones 1 to 4. In addition, several other amino acid substitutions specific to each clone were observed. No substitution or truncation was observed in the vpu gene in clones 5 to 9, although a minor nucleotide substitution, from A to G, at nucleotide 6204 (phenotypically silent) was observed in clone 5. In contrast to the sequences derived from these persistently infected cells, no change was observed
The cell surface expression of gp120 and CD4 molecules and the syncytium-inducing ability of M10 cells acutely or persistently infected with serial passages of wild-type or nef- HIV-1. M10 cells were mock-infected or were acutely infected with wt-20 [wt-20(A)] or nef-50 [nef-50(A)] at an m.o.i. of 0.01 and cultured as described in the legend to Fig. 1. After incubation for 3 days, the cells were analysed by flow cytometry (a). Persistently wt-20-infected cells [wt-20(P)] were also similarly analysed (a). The antibodies used were 10 μg/ml of 0.5% MAb for gp120 and OKT4 MAb for CD4 ( ). As a control antibody, the same concentration of normal mouse IgG was used ( ··· ). The fluorescence intensity of the positive reaction in each figure was calculated. The relative percentages of these positive reactions are shown in parentheses. The same acutely and persistently infected cells were co-cultured with MOLT-4 clone number 8 at a ratio of 1:10 for 24 h (b). The relative percentages of syncytia, calculated from the average of triplicate assays, are shown.

HIV-1 protein analysis of cells acutely or persistently infected with HIV-1 serially passaged virus

We looked for a correlation between the HIV-1-expressed protein profile and the generation of persistent infection by higher numbers of passages of wild-type HIV-1. The HIV-1 proteins in persistently infected cells were compared with those in M10 cells acutely infected with different passages of wild-type and nef- HIV-1 stocks. HIV-1 proteins were analysed by immunoprecipitation with a polyvalent serum followed by SDS-PAGE (Fig. 6). The results showed that the Env proteins present in M10 cells, both acutely and persistently infected with wild-type or nef- HIV-1 stocks and irrespective of their in vitro passage history, were similarly cleaved into mature Env proteins. A slight increase in the mobility of
Env gp120 in acutely infected cells was observed, which correlated with passage number in wild-type HIV-1 (Fig. 6). In contrast, the mobility of gp120 in cells acutely infected with nef-50 was similar to that of gp120 in cells acutely infected with lower passages of wild-type HIV-1. Thus, the difference in size of gp120 appeared to be linked with serial passage of the wild-type HIV-1, but not with nef- HIV-1. However, this difference was also observed in cells persistently infected with the same passage numbers of virus (Fig. 6). Therefore, although this difference could be induced with serial passage, it cannot be the major reason for the change in cytopathogenicity.

Cell surface expression of Env gp120 and receptor CD4 on acutely or persistently infected cells

Several studies have strongly implicated the viral envelope as a key determinant of cytopathogenicity (Sodroski et al., 1986; Cheng-Mayer et al., 1988; Kowalski et al., 1991). Generally, there are two mechanisms for the killing of cells in HIV-1 infection: the killing of single cells or syncytium formation. Both of the mechanisms are mediated by an interaction between Env and the CD4 glycoprotein, which results in membrane fusion that destroys the integrity of the cell membrane and kills cells. The killing of a single cell, which is the mechanism predominantly observed in the killing of M10 cells, could occur by an autofusion reaction which might be induced in stocks of virus capable of persistent infection. Accordingly, the cell surface expression of gp120 and CD4 were compared in both persistently and acutely infected cells by flow cytometry (Fig. 7a). The results showed that the expression of gp120 was marginally lower on the surface of the M10 cells acutely infected with wt-20 but much lower on the surface of M10/wt-20 cells than on the surface of cells acutely infected with nef-50. In addition, the ability of these infected cell cultures to form syncytia showed an unexpected greater disparity compared to the difference in their cell surface expression levels. This is demonstrated by relative syncytium numbers of 64.3 and 100 % in acutely wt-20- and nef-50-infected cells, respectively, compared to only 6.3 % in M10/wt-20 cells (Fig. 7b). Furthermore, the partial down-regulation of CD4 on the cell surface following infection with either wt-20 or nef-50 was similar (Fig. 7a). By contrast, there was almost complete down-regulation of CD4 on the cell surface of cells persistently infected with wt-20 (Fig. 7a). Thus, the increased incidence of persistent infection with serial passages of wild-type HIV-1 correlates with a lower level of Env−CD4 interaction as a result of the reduced level of Env expression and a further disproportionate reduction in Env function at the cell surface.

Discussion

We have clearly demonstrated here a correlation between in vitro serial passage and the generation of persistent infections of M10 cells (Fig. 1 and 2). In addition, the HIV-1 particles produced by persistently infected cells were greatly reduced in their cytopathogenic character when compared with the original serial passages (Fig. 3). These results imply that one or more mutations in the HIV-1 gene or genes necessary for cell killing may accumulate during serial passage in vitro. Functional heterogeneity among HIV-1 isolates has been reported, including differences in replicative potential (Fenyo et al., 1988), cytopathogenicity (Fenyo et al., 1988; Tersmette et al., 1988) and host range (Evans et al., 1988). Our finding that acquisition of the capacity to generate persistent infection is dependent on in vitro serial passage leads us to suggest that there is a positive trend towards the selection of non-cytopathogenic or less cytopathogenic HIV-1 during their propagation.

Less cytopathogenic HIV-1 could be generated by the process of reverse transcription during serial passages of wild-type HIV-1, as a high error rate during reverse transcription is a characteristic feature of the life cycle of retroviruses (Preston et al., 1988). However, the rate of generation of less cytopathogenic HIV-1 is quite low even after 20 serial passages, as indicated by the kinetics of the appearance of persistently infected cells (Fig. 1). To determine the underlying cause of the increased likelihood of persistence with serial passage, we characterized the HIV-1 genome structure in persistently infected M10/wt-20 cells by selected PCR amplification followed by nucleotide sequencing (Fig. 5). The data showed that the HIV-1 genome derived from M10/wt-20 had accumulated mutations, at least in the accessory genes vif and vpr. Our recent results show that more extensive mutations in this region, including a large deletion of the vif to vpr region, predominate within persistently infected cultures derived from further serial passage of wild-type HIV-1 (T. Nakaya et al., unpublished results). These observations are in keeping with our previous findings that HIV-1 with mutations in the accessory genes vif, vpr or vpu lead to the generation of persistent infections in M10 cells (Nishino et al., 1991; Kishi et al., 1992, 1993). Moreover, these results imply that accessory gene products modulate the maturation and/or expression of Env proteins, as these are the eventual effectors of cell killing by their interaction with the CD4 receptor molecule (Koga et al., 1990). Consistent with this, in the experiments described here, persistent infections by passaged wild-type HIV-1 reduced the level of cell surface Env expression (Fig. 7a), although its maturation from gp160 to gp120–gp41 was not affected (Fig. 6). Syncytium formation assays using
persistently infected cells showed a far greater reduction in Env-mediated fusion than the level of Env antigen (Fig. 7b) would have suggested, possibly due to a threshold effect for the triggering of fusion. Finally, the level of down-regulation of the CD4 molecule on the surface of cells persistently infected with serially passaged wild-type HIV-1 was almost complete (Fig. 7a). Thus, it seems plausible that the accessory gene mutations we have observed following serial passage of wild-type HIV-1 resulted, indirectly, in a lower level of interaction between Env and CD4 on the cell surface and this, in turn, allowed the generation of persistent HIV-1 infection.

HIV-1 viral stocks with reduced cytopathogenicity were generated during serial passage only in the presence of Nef protein, as nef− virus did not generate persistent infections even after 50 passages (Fig. 1). The corollary observation, that Nef expression was maintained in cells persistently infected with wild-type HIV-1 at similar level to cells acutely infected with wild-type HIV-1, was also clear (Fig. 4). The nef gene has been reported to be involved in the down-regulation of CD4 from the cell surface (Garcia & Miller, 1991). In addition, it has also been shown that, although the presence of Nef protein does not modify the processing of Env gp160, it does result in a significant decrease in cell surface levels of gp120 and an associated dramatic reduction in fusion-mediated cell death (Schwartz et al., 1993). The data presented here are entirely in accord with these reports and, taken together, it would seem that Nef has a role in the down-regulation of both Env and CD4 from the cell surface and that this can prevent fusion-mediated cell death. However, in our experiments, CD4 down-regulation during acute infections was marginally stronger in the absence of Nef, as revealed by flow cytometry (Fig. 7a). This result appears to contradict the recent data of Aiken et al. (1994), who showed that nef− failed to induce any down-regulation of cell surface CD4 at early times after infection. This discrepancy is presumably due to the action of Env (Jabbar & Nayak, 1990) and Vpu (Chen et al., 1993; Vincent et al., 1993), because a higher level of Env protein was identified in cells acutely infected with nef− passaged virus, compared to cells acutely infected with wild-type passaged virus (Fig. 6 and 7). In persistently infected M10/wt-20 cells however, surface CD4 was almost completely down-regulated (Fig. 7a), most probably as a result of combined Nef, Env and Vpu interactions with CD4. Similarly, Env protein levels on persistently infected cells were reduced and were disproportionately less functional when compared with those on the cells acutely infected with wild-type or nef− passaged virus. The observation that less cytopathogenic HIV-1 forms were only minor components in the passaged acute phase virus (Fig. 1) and that accessory gene mutations increased this proportion suggests that the failure to down-regulate completely CD4 from the cell surface might be due to a direct role of the vif and/or vpr gene products in CD4 down-regulation. However, an indirect effect through Env antigen is, perhaps, more likely.

Kestler et al. (1991) have reported that a nef mutation in simian immunodeficiency virus (SIVmac) had no significant influence on its replication in cultured cells. In contrast, when rhesus monkeys were infected with a mutant that had a deletion in the nef gene, the virus failed to cause simian AIDS. Their conclusion, that nef is required for the maintenance of high virus loads during the course of persistent infections in vivo, which seems to be a prerequisite for AIDS development, is supported and extended by our data. In the cell model we have described, the Nef gene product is essential for the generation of persistently infected cultures by a small proportion of the virus population and this proportion increased when the virus accumulated accessory gene mutations. The exact role of Nef in allowing the accumulation of accessory gene mutations is still under study in our laboratory and the further study of our cell model should shed more light on the requirement for persistent infections and, more importantly, the factors required to trigger the onset of AIDS.

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Cytosvin of HIV-1 in serial passage 2249


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