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Role of CD4 Endocytosis in Human Immunodeficiency Virus Infection

ANNEGRET PELCHEN-MATTHEWS,[1] PAUL CLAPHAM,[2] AND MARK MARSH[1]*

Medical Research Council Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, London WC1E 6BT,[1] and Chester Beatty Laboratories, Institute of Cancer Research, London SW3 6JB,[2] United Kingdom

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We have analyzed the role of CD4 endocytosis in human immunodeficiency virus (HIV) entry by measuring the infection of HeLa cells expressing various CD4 constructs with endocytosis rates of between 0.2 and 30%/min in a quantitative infectious focus assay. For a number of laboratory-adapted HIV-1 and HIV-2 strains, the highest levels of infection were found on cells with very limited CD4 endocytosis, while cells with efficient CD4 uptake were only poorly infectable, suggesting that CD4 internalization is not required for HIV entry. This was confirmed in a modified assay involving prebinding of HIV-1_{LAI} to HeLa-CD4 cells at 4°C, synchronized virus entry during warming to 37°C, and neutralization of virions remaining at the cell surface with anti-V3 loop antibodies. Warming cells in hypertonic medium inhibited CD4 endocytosis but did not affect the rate or the extent of infection. These studies confirm that HIV infection does not require endocytosis and that laboratory-adapted virus strains can enter HeLa-CD4 cells by fusion at the plasma membrane.

The cell surface glycoprotein CD4, which has been identified as the principal receptor of the primate immunodeficiency viruses (human immunodeficiency virus type 1 [HIV-1] and HIV-2 and simian immunodeficiency virus [10, 20, 23, 28, 38]), can be internalized via the endocytic pathway in all cell lines examined to date (26). However, the rate and extent of CD4 endocytosis vary with the cell type in which CD4 is expressed, and CD4 can be modulated under various physiological or experimental conditions (26). Differences in constitutive CD4 endocytosis in various cells are primarily due to the interaction of CD4 with the *src*-related protein tyrosine kinase $p56^{lck}$. Thus, in $p56^{lck}$-negative nonlymphoid cells (e.g., cells of the macrophage/monocyte lineage or CD4-transfected HeLa cells) CD4 is internalized efficiently into early endosomes and is recycled to the cell surface (31, 32). In contrast, in lymphoid cells, $p56^{lck}$ interacts with the cytoplasmic domain of CD4 and prevents CD4 endocytosis (31, 33). The interaction of CD4 and $p56^{lck}$ and the endocytic trafficking of CD4 are controlled by phosphorylation events. In particular, activation of protein kinase C by various physiological or experimental stimuli (e.g., treatment with phorbol esters) leads to dissociation of $p56^{lck}$ and CD4 and enhanced CD4 endocytosis (see reference 26).

Despite these trafficking properties of CD4, the observations that (i) HIV *Env*-expressing cells can form syncytia with uninfected $CD4^+$ cells (see, for example, reference 27), (ii) HIV entry is pH independent (27, 45), (iii) cell lines expressing CD4 molecules that do not undergo phorbol ester-mediated down-regulation can be infected by HIV (2, 24), and (iv) entry of HIV does not appear to induce the internalization or down-regulation of cell surface CD4 (29) have led to the assumption that HIV and simian immunodeficiency virus infect cells by fusion at the cell surface. However, the experiments reported to date did not examine infectious virus particles during entry and did not exclude endocytosis as a component of the entry mechanism of these viruses. First, mutational and other studies indicate, for example, that the molecular requirements for the fusion events involved in syncytium formation between HIV-infected and CD4-expressing cells differ from those required for virus fusion (see, for example, references 21, 42, and 46). Second, the pH independence of HIV infection simply indicates that an acidic environment is not essential to trigger fusion and that fusion may occur at either endosomal or neutral pH (27). Third, it is now apparent that the ability of CD4 mutants to be down-modulated after phorbol ester treatment is not a good indicator of whether these molecules can be constitutively internalized. Thus, CD4 molecules lacking the entire cytoplasmic domain will undergo constitutive endocytosis, albeit at rates slower than those measured with phorbol ester-treated cells (31, 32, 34), and in T cells expressing $p56^{lck}$, tailless CD4 molecules are internalized more efficiently than full-length CD4 (31). Furthermore, CD4 and $p56^{lck}$ can be dissociated by agents such as phorbol esters (15, 34) or HIV gp120 (4, 16), leading to enhanced CD4 endocytosis. Finally, although fusion figures for HIV have been observed at the cell surface by electron microscopy (13, 35, 45), HIV virions are also frequently associated with endocytic clathrin-coated vesicles, and internalized virions can be observed in the process of fusion (1, 13, 30, 35, 37). Since only a small proportion of the virions in HIV preparations are actually infectious (22), it is unclear whether the viruses observed in these images would have gone on to establish productive infections. Thus, an infection pathway involving endocytosis remains a formal possibility for HIV; indeed, an endocytic route might present advantages to the virus under certain circumstances (see reference 25).

Here, we have directly addressed the question of whether endocytosis plays a role in the infectious pathway of HIV, either by attempting to correlate CD4 endocytosis rates with infection efficiencies or by assaying infection under conditions in which CD4 endocytosis was inhibited.

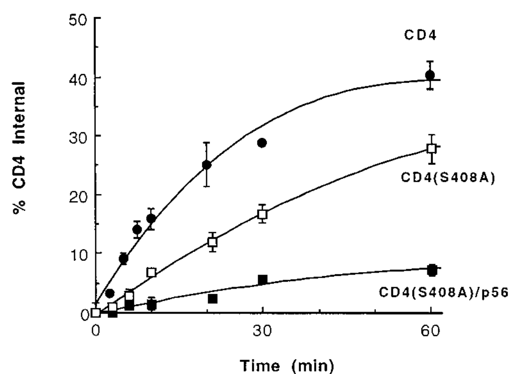
HIV infection of HeLa cell lines expressing various CD4 constructs. To determine whether the endocytic properties of CD4 influence HIV entry, we have studied HeLa cell lines stably transfected with wild-type CD4 (23) or a mutant CD4 in which Ser-408 was replaced by Ala [CD4(S408A)] (24) and

* Corresponding author. Mailing address: Medical Research Council Laboratory for Molecular Cell Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom. Phone: 171 380 7807. Fax: 171 380 7805. Electronic mail address: m.marsh@ucl.ac.uk.

grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% fetal calf serum, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, and 1 mg of G418 per ml (Gibco BRL Life Technologies, Paisley, Scotland). In addition, a HeLa-CD4(S408A)/p56^{lck} cell line was generated by transfection of the expression plasmid pSM containing the murine p56^{lck} cDNA (47) into HeLa-CD4(S408A) together with the pBabe/hygro vector containing the gene for hygromycin resistance essentially as described previously (33). Clones were selected by growth in media containing 0.2 mg of hygromycin B per ml (Sigma Chemical Company Ltd., Poole, Dorset, United Kingdom) and were screened for p56^{lck} expression by immunofluorescence and Western blotting (immunoblotting) essentially as described previously (33).

CD4 endocytosis on these cell lines was measured with the anti-CD4 monoclonal antibody (MAb) Leu3a (Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom), which was radioiodinated as described previously (31). Briefly, cell surface CD4 was labelled by incubating of the cells for 2 h on ice with 0.3 nM ^{125}I-Leu in binding medium (RPMI 1640 medium containing 0.2% bovine serum albumin and buffered with 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] to pH 7.4) and washing and warming to 37°C for various times to permit endocytosis. For analysis, cells were chilled again on ice, the ^{125}I-Leu3a remaining at the cell surface was removed by washing in medium acidified to pH 2, and the amount of acid-resistant (internalized) ^{125}I-Leu3a was determined by γ-counting (Fig. 1A). HeLa cells expressing the full-length CD4 molecule showed constitutive CD4 endocytosis at rates of approximately 2 to 3%/min, and at steady state, about 40% of the cellular CD4 was found in endosomes (see also reference 32). The mutant CD4 molecule in which the most prominently phosphorylated cytoplasmic serine residue, Ser-408, is replaced by alanine [CD4(S408A)] showed reduced constitutive endocytosis: uptake was at 0.8 to 0.9%/min but still reached 30 to 40% within 1 h. In the HeLa cells expressing CD4(S408A) together with murine p56^{lck}, CD4 endocytosis was almost completely inhibited (Fig. 1A). Further analysis demonstrated that while endocytosis of the wild-type CD4 molecule can be stimulated, the S408A mutation removes a critical residue in the cytoplasmic domain which has been shown to be phosphorylated in response to phorbol ester (41) and is involved both in CD4 endocytosis and in the dissociation of p56^{lck} from CD4 (3, 43). Thus, CD4(S408A) exhibits only a little and CD4(S408A)/p56^{lck} exhibits no phorbol ester-induced down-regulation (data not shown).

The susceptibility of these CD4-transfected HeLa cell lines to HIV was examined with an infectious focus assay (5) adapted for β-galactosidase staining. Cells were plated onto 24-well plates (16-mm-diameter tissue culture wells) at 1×10^4 to 2×10^4 cells per well 1 to 2 days before the experiment. Cells were washed in DMEM before incubation in a final volume of 200 μl of serial 10-fold dilutions of tissue culture supernatant virus stocks of HIV-1$_{LAI}$ (IIIb [49]), HIV-1$_{RF}$ (36), HIV-1$_{CBL-4}$ (50), HIV-2$_{CBL-20}$ (40), and LAV-2$_{ROD}$ (8) prepared as described previously (6, 7, 40) and stored in aliquots under liquid N_2. After 1 h of infection, cells were washed extensively in DMEM before being recultured for 4 days. Infected cells were identified after fixation in methanol-acetone (1:1 [vol/vol]) at −20°C for 10 min and staining for p24 with a mixture of the anti-HIV-1 gag MAbs 38:96:K and EF7 (provided by B. Wahren through the Medical Research Council AIDS Directed Programme Reagents Programme, reference no. ADP365 and ADP366) or HIV-2-positive human serum from The Gambia (Medical Research Council AIDS Directed Programme Reagents Programme) followed by sheep anti-



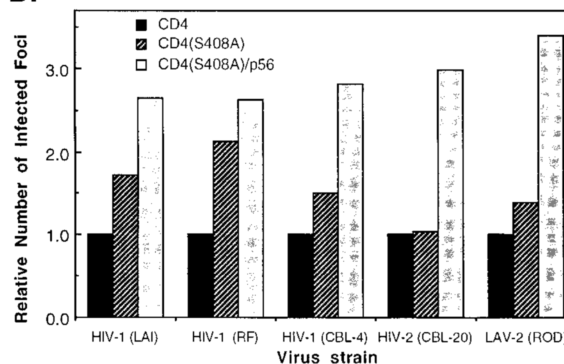


FIG. 1. (A) Time courses of CD4 endocytosis on HeLa cell lines expressing wild-type CD4 (●), CD4(S408A) (□), or CD4(S408A)/p56^{lck} (■). The plots show the ratios of acid-resistant (internal) ^{125}I-Leu3a to the total cell-associated label after various times at 37°C. There was no significant degradation of the ^{125}I-Leu3a tracer during the course of the experiment: trichloroacetic acid soluble counts in the warm-up medium after 1 h accounted for 1.6, 2.1, and 1.2% of the activity initially bound for cells expressing wild-type CD4, CD4(S408A), and CD4(S408A)/p56^{lck}, respectively. (B) Infection of these HeLa cell lines by various HIV-1 and HIV-2 strains. HeLa cell lines expressing wild-type CD4, CD4(S408A), and CD4(S408A)/p56^{lck} were incubated with serial 10× dilutions of virus-containing tissue culture supernatants for 1 h at 37°C, washed, and recultured for 4 days before analysis of infected cell foci by immunostaining. The numbers of infected foci per well were counted and are shown relative to the number of foci observed on HeLa-CD4 cells (41,500, 61,000, 520, 5,700, and 61,000 for HIV-1$_{LAI}$, HIV-1$_{RF}$, HIV-1$_{CBL-4}$, HIV-2$_{CBL-20}$, and LAV-2$_{ROD}$, respectively).

mouse or anti-human F(ab')$_2$ fragments linked to β-galactosidase (Amersham International, Little Chalfont, United Kingdom). Development with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Novabiochem, Nottingham, United Kingdom) thus yielded blue-stained infected cell foci.

For quantitative analysis of infection, the numbers of stained cell foci (single or small groups of stained cells separated from further stained cells by at least one layer of unstained cells) in the entire 16-mm-diameter tissue culture well were counted by scanning the well under low magnification (×100) with a tissue culture microscope. Only focus densities up to about 250 foci per well can be accurately counted by this method. To compare the infection frequencies in different wells, the number of foci observed in a given well was multiplied by the virus dilution used to give an extrapolation to the total infectious potential of the virus in number of foci per well (if cell number had not been limiting). In most experiments, optimum focus numbers (5 to 250 foci per well) were obtained for two virus dilutions, and the estimates of the total infectious potential for these

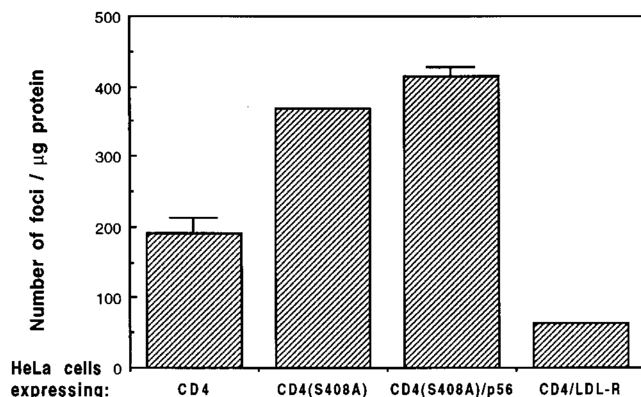


FIG. 2. HIV infection of HeLa/LDL-R cells. Infection of HeLa cell lines expressing wild-type CD4, CD4(S408A), CD4(S408A)/p56^{lck}, or CD4/LDL-R by HIV-1_{LAI} is shown. Cells were incubated for 1 h on ice with serial 10× dilutions of virus-containing tissue culture supernatants, washed, and cultured for 4 days at 37°C. AZT was added after 18 to 20 h to prevent secondary infections. Infected cells were identified by immunostaining and counted as the number of foci per well. The fixed and stained cell monolayers were then scraped into 250 μl of a prewarmed (≈50°C) mixture of 2% sodium dodecyl sulfate in 1 M NaOH, pooled with a 250-μl H_2O rinse of the tissue culture wells, and heated for 2 h at 80°C. Aliquots of these cell lysates were assayed for protein with the bicinchoninic acid assay (44). The data are shown relative to the protein content per well to correct for variations in confluence.

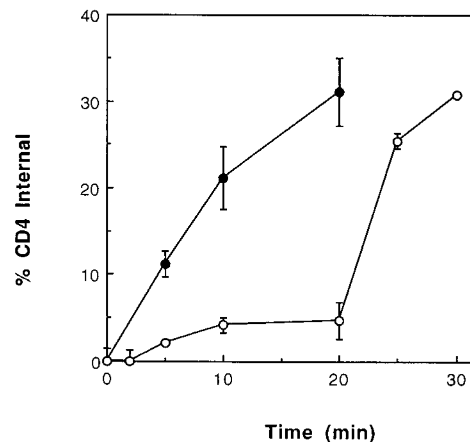


FIG. 3. Inhibition of CD4 endocytosis in hypertonic medium. CD4 endocytosis in DMEM (●) or hypertonic DMEM containing 0.45 M sucrose (○) was measured with the anti-CD4 MAb Q4120 (14) (supplied by Q. J. Sattentau through the Medical Research Council AIDS Directed Programme Reagents Programme and radioiodinated essentially as described previously [32]). After 20 min, the sucrose was washed away and the cells were reincubated in normal DMEM. The plot shows the ratios of acid-resistant (internal) ^{125}I-Q4120 to the total cell-associated label after various times at 37°C.

wells were usually in reasonable agreement. The extrapolated infectious potential on the basis of focus counts from several wells incubated with different virus dilutions under otherwise identical conditions could therefore be averaged to give the number of total foci per 16-mm-diameter well.

By these assays with the tissue culture-adapted HIV-1 and HIV-2 strains, the levels of infection were found to be inversely related to the amount of CD4 endocytosis on the different cell lines (Fig. 1B). Thus, with the exception of HIV-2_{CBL-20}, the number of foci on HeLa-CD4(S408A) cells was 1.5 to 2× that on HeLa-CD4 cells, even though the two cell lines expressed comparable numbers of CD4 molecules (approximately 2 × 10^5 per cell). The HeLa-CD4(S408A)/p56^{lck} cells expressed twice the level of CD4 at the cell surface and showed 2.5 to 3.5 times the number of foci as HeLa-CD4 cells. Hence, virus infection was enhanced on the cell lines showing the lowest levels of constitutive CD4 endocytosis, independently of the level of receptor expressed at the cell surface.

To extend these observations, we studied the infectability of a HeLa cell line expressing a chimera consisting of the extracellular and transmembrane domains of CD4 fused to the cytoplasmic domain of the low-density lipoprotein receptor (LDL-R [provided by J. Hoxie, University of Pennsylvania, Philadelphia, and grown in DMEM containing 10% fetal calf serum]). This molecule is internalized very efficiently at a rate of about 30%/min (39). Cells were seeded in 16-mm-diameter tissue culture wells, cooled on ice, and incubated with serial dilutions of HIV-1_{LAI} for 1 h before being washed and recultured at 37°C. To prevent cycles of secondary infections, zidovudine (AZT [5 μg/ml]) was added to the cultures after 18 to 20 h. After 4 days, cells were fixed and stained for p24 expression and developed with X-Gal. As shown in Fig. 2, the CD4/LDL-R chimera did act as a receptor for HIV-1_{LAI}, although the number of infected cell foci observed was much lower than that found on any of the other CD4-transfected HeLa cell lines. One consequence of the rapid endocytosis of CD4/LDL-R is that the cell surface expression levels of the chimera are very low, and this molecule is located primarily in early endosomes (data not shown). Furthermore, the HeLa cells transfected with the CD4/LDL-R chimera are of a more rapidly replicating strain than the other HeLa cell lines. Thus, although these data are consistent with the observation that HIV infection is less efficient on cells that exhibit rapid CD4 uptake, the result may at least in part be explained by the different growth rate of these cells or the lower levels of CD4 expression.

Thus, despite extremely low endocytosis rates in some cases, the CD4-expressing HeLa cells could be infected with a number of HIV-1 and HIV-2 strains. Infection did not correlate with differences in the density of CD4 expressed on the cells. Instead, infection was most efficient on the cells with the slowest rates of CD4 endocytosis, suggesting not only that endocytosis was not required for infection but also that high rates of CD4 endocytosis might reduce the infectivity of the viruses. While a number of virus strains were used in these experiments, all were tissue culture-adapted isolates which can replicate in a wide range of cell lines. The possibility that primary isolates or nonsyncytial HIV strains, which infect a more restricted set of target cells, may exploit the endocytic pathway cannot be excluded.

Inhibition of CD4 endocytosis during HIV infection. In order to focus directly on the effects of CD4 endocytosis on HIV infection, we designed experiments to measure the infection efficiencies of HIV-1_{LAI} under conditions in which CD4 internalization was inhibited. CD4 endocytosis occurs through clathrin-coated pits (17, 31), and treatments which disrupt coated pit function (see reference 48) inhibit CD4 endocytosis (34). In our hands, the most reliable method of inhibiting CD4 endocytosis is incubation in hypertonic medium (containing either 0.45 M sucrose or 0.225 M NaCl [33a]). For example, on HeLa-CD4 cells, CD4 endocytosis can be inhibited by about 90% when cells are warmed in hypertonic medium, and yet CD4 uptake quickly recovered when the sucrose was washed away and the cells were returned to normal medium (Fig. 3). Incubation in medium containing 0.45 M sucrose also inhibits phorbol ester-induced endocytosis of CD4 (34), the receptor-mediated endocytosis of transferrin, and fluid-phase endocytosis (33a), indicating that, in contrast to some other cells (9),

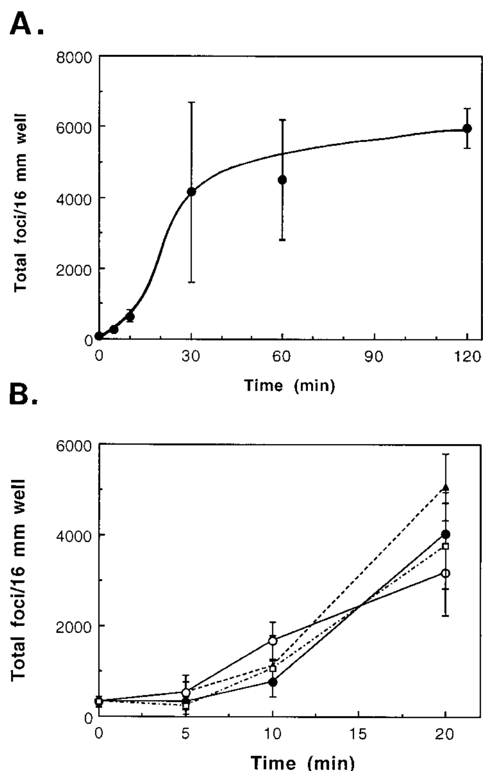


FIG. 4. Infection of HeLa-CD4 cells warmed in DMEM or in hypertonic DMEM. Data from focus analysis are shown. (A) HeLa-CD4 cells were incubated on ice with serial 3× dilutions of HIV-1_{LAI}, unbound virus was removed by washing, and the cells were incubated at 37°C. At various times, the warm medium was withdrawn and the cells were incubated in 200 μl of a 1:100 dilution of ALAV-110.5 ascites for 1 h. The cells were washed and recultured at 37°C for 4 days (with AZT added after 18 to 20 h) before fixation and infected cell focus analysis. (B) HeLa-CD4 cells were infected with HIV-1_{LAI} as described above, but warmed to 37°C for various times in normal DMEM (●, ▲, □) or in DMEM containing 0.45 M sucrose (○). To control for cell death caused by the hypertonic medium, infection time courses for cells treated with 0.45 M sucrose 30 min before (▲) or after (□) the infection experiment are also shown.

HeLa-CD4 cells are not able to compensate for the loss of clathrin-mediated endocytosis in the short term. Unfortunately, treatments that inhibit coated pit function and endocytosis can have pronounced effects on long-term cell survival. Thus, cells treated at 37°C for as little as 30 min recovered normal endocytic properties when returned to DMEM, but many of these cells died during the following 24 h of culture.

These difficulties with cell survival after treatment with hypertonic medium required an assay in which the exposure of cells to the sucrose medium could be minimized. To achieve this, cells were incubated with serial 3-fold dilutions (10×, 30×, 90×, 270×, and 810×) of HIV-1_{LAI}-containing tissue culture supernatant on ice, in which both CD4 endocytosis and HIV fusion are inhibited (11, 12). Virus binding to HeLa-CD4 cell monolayers, as assayed by the production of infected cell foci, appeared to saturate within 1 h at 4°C and was not pH dependent, since comparable levels of infection were observed when cells were incubated with the virus in media adjusted to pH 5.5 to 8 (data not shown). The infections observed were reduced by >95% when a 1:100 dilution of ascites of the neutralizing anti-V3 loop MAb 110.5 (19) (purchased from Genetic Systems, Inc., Redmond, Wash.) was included in the reculture medium (Fig. 4).

To assess the role of endocytosis in HIV infection, HeLa-CD4 cells were therefore incubated with HIV-1_{LAI} for 1 h on ice, and unbound virus was washed away before the cells were warmed to 37°C for defined periods. After this incubation, any unfused virions remaining at the cell surface were neutralized by incubation with the MAb 110.5. Cells were recultured for 4 days, with AZT (5 μg/ml) added after 18 to 20 h to prevent secondary infections, before fixing and identification of infected cell foci by staining for p24 as described above. Each focus of stained cells can thus be assumed to be derived from a single infection event during the 37°C virus entry time window. A typical infection time course is shown in Fig. 4A. This entry time course fits a sigmoid curve; thus there was a lag of 5 to 10 min before significant numbers of infected foci could be detected, while maximal levels of infection were observed after 30 min to 1 h of warming. When cells were warmed in the presence or absence of hypertonic medium, escape from the anti-V3 loop antibody via endocytosis or via direct fusion with the plasma membrane could be distinguished. There was no difference in the number of infected cell foci observed if cells were warmed for short periods (5, 10, or 20 min) in normal DMEM or in medium containing 0.45 M sucrose (Fig. 4B). To control for other effects of the hypertonic medium (e.g., on cell growth), cells were either pretreated with hypertonic medium before the experiment or were exposed to hypertonic medium after the virus entry step. Neither of these protocols had a significant effect on the level of infection observed (Fig. 4B). Longer incubations in hypertonic medium led to significant cell death and precluded further analysis.

These data, together with previous studies (2, 24, 29), indicate that, at least in this experimental system (HIV-1_{LAI} on HeLa-CD4 cells), endocytosis is not required for virus entry, and the fusion events leading to productive infection occur at the cell surface. The lag observed in the infection time course (Fig. 4A) therefore cannot be due to endocytosis but must reflect the time required for the molecular rearrangements at the cell surface that are a prerequisite for fusion.

Interestingly, inhibition of CD4 endocytosis by hypertonic medium did not increase the number of infected cell foci on HeLa-CD4 cells. The finding that HIV infection was more efficient on cell lines expressing poorly endocytosed CD4 constructs or in the presence of $p56^{lck}$ therefore suggests that CD4 engagement and the induction of the conformational changes that lead to fusion require a more stable expression of CD4 at the cell surface. Thus, reduced CD4 endocytosis, which lengthens the residence time of CD4 in the plasma membrane, perhaps together with the proposed $p56^{lck}$-mediated anchoring to the cytoskeleton (18, 33), may be advantageous for HIV entry.

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