Chemically Induced Infection of CD4-Negative HeLa Cells with HIV-1

Shinji Harada*,1,2, and Yosuke Maeda1

1Department of Biodefence and Medical Virology, Kumamoto University School of Medicine, and 2Center for AIDS Research, Kumamoto University, Kumamoto, Kumamoto 860-0811, Japan

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Abstract: Infection with human immunodeficiency virus type-1 (HIV-1) requires the presence of a CD4 molecule and chemokine receptors such as CXCR4 or CCR5 on the surface of target cells. However, it is still not clear how the virus enters the cells. Although CD4 was initially identified as the primary receptor for HIV-1, the expression of CD4 or one of the chemokine receptors alone is not sufficient to render susceptibility to infection with the virus. To ascertain whether or not adsorption of the virus needs charge-to-charge interaction between viral envelope and host cell membrane protein(s) and if binding alone promotes penetration of the virus into the cells, we have developed a chemically induced infection system targeting a CD4-negative and CXCR4-positive HeLa cell clone (N7 HeLa) which is usually not susceptible to infection with the LAI strain of HIV-1. Use of a poly-L-lysine (PLL)-coated culture plate to enhance the attachment of the virus to the cells made N7 HeLa cells infectable with HIV-1 at very low efficiency. PLL alone cannot fully substitute for the function of the CD4 molecule. However, trypsin-treated viruses, which have largely lost infectivity to CD4-positive MT-4 cells that are highly susceptible to HIV-1 infection, enhanced infectivity against N7 HeLa cells when the PLL-coated plate was used. These results provide evidence that infection with HIV-1 requires both high binding affinity between viruses and cells, and then needs a modification of the viral envelope such as cleavage of gp120/160 to enhance the infection, probably resulting in exposure of the hydrophobic fusion domain of gp41. HIV-1 infection of N7 HeLa cells was also enhanced by treatment with low pH, 12-O-tetradecanoylphorbol-13-acetate (TPA) and some factor(s) from the MT-4 cell culture supernatant. Not only tight viral adsorption with cleavage of the viral envelope but also some activated status of the cells may be required for sufficient HIV-1 infection in this artificial condition.

Key words: HIV-1, AIDS, Receptor, Poly-L-lysine

Many viruses have evolved to utilize molecules on cell membrane for adsorption on and penetration into the cell. The discovery that several chemokine receptors can serve as coreceptors for HIV-1, HIV-2 and simian immunodeficiency virus (SIV) highlighted the importance of their functions as an entry cofactor for envelope proteins from macrophage (M)-tropic, T-cell (T)-tropic and dual tropic strains of HIV and SIV (8, reviewed in reference 17). Although coreceptors may have initially served as primary receptors and use of CD4 is a more recent adaptation, the role of CD4 and cofactor with respect to entry into the cell remains unknown. In general, the high affinity interactions between virions and receptors induce both the attachment and conformational changes needed for penetration of the virus into the cell (reviewed in reference 7). In contrast, the low affinity binding requires a low pH trigger for the viral entry such as the spring-loaded mechanism to relocate fusion peptides as demonstrated in influenza virus (3, 4).

In the case of HIV-1, it was hypothesized and demonstrated recently by X-ray analysis (22, 34, 40) that the virus binds to a CD4 molecule and then induces a conformational shift of gp120 to uncover the binding site of the chemokine receptor. This is supported by the finding...
that the binding of gp120 to CXCR4 is enhanced by soluble CD4 (1). The oligomeric HIV envelope proteins are thereafter crosslinked by the CD4 and chemokine receptor leading to formation of a ternary complex consisting of gp120, coreceptor and CD4 on the surface of the target cell. This complex can induce further conformational change to release gp120 from virion (12, 27, 37, 39), which may expose the fusion domain of gp41 (19).

HeLa cells are not usually susceptible to HIV infection due to the lack of CD4 molecule on their surface. Recently, we have cloned HIV-1 susceptible (P6 HeLa) and non-susceptible (N7 HeLa) cells (29). The P6 HeLa cells exhibited both HIV-1 infection and transcytosis, which may be mediated by a glycolipid-like molecule recognized by C57 a9-9 monoclonal antibody (29). Since the activated state of adhesion molecule LFA-1 on the cell surface was reported to increase susceptibility to infection by ICAM-1-bearing HIV-1 (10, 11), the molecule on the P6 HeLa cells could positively affect the binding affinity of HIV-1 to the target cells.

These observations prompted us to examine whether non-susceptible N7 HeLa (CD4-negative and CXCR4-positive) cells could become infected with HIV-1 after the affinity of the binding of viruses or cells was enhanced by a chemical agent. This artificial infection system would be useful for step by step analyses of the early stage of HIV-1 infection.

Materials and Methods

Reagents. Poly-L-lysine (PLL; MW, 99,500) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma. PLL was dissolved in phosphate-buffered saline (PBS) at the concentration of 5 mg/ml, aliquoted in small volumes and stored at −20 C (15). TPA was stored in a small volume (10 µl/tube) at −80 C as a stock solution of 1.0 mg/ml in dimethyl sulfoxide until use (14). Trypsin (DIFCO) was also dissolved in PBS to make a 5% concentration as a stock solution and stored at −20 C. Fibronectin (FN) (RetroNectinTM, Takara Shuzo Co., Ltd., Japan) at the concentration of 1.0 mg/ml in double-distilled water was stored at −20 C. Polybrene (PB) was purchased from Aldrich Chemical Company Inc. and stored at the concentration of 5.0 mg/ml in PBS at 4 C until use.

Cells and viruses. N7 HeLa cells (29), HeLa-CD4-LTR-β-gal cells (20), human T-cell leukemia/lymphoma virus type-I (HTLV-I)-carrying MT-4 cells (15), MOLT-4 cells (25), U937 cells (35) and HIV-1 (LAI strain)-producer MOLT-4/HIV-1 cells (21) were maintained at 37 C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml of penicillin and 100 µg/ml of streptomycin.

MOLT-4/HIV-1 culture medium containing the LAI strain of HIV-1 was harvested after culturing for 4 days, filtered through a 0.45 µm filter to remove cells and centrifuged at 100,000×g for 1 hr at 4 C in a TLA-100.3 Beckman rotor. The viral pellet was resuspended in PBS to make approximately 7-fold-concentrated viral samples and applied to a PLL-coated culture plate. The amount of HIV-1 p24 core antigen in these concentrated viral samples was 500–600 ng/ml.

Trypsinization of HIV-1 was conducted by adding 5% trypsin into a filtered medium with 10% FCS from MOLT-4/HIV-1 culture to make a final concentration of 0.4 to 0.025%. Immediately after trypsin was added, the medium was ultracentrifuged for 1 hr at 4 C to obtain the viral pellet, which was then resuspended in PBS to make 7-fold-concentrated viral samples.

Cell-free supernatants were obtained after filtering 4-day-old culture medium from MT-4 cells, MOLT-4 cells and U937 cells through a 0.45 µm filter.

Multinuclear activation of a galactosidase indicator (MAGI) assay. Three-hundred microliters of 50 µg/ml PLL, PB or FN was added to each well of a tissue culture plate with 48 flat wells (Iwaki Glass, Japan) and incubated at room temperature for 1 hr. The treated plate was washed twice with PBS and 50 µl/well of concentrated virus in PBS was inoculated. The plate was then incubated at 4 C for 1 hr with rocking. HeLa-CD4-LTR-β-gal cells were adjusted to 4×10⁴/ml, and 500 µl of the cell suspension was seeded into each well (20, 31). The infected cells were then cultured for 2 days. After removing the culture medium, the cells were fixed with 1% formaldehyde-0.2% glutaraldehyde in PBS, washed twice with PBS and incubated with the staining solution (500 µg/ml of X-gal, 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide and 1 mM MgCl₂ in PBS). The number of infected and blue-stained cells was counted under a light microscope.

PLL-supported infection of N7 HeLa cells. A 24 flat-well plate (Iwaki Glass) was used. PLL was adjusted to 50 µg/ml, and 0.5 ml of the PLL solution was added to each well. After incubating for 1 hr at room temperature, the plate was washed twice with PBS and 100 µl of concentrated HIV-1 or trypsin-treated virus was applied in each well. Then, the plate was incubated at 4 C for 1 hr with rocking. One milliliter of N7 HeLa cells (4×10⁴/ml) was seeded into each well, incubated at 37 C for 1 day in a CO₂ incubator, and the medium was replaced. The cells were then cultured for 2 days in a CO₂ incubator. HIV-1 infection was determined by immunostaining and measurement of the amount of p24 in the medium.

Immunostaining. The immunostaining of HIV-1-
infected N7 HeLa cells was performed as previously described (29, 36). Three days after infection, the medium was removed and cells were washed once in PBS before being fixed with a 50:50 mixture of methanol and acetone at −20 C for 3 min. After drying, the fixed cells were washed once in PBS followed by washing in PBS containing 1% FCS. The primary antibody (1:200 dilution of HIV-1-positive serum; 250 µl per well) was added to the fixed cells, allowed to bind for 1 hr at 37 C and washed twice in PBS containing 1% FCS. Goat anti-human immunoglobulin G conjugated with β-galactosidase (BIOSYS) at a 1:500 dilution of HIV-1-positive serum; 250 µl per well) was added to the fixed cells, allowed to bind for 1 hr at 37 C and washed twice in PBS containing 1% FCS. Then, the cells were washed twice in serum-free PBS. The X-gal substrate described above, in MAGI assay, was added in each well. Infected cells were stained blue within 4 to 5 hr of substrate addition.

**P24 assay.** The amount of HIV-1 produced from the infected cells was assessed by measuring the amount of viral core p24 using the HIV-1 p24 antigen ELISA (Cellular Products Inc., Buffalo, N.Y., U.S.A.) according to the protocol provided by the manufacturer.

**Infection to MT-4 cells.** MT-4 cells were adjusted at the concentration of 30 × 10^6/ml, and 1 ml of the cell suspension was seeded into each well of a 24 flat-well plate (Iwaki Glass). Then, 50 µl of concentrated viral sample or trypsin-treated virus was added. The cells were cultured at 37 C in a CO2 incubator for 2 days and smeared on a slide glass. After the cells were fixed with a 50:50 mixture of methanol and acetone at −20 C for 5 min, infected cells were stained by the indirect immunofluorescence (IF) method as described previously (15).

**Flow cytometric analysis.** In preparation for flow cytometry, trypsin-detached cells were washed twice in cold PBS containing 1% FCS and then resuspended in anti-CD4 monoclonal antibody (OKT4a; Ortho Diagnostics, Raritan, N.Y., U.S.A.), rabbit anti-galactocerebroside polyclonal antibody (Chemicon International Inc., Temecula, Calif., U.S.A.) or anti-CXCR4 monoclonal antibody (12G5; Pharmingen). After incubating for 1 hr at 4 C, the cells were washed twice with PBS containing 1% FCS. Then, the cells were treated with fluorescein isothiocyanate-conjugated goat anti-serum to mice or rabbits (Organon Teknika) for 1 hr at 4 C. After washing twice, the cells were fixed with PBS containing 1% paraformaldehyde to prepare for analysis on a Becton Dickinson FACScan system (29).

**PCR analysis.** DNA was extracted by using the InstaGene DNA purification matrix (Bio-Rad) according to the manufacturer’s protocol. PCR was performed using 20 µl of sample DNA template in a 50 µl final volume. DNA extracted from MOLT-4/HIV-1 cells was used as a positive control for cellular DNA containing the HIV-1 proviral genome. The final PCR mixture per reaction contained 1.5 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate, 1 × PCR buffer (GIBCO-BRL), 0.5 pmol of each oligonucleotide primer and 0.05 U of AmpliTaq gold DNA polymerase (Perkin Elmer). The PCR consisted of 40 cycles of 94 C for 0.5 min, 55 C for 1 min and 72 C for 1 min. The M661/M667 primer pair was used to detect 161 bp DNA of the HIV-1 LTR/Gag region. As an internal control to standardize the DNA content in each DNA sample, the human β-globin gene was amplified as described elsewhere (41). Amplified DNA products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

**Results**

**PLL Used as an Enhancing Agent of CD4-Dependent HIV-1 Infection**

Polycations (38) and fibronectin (FN) (13) are known to enhance the infectivity of retroviruses. A tissue culture plate coated with 50 µg/ml of PLL, polybrene (PB) or FN was used for the LAI strain of HIV-1 for adherence. Then, HeLa-CD4-LTR-β-gal cells were seeded and cultured for 48 hr. After adding X-gal staining solution, the number of infected, blue-staining cells was counted. Figure 1 shows that PLL and FN increased the HIV-1 infectivity by 4.2 and 1.6 times, respectively, but PB had no effect on infection by this pretreatment method.

**HIV-1 Infection of CD4-Negative N7 HeLa Cells by PLL Pretreatment**

Since PLL caused the most significant augmentation of the infectivity to CD4-positive HeLa cells in the pretreatment, this agent was selected to infect the CD4-negative and CXCR4-positive N7 HeLa cells (Fig. 2) with HIV-1. CD4-negative N7 HeLa cells were cultured with HIV-1 pretreated with PLL on the plate. Figure 3C shows aggregation of the cells observed when viruses in the PLL-treated plate were applied. No aggregation was seen when N7 HeLa cells were simply mixed with HIV-1 (without PLL in Fig. 3A) or when N7 HeLa cells were cultured only in the PLL-coated plate without adding the virus (data not shown). The degree of cell aggregation was seemingly dependent on the amount of the virus applied to the PLL-coated plate. After cultivation for 3 days, the infected cells were visualized by immunostaining. A few (50–60 cells per well) of the N7 HeLa cells infected with viruses using the PLL-treated plate were positive (Fig. 3D), whereas almost no positive cells were detected among the HIV-1-infected N7 HeLa cells with the non-treated plate (Fig. 3B). We also assessed the p24 amount of the virus in the culture
supernatant. It was found that 592.7 ± 11.3 pg/ml of p24 was released from the cells infected with viruses using the PLL-coated plate as compared with 87.8 ± 3.4 pg/ml in the control cells infected with HIV-1 without PLL pretreatment (Table 1). To exclude the possibility that the viruses just remain on the PLL-coated plate or on the cells, the infected cells were briefly washed with 0.1% trypsin after 24 hr post-infection and cultured for 2 days. Unexpectedly, more p24 (1,038.3 ± 16.8 pg/ml) was detected from the trypsin-treated cells than from the untreated control (Table 1).

**Effect of Trypsin-Treated Viruses on Infectivity to N7 HeLa Cells Using PLL-Coated Plate and to MT-4 Cells**

To determine the direct effect of trypsin on HIV-1, virus-containing supernatants from MOLT-4/HIV-1 cells were harvested and 5% trypsin was added to make a final concentration of 0.4 to 0.025% trypsin. The viral supernatants with trypsin were then ultracentrifuged for 1 hr at 4°C to obtain viral pellets, which were resuspended with PBS and used as trypsin-treated HIVs-1. Subsequently, the trypsin-treated HIVs-1 were applied to a PLL-coated plate, and then N7 HeLa cells were seeded to evaluate the CD4-independent infectivity. At the same time, the same viral sample was used to infect MT-4 cells on a normal plate to assess the CD4-dependent infectivity of the trypsin-treated virus. Viral p24 of each trypsin-treated sample was also measured to evaluate trypsin-induced destruction of the virion.

Infection to N7 HeLa cells was enhanced by 0.025 or 0.05% trypsin-treated HIV-1 (Fig. 4). This enhancement was consistently observed in repeated experiments. The trypsin-treated viruses induced cell clumps (Fig. 3E), and the HIV-1-positive cells were found in the center of these cell clumps (Fig. 3F). In contrast, the trypsin-treated viruses lost their infectivity to MT-4 cells dose-dependently (Fig. 4). Viruses treated with 0.05% trypsin showed decreased infectivity to MT-4 cells by 72%, but retained infectivity against N7 HeLa cells cultured on the PLL-treated plate by 125%. Viruses were not directly ruptured by 0.025 to 0.1% trypsin, since the p24 amount of each sample was around 500 ng/ml. However, the amount of p24 from the 0.2 and 0.4% trypsin-treated samples showed rapid decreases of 91 and 93%, respectively.

**Table 1. Effect of trypsin on HIV-1 production after the viral attachment using PLL-coated plate**

<table>
<thead>
<tr>
<th>PLL-coating</th>
<th>Trypsin treatment</th>
<th>p24 (average ± SEM) pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1%</td>
<td>−</td>
<td>592.7 ± 11.3</td>
</tr>
<tr>
<td>0.05%</td>
<td>−</td>
<td>87.8 ± 3.4</td>
</tr>
<tr>
<td>0.025%</td>
<td>+</td>
<td>1,038.3 ± 16.8</td>
</tr>
<tr>
<td>0.01%</td>
<td>+</td>
<td>104.9 ± 1.4</td>
</tr>
</tbody>
</table>

* After 0.1% trypsin treatment for 1–2 min, a few cells were detached from the plate.
* SEM: standard error of the mean from triplicate wells.

Fig. 1. Effect of poly-L-lysine (PLL), fibronectin (FN) and polybrene (PB) on HIV-1 infectivity to HeLa-CD4-LTR-β-gal cells. Tissue culture plate coated with 50 µg/ml of PLL, FN or PB was used for HIV-1 infection to HeLa-CD4-LTR-β-gal cells. The infected cells were counted 48 hr post-infection after staining by X-gal. Error bars indicate results determined for triplicate wells.

Fig. 2. Phenotype analysis of N7 HeLa cells. The cells were stained by anti-CXCR4 (A), anti-CD4 (B) and anti-galactocerebroside (C).
Effect of Low pH, TPA and MT-4 Supernatant on HIV-1 Production from Infected N7 HeLa Cells Using PLL-Coated Plate

N7 HeLa cells were infected with HIV-1 using a PLL-coated plate. After 24 hr-incubation, the infected cells were washed once and the medium was replaced with one adjusted to a different pH (7.5, 6.5 and 5.5). After 6 hr of treatment, the medium was again changed to the normal pH (7.5), and the cells were cultured for 3 days. Table 2 shows that the treatment at a lower pH released more p24 and also induced a higher number of HIV-1-positive cells. After an 18 hr-treatment at pH 5.5, the amount of p24 (998.0 ± 88.7 pg/ml) was also increased by 1.2 times compared with p24 (825.5 ± 28.5 pg/ml) at pH 7.5, although the cells incurred some damage by the prolonged treatment at pH 5.5.

TPA enhanced the p24 release and number of HIV-1-positive cells in a dose-dependent manner (Fig. 5). Infected N7 HeLa cells treated by 10 ng/ml of TPA released 1,816.1 pg/ml of p24 compared with 1,372.6

Fig. 3. HIV-1 infection to N7 HeLa cells. The cells were infected with HIV-1 alone (A and B), HIV-1 using PLL-coated plate (C and D) and 0.05% trypsin-treated HIV-1 using PLL-coated plate (E and F). The infected (blue) cells were immunostained (B, D and F) and photographed 3 days post-infection under an original magnification of ×40.
pg/ml from control non-treated cells. TPA also induced 135.3 HIV-1-positive cells per well, while infected control cells showed 30.3 positive cells per well.

Fig. 4. Effect of trypsin-treated HIV-1 on infectivity to N7 HeLa cells and MT-4 cells. Infectivity to N7 HeLa cells was assessed by quantifying p24 antigen in the supernatant of triplicate cultures by ELISA. Average ± standard error (barr) of the means are given. Infectivity to MT-4 cells was determined by counting the HIV-1 antigen-positive cells by IF. Percent positivity of MT-4 cells (30 X 10³/well) infected with 50 µl/well of 0, 0.025, 0.05, 0.1, 0.2 and 0.4% trypsin-treated HIV-1 was 39.4, 30.1, 11.2, 4.3, 0 and 0%, respectively. Infectivity (39.4%) of non-treated HIV-1 was designated 100% in this figure.

Fig. 5. Effect of TPA on PLL-supported HIV-1 infection to N7 HeLa cells. N7 HeLa cells were infected and cultured with HIV-1 using PLL-coated plate for 24 hr, and the medium was replaced with 0.1 to 10 ng/ml of TPA. The cells were then cultured for 2 days. Enhanced infection was assessed by measuring p24 antigen released in the supernatant and by counting the HIV-1-positive cells. Results are averages from triplicate wells.

Table 2. Effect of acidic medium on HIV-1 production from N7 HeLa cells using PLL-treated plate

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Treatment</th>
<th>Amount of p24 released (Ave ± SEM) pg/ml</th>
<th>HIV-positive cell/well (Ave ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>pH 7.5</td>
<td>1,240.3 ± 60.8</td>
<td>83.3 ± 6.9</td>
</tr>
<tr>
<td>(6 hr-treatment)</td>
<td>pH 6.5</td>
<td>1,405.0 ± 152.1</td>
<td>92.3 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>pH 5.5</td>
<td>1,635.9 ± 83.2</td>
<td>114.3 ± 21.3</td>
</tr>
<tr>
<td></td>
<td>pH 7.5 (without PLL treatment)</td>
<td>86.7 ± 3.1</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>II</td>
<td>pH 7.5</td>
<td>825.5 ± 28.5</td>
<td>NT</td>
</tr>
<tr>
<td>(18 hr-treatment)</td>
<td>pH 5.5</td>
<td>998.0 ± 88.7</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>pH 7.5 (without PLL treatment)</td>
<td>53.8 ± 1.8</td>
<td>NT</td>
</tr>
</tbody>
</table>

SEM: standard error of the mean from triplicate wells.

Table 3. Effect of culture supernatants on PLL-induced infection to N7 HeLa cell

<table>
<thead>
<tr>
<th>Culture supernatant</th>
<th>Amount of p24 released (Ave ± SEM) pg/ml</th>
<th>HIV-positive cell/well (Ave ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% MT-4 sup.</td>
<td>1,623.1 ± 9.6</td>
<td>124.7 ± 2.5</td>
</tr>
<tr>
<td>50% MOLT-4 sup.</td>
<td>1,487.9 ± 77.4</td>
<td>25.0 ± 22.0</td>
</tr>
<tr>
<td>50% U937 sup.</td>
<td>1,444.7 ± 38.3</td>
<td>42.7 ± 18.8</td>
</tr>
<tr>
<td>Medium</td>
<td>1,372.6 ± 142.9</td>
<td>49.3 ± 15.1</td>
</tr>
</tbody>
</table>

SEM: standard error of the mean from triplicate wells.
Culture supernatants from MT-4, MOLT-4 or U937 cells were examined regarding the infectivity of HIV-1 adsorbed on N7 HeLa cells by PLL pretreatment. Increased p24 release and HIV-1-positive cells were observed only when 50% MT-4 supernatant was used (Table 3).

It is well known that TPA induces NFκB transcription factor and subsequently enhances the transcription of proviral DNA through NFκB binding of HIV-1 LTR (14, 30). Supernatant from MT-4 cell culture may contain a substance like NFκB. To test this possibility, persistently infected (MOLT-4/HIV-1) cells which show 100% HIV-1 antigen-positive by IF were treated by 10 ng/ml of TPA or a 50% supernatant of MT-4 cells. TPA enhanced virus production 18-fold from the proviral DNA of MOLT-4/HIV-1 cells, while the MT-4 supernatant had no effect (Fig. 6).

Detection of HIV-1 DNA by PCR

HIV-1 DNA was amplified in lysate from HIV-1-infected N7 HeLa cells cultured on a PLL-coated plate, but not without the virus (Fig. 7). No viral DNA was amplified in the case of PLL-nontreated and HIV-1-infected N7 HeLa cells (data not shown). Figure 7 also displays that increased DNA was amplified when the cells were treated with 10 ng/ml of TPA.

Discussion

In the present study, we first described that CD4-negative cells are infectable with HIV-1 under artificial conditions. First, charge-to-charge binding of the HIV-1 with cells by PLL did not fully substitute for the role of CD4 and coreceptor, although PLL treatment induced virus-dependent aggregation of the cells and apparent infection, albeit at very low efficiency. Second, degradation of gp120 was essential for efficient infection after binding of the virus on the cell membrane. Trypsinization (0.025 to 0.05%) of the virion enhanced PLL-supported infection of HIV-1. Cleavage sites of the trypsin would be viral envelope glycoproteins. In fact, trypsin ablated the CD4 binding site of gpl20, since trypsin-treated viruses markedly lost their CD4-dependent infectivity to MT-4 cells (Fig. 4). The amino acid sequence of HIV-1 gp160 revealed that there are several cleavage sites at and around the N-terminal portion of gp41 (Fig. 8). Thus, hydrophobic fusion domain is expected to be exposed after trypsinization. According to this observation, we can speculate that HIV-1 receptors show biphasic functions. The binding of the virus and conformational changes leading to increased expression of the hydrophobic domain (5, 19) on the virus are requisite for virus infection and are separable functions of the receptor molecules for HIV-1. The importance of envelope cleavage by cellular proteolytic enzymes in infection (16) should be emphasized but is still not known.

The mechanism of PLL-mediated potentiation of virus infection needs to be further studied.
infectivity is not clearly understood. Polycations are known to enhance the infectivity of retroviruses and have been used in assays of infectious retroviruses (38). PLL is one of the polycations and is thought to enhance infectivity through the interaction of negatively charged cell surface and viral envelope with positively charged PLL. The adsorption of the enveloped viruses might need charge-to-charge interaction, since negatively charged heparan sulfate on cell membrane is involved in the initial attachment phase of alphaherpesviruses through positively charged glycoprotein structures (23) and since infectivities of lentiviruses are activated by desialylation of the virion surface (18). However, it was recently reported that pretreatment of HeLa or HeLa-CD4 cells with 10 μg/ml of PLL inhibited HIV-1 attachment to both cell lines by 70 to 90% and reduced the infection of HeLa-CD4 cells by 75% (26). This contradictory finding may be due to the direct treatment of cells with PLL in which the entire cell surface is covered. Thus, the mode of the PLL treatment seems to be critical for this kind of experiment.

The possibility that other factors such as low pH, TPA and MT-4 supernatant are involved in the enhanced infection of HIV-1 after viral interaction with cells by PLL was suggested. In the case of HIV-1, raising the pH in cellular components by chloroquine and NH4Cl does not affect virus infection (33). It is believed that the HIV-1 envelope can fuse directly with the cell membrane and not necessarily within endocytotic vesicles (24) because HIV-1 entry through the receptors is pH-independent. However, in our study, a low pH environment enhanced PLL-supported HIV-1 infection, suggesting that HIV-1 attachment to cells by PLL needs endocytotic involvement or conformational changes induced by low pH in order to complete infection.

The questions of whether engagement of the HIV-1 receptors by the viral envelope glycoprotein activates intracellular signaling pathways and whether the activated signaling pathways prepare the target cells for viral replication require further study (2, 6, 9, 32). In this context, we used TPA (14) for activating HIV-1-attached N7 HeLa cells by PLL. Figure 5 shows that TPA increased the infectivity of the HIV-1 in our experimental system, indicating that TPA activation of the cells directs penetration of the virus and events in the intracellular viral life cycle. The same enhancement was also observed by the treatment with the 50% supernatant of MT-4 culture (Table 3). However, in contrast to TPA, the MT-4 supernatant did not induce transcription of the proviral DNA through activation of NFkB (Fig. 6). Moriuchi et al reported that MT-2 supernatants contained chemokines as the suppressive factors for M-tropic HIV-1 but enhanced T-tropic HIV-1 infection (28). Soluble Tax protein increased susceptibility to both M- and T-tropic HIV-1. They also demonstrated that Tax protein is able to enhance HIV-1 fusion and entry (28). Since both MT-2 and MT-4 cells are cell lines transformed by human T-cell leukemia/lymphoma virus type-I (HTLV-I), factors from MT-4 cells are able to modulate HIV-1 infection by the same mechanism such as induction of fusion and entry. Further study is needed to identify whether Tax or other factors are responsible for this phenomenon using our artificial infection system.

The aim of our experiment is to establish an artificial infection system over the barrier of natural receptor functions. Using this system, we can analyze independently the biological meaning of early events in the viral life cycle such as adsorption, fusion, and penetration. The same method could be applied to enhance the rate of transduction of target cells with retroviral vectors. Moreover, it could be used to widen the tropism of retroviral vectors. We are now exploring more convenient and less toxic agents than PLL for this purpose.

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