Syncytium Formation and HIV-1 Replication Are Both Accentuated by Purified Influenza and Virus-associated Neuraminidase*

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The degree of sialylation has been shown previously to modulate the process of human immunodeficiency virus type-1 (HIV-1) infection by affecting the interaction between the virus and CD4-expressing target cells. In the present study, we investigated whether HIV-1 replication cycle was affected by neuraminidase (NA) derived from the human influenza (flu) virus. We first demonstrate that the level of HIV-1-mediated syncytium formation was greatly enhanced in the presence of purified flu NA. Pretreatment of established monocytic and lymphocytic cell lines as well as primary mononuclear cells with purified flu NA augmented also the process of virus infection. A comparable up-regulating effect was observed when using several strains of UV-inactivated whole flu virus, thereby suggesting that virus-anchored NA enzymes positively modulate the HIV-1 life cycle. Furthermore, flu NA-mediated positive effect on HIV-1 biology was abrogated with zanamivir, a specific flu NA inhibitor. Our results provide a new model allowing the investigation of the potential benefit of using NA inhibitors in the treatment of HIV-1-infected patients suffering from coinfection with NA-bearing pathogens.

HIV-1 infection is dependent on a now well established interaction between the external viral envelope glycoprotein gp120 and CD4/chemokine receptors (1). Infected T cells expressing gp120 molecules on their surface, when fusing with uninfected T cells (a process known as syncytium formation), equally need the same intercellular interaction for such a virus-mediated cytopathic effect to resume (2–5). However, HIV-1 attachment to host cells often occurs under suboptimal conditions because of a low frequency of gp120/CD4 interaction events (6–8). Several factors have been proposed to explain this phenomenon. First, most cell types that are permissive for HIV-1 infection express little CD4 (9, 10). Second, the weak association between gp120 and gp41 results in a rapid gp120 shedding and, consequently, a loss of virus infectivity (11, 12). Third, the efficient attachment of circulating virions to target cells has to take place despite the presence of neutralizing antibodies that are directed predominantly against gp120 (13). Finally, the electrostatic repulsive forces that result from net negative charges present on the surface of both virion and target cell represent an obstacle to the initial virus attachment process (14). Thus, it has been proposed that other interactions between the virus and the cell surface are necessary to overcome the various factors that might jeopardize the first step in the life cycle of an intracellular parasite such as HIV-1. Accumulating evidence suggest that the activity of bacterially derived neuraminidase (NA, also termed sialidase) can also modulate replication of this retrovirus, including the attachment process, by reducing the level of sialylation of glycoconjugates expressed on the surface of viruses and target cells (15–17). Sialic acids are monosaccharides transferred onto glycoproteins and glycoproteins which travel through the secretory pathway (18, 19). One of the distinct features of sialic acid is its outermost cellular location and its negative charge that increases the net negative charge present on the cell surface. In animals, NAS have been found in several tissues, where the enzymes play various roles in regulation of the surface sialic acid profile of cells. Thus, the balance between sialic acid content and sialidase activity is often found to affect many biological phenomena in animal cells, such as T and B cell activation, hematopoietic cell differentiation, apoptosis, and particularly the regulation of various cell-cell and cell-substrate interactions (20–25). As the surface of both HIV-1 and its natural target cells contain highly sialylated glycoconjugates, previous works have scrutinized the potential implication of sialic acid and neuraminidase activity in the HIV-1 life cycle. For example, Hu et al. (15) reported that desialylation of HIV-1 increases virus infectivity, whereas others have reported that desialylation of freshly isolated human peripheral blood mononuclear cells (PBMCs) creates a cellular environment more suitable for virus growth (16, 26). More recently, we reported that Arthrobacter-derived NA augmented HIV-1-mediated syncytium formation and the initial steps in the virus life cycle (i.e. binding and entry) (17). Human influenza (flu) virus represents a pathogen that

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The abbreviations used are: HIV, human immunodeficiency virus; flu, influenza; NA, neuraminidase; PBMC, peripheral blood mononuclear cell; MBS, 2-(N-morpholino)ethanesulfonic acid; MUN, 4-methylumbelliferyl-N-acetyl neuraminic acid; FBS, fetal bovine serum; IL, interleukin; MEM, minimal essential medium; DMEM, Dulbecco’s modified Eagle’s medium; MDCK, Madin-Darby canine kidney; LTR, long terminal repeat; m.o.i., multiplicity of infection; PFU, plaque-forming unit(s).
**Flu NA Desialylation in HIV-1 Infection, Syncytium Formation**

**Production, Purification, and Titration of Fully Competent or UV-inactivated Flu Virus Stocks**—Human flu virus strains including A/H3N2/England/427/88, A/H3N2/Sydney/05/97, A/H1N1/Beijing/262/95, and B/Harin/07/94 were used. Stocks were prepared by infecting MDCK cells (90% confluent) with 0.01 plaque-forming unit (PFU)/cell and subsequently infecting the supernatant 42 h postinfection. The supernatant was separated from cellular debris by low speed centrifugation and filtration through a 0.45-µm pore size filter. Such virus preparations constituted partially purified viral preparations, which were aliquoted and stored at −85°C until use. Virus crude preparation of strain A/H3N2/England/427/88 was purified by a modification of a centrifugation procedure as described previously (42). Flu virus was titrated by a standard plaque assay as described elsewhere (43). Partially purified flu preparation commonly achieved 10⁷ PFU/ml. For UV inactivation, virus stocks were held in open culture dishes and exposed to a UV lamp (providing an intensity of 100 microwatts/cm² falling on the horizontal plane defined by the bottom of the work surface) at a distance of 0.7 m for 20 min in a ventilated laminar flow hood. Complete loss of infectivity was confirmed by plaque assay.

**NA Activity**—To evaluate NA activity, partially purified and purified flu A/H3N2/England/427/88 were first serially diluted in an enzyme buffer containing 162.5 mM MES and 5 mM CaCl₂. Duplicate samples of each virus dilution (10 µl) were subsequently mixed with 30 µl of a substrate made of 0.5 µmol 4-methylumbelliferyl-N-acetyl neuraminic acid (MUN). Plates (Falcon black, 24-well plates) were sealed and incubated with shaking at 37°C for 15 min before addition of 150 µl of stop solution made of ethanol and 0.5 mM NaOH (4:1). For zanamivir sensitivity test, 10 µl of virus dilutions (1:32 dilution for partially purified virus and 1/6 dilution for purified flu virus) were incubated with enzyme buffer and substrate mix in the absence or the presence of increasing concentrations of zanamivir (i.e. from 10 to 160 nM) at 37°C for 1 h. The enzymatic reaction was then stopped by addition of 150 µl of stop solution. Finally, after the enzymatic reaction, MUN was quantified by fluorometric determination with a fluorescence reader (FL600, Biotec Instruments).

**Syncytium Assay**—Luciferase-based quantitative syncytium assay was performed according to a previously described protocol with slight modifications (44). Briefly, cells were first adjusted at a concentration of 1 × 10⁶ cells/ml in DMEM (without FBS) containing CaCl₂ (4 mM) and incubated at 37°C for the indicated time periods either in the absence or the presence of different concentrations of purified flu NA or UV-inactivated flu virus (ranging from 0.1 to 10 m.o.i.). In some experiments, zanamivir (0.01–1 µM) was introduced into the cell culture along with purified NA or inactivated virions. Then, calcium, NA, and cell-free filtrates were removed and the cells were washed and then incubated with a mixture of flu virus and serum-free DMEM. Pelleted uninfected and cells chronically infected with HIV-1 (i.e. J1.1) were resuspended in complete RPMI 1640 culture medium at a concentration of 2 × 10⁶/ml and 1 × 10⁶/ml, respectively. For the samples pretreated with zanamivir, the concentration of the NA inhibitor was maintained in the culture medium to completely inhibit the effect of flu virus-associated NA activity. An aliquot of each cell suspension (25 µl) was then added to 1 × 10⁶ HIV-positive Jurkat cells/mL in 96-well plates. In some experiments, SIM2 and SDF-1 were added to cells to block HIV-1-mediated syncytium formation. After 16 h of incubation at 37°C, syncytia were first visualized and photographed through an inverted microscope. Cells were then lysed with 1% Triton X-100 and luciferase activity (expressed in relative light units) in cellular lysates was assessed with a microplate luminometer device (MLX, Dynex Technologies, Chantilly, VA).

**Production of HIV-1 Preparations and Virus Infection**—HIV-1 particles were produced by transient transfection of 293T cells as previously described (45, 46). Transfection of pNL4-3 or pXHB-LUC led to the production of virus stocks called NL4-3 and XHB-LUC, whereas co-transfection of pNL4-3-LUC-E’ R’ with vectors encoding HIV-1 envelope pXHB-2-env or pAda-M-env generated the pseudotyped virus stock HXB-2 and Ada-M, respectively. Virus stocks were normalized for virion content using an in-house sensitive double antibody sandwich enzyme-linked immunosorbent assay specific for the major core viral protein p24 (47). Infections were done by using appropriate amounts of virus (standardized in terms of p24 protein) to infect 10⁶ cells. Cells were either left untreated or were treated with 0.01 unit of flu NA or flu virus pretreated with 1 µM of a known NA inhibitor, given in a m.o.i. of 1. NA activity was determined by exposing target cells to a known concentration of infectious particles per target cell at 37°C (puredified flu NA enzyme for 60 min, UV-inactivated flu virus for 30 min). To address the NA-mediated specific effect on HIV-1 infection, NA was added to the cell culture in the presence of the specific flu NA inhibitor zanamivir at several concentrations ranging from 0.01 to 1 µM. Next, soluble purified flu NA enzyme, UV-inactivated flu virus particle and NA inhibitor were

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**Materials and Methods**

**Cell Lines and Media**—Cell lines were cultured in complete culture medium made of RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen), glutamine (2 mM), penicillin G (100 units/ml), and streptomycin (100 µg/ml) except where indicated.

**Plasmids and Reagents**—The proviral plasmid pHXB-LUC, kindly provided by Dr. I. S. Y. Chen (UCLA AIDS Institute, Los Angeles, CA), was originally derived from pHXB-2D into which a part of the nef gene was deleted and replaced with the luciferase reporter gene (41). pNL4-3 is a full-length infectious molecular clone of HIV-1 and was provided by the AIDS Repository Program (NIAID, National Institutes of Health, Bethesda, MD). pNL4–3–LUC-E’ R’ (NL4–3 backBone), pHXB-2-env, and pAda-M-env were generously provided by Dr. Landau (Salk Institute for Biological Studies, La Jolla, CA).

NA purified from flu B/Beijing/31/87 and flu NA-specific inhibitor zanamivir (Glaxo Wellcome, Stevenage, United Kingdom) were used. Bacterial *Arthrobacter*-derived NA was purchased from Nacalai Tesque Inc. (Japan). Stomal cell-derived factor-1 (SF-1) was a kind gift from Dr. I. Clark-Lewis (Biomedical Research Center, Vancouver). Anti-CD4 antibody SIM.2, a potent inhibitor of HIV-1-mediated syncytium formation, has been supplied by the AIDS Repository Program.
eliminated by washing cells with serum-free DMEM. Pelleted cells were resuspended in 200 μl of RPMI containing 10 ng of p24 for each HIV-1 virus preparation (for PBMCs, the culture medium was further supplemented with 30 units/ml rhIL-2) and incubated at 37 °C in 96-well tissue culture plates for the indicated time periods.

RESULTS

Treatment of Cells with Purified NA from Human Flu Virus Increases Cell-Cell Interaction and HIV-1-mediated Syncytium Formation

The CD4-positive T-lymphoid cell line 1G5 and chronically HIV-1-infected cell line J1.1 were used in a quantitative assay to measure HIV-1-dependent syncytium formation. Both cell lines were either left untreated or were treated with purified NA from human flu virus strain B/Beijing/1/87. As shown in Fig. 1A, higher levels of cell aggregation were discernible when cells were pretreated with purified flu NA. Concomitant with an increase in cellular aggregation, a higher level of cell-cell fusion was noticed, which resulted in a remarkable increase in both the number and size of syncytia. A similar observation was made when PBMCs from healthy donors were co-cultured with J1.1 cells in the presence of purified flu NA (Fig. 1B). The direct involvement of flu NA in the observed enhancement of HIV-1-induced syncytium formation was provided by the finding that zanamivir, a specific inhibitor of flu NA, blocked this effect.

A quantitative determination of flu NA-mediated induction of syncytium formation was also carried out using the 1G5 and J1.1 co-culture system (44). The presence of purified flu NA resulted in a dose-dependent increase in HIV-1 LTR-driven reporter gene activity (Fig. 2A). Treatment of 1G5 cells alone with similar increasing concentrations of flu NA did not lead to any detectable modification of HIV-1 LTR activity, indicating that flu NA by itself has no direct potentiating effect on the virus regulatory domain. Data from these experiments indicate that cellular desialylation mediated by purified flu NA enzyme results in a higher rate of cell-cell interaction and, consequently, HIV-1-dependent multinucleated giant cell formation. The observed enhancement of luciferase activity was totally abrogated when NA treatment was performed in the presence of zanamivir (Fig. 2B). No such inhibitory effect was seen when bacterially derived NA (i.e. Arthrobacter) was instead used.

The importance of the gp120-CXCR4 chemokine co-receptor interaction in the flu NA-mediated increase in syncytium formation was next analyzed because cellular desialylation by NA treatment can potentially alter a large array of cellular functions. To this end, the anti-CXCR4 monoclonal antibody SIM.2 and the CXCR4 natural ligand SDF-1 that compete for the binding of gp120 to CD4 and chemokine co-receptor, respectively, were added to the 1G5/J1.1 co-culture system. As expected, both agents almost completely blocked the observed HIV-1-mediated syncytium formation (Fig. 2C). Moreover, the presence of either SIM.2 or SDF-1 reduced luciferase activity of both untreated and flu NA-treated cells to a similar extent. These data indicated that the flu NA-induced increase in HIV-1-mediated syncytium formation was dependent on the interaction between gp120 and CD4/CXCR4.

Desialylation of CD4-expressing Human Cells by Flu NA Leads to an Enhancement of HIV-1 Infection—Treatment of HIV-1 particles or cells with NAs from a bacterial source results in an increase of both HIV-1 infectivity and susceptibility of target cells to virus infection (15, 17). To define whether the same phenomenon can be observed with flu NA, uninfected T lymphoid cells as well as the monocytoid cell line Mono Mac 1 were first pretreated with flu NA and then incubated with either replication-competent virus (i.e. NL4–3) or X4 single-cycle reporter virus (i.e. HXB-LUC). In this set of experiments, luciferase activity was used as an indicator of HIV-1 infection. Upon the treatment of cells with flu NA, HIV-1 infection was augmented for all tested virus/cell line combinations (Fig. 3A).
Arthrobacter-derived NA was added to cocultured 1G5/J1.1 cells at a final concentration of 0.01 unit/ml either in the absence or the presence of zanamivir (0.1 \mu M).

C, monoclonal anti-CD4 SIM.2 antibody (20 \mu g/ml) or SDF-1 (5 \mu g/ml) was also added to the coculture system. In all instances, cells were lysed 16 h after the start of the coincubation.

Luciferase activity for all samples was read with a Dynex luminometer apparatus. The results are shown as the mean \pm S.D of quadruplicate samples and are representative of three independent experiments.

RLU, relative light units.

**Fig. 2.** Purified flu NA increases HIV-1 LTR-driven reporter gene activity in the 1G5/J1.1 syncytium formation assay. **A**, 1G5 and J1.1 cells (1:2 ratio) were mixed together and incubated in the presence of increasing amount of purified flu NA. **B**, purified flu NA or Arthrobacter NA was added to cocultured 1G5/J1.1 cells at a final concentration of 0.01 unit/ml either in the absence or the presence of zanamivir (0.1 \mu M). C, monoclonal anti-CD4 SIM.2 antibody (20 \mu g/ml) or SDF-1 (5 \mu g/ml) was also added to the coculture system. In all instances, cells were lysed 16 h after the start of the coincubation.

Luciferase activity for all samples was read with a Dynex luminometer apparatus. The results are shown as the mean \pm S.D of quadruplicate samples and are representative of three independent experiments. RLU, relative light units.

**Fig. 3.** Purified flu NA also enhances infection with cell-free HIV-1 particles. A, Jurkat, 1G5 and Mono Mac 1 cells (10^5) were first pretreated or not with purified flu NA (0.01 unit/ml) for 1 h. After washing the cells with serum-free DMEM, cells were inoculated either with fully infectious HIV-1NL4–3 or luciferase reporter viruses (i.e. HXB-LUC) (10 ng of p24). Cells were lysed 48 h after the start of the culture. B, human PBMCs were first pretreated or not with purified flu NA (0.01 unit/ml) in the presence or absence of zanamivir (0.1 \mu M). Cells were next inoculated with HXB-LUC (X4) or R5 HIV-1 pseudotypes bearing Ada-M envelope protein (10 ng of p24). Cells were lysed 72 h after the start of the culture. The results are shown as the mean \pm S.D of quadruplicate samples and are representative of three independent experiments.

RLU, relative light units.
To more closely parallel physiological conditions, mitogen-stimulated PBMCs from healthy donors were pretreated with flu NA and then infected either with X4 or R5 reporter viruses. Replication of HIV-1 was also augmented in primary human cells following NA treatment (Fig. 3B). The noticed enhancement in single-round virus infection was solely attributable to flu NA enzymatic activity as it was completely abrogated when the influenza NA-specific inhibitor zanamivir was added along with flu NA.

Flu virus-associated NAs Are Enzymatically Active and Can Enhance HIV-1-mediated Syncytium Formation and HIV-1 Replication—We next used fully competent human flu virus preparations to study the action of pathogen-associated NA on HIV-1-mediated syncytium formation and virus infection. To eliminate the possible replication of flu virus in studied target cells, viruses were first inactivated by UV light radiation. The complete loss of infectivity of flu virus was confirmed by plaque assay (data not shown). Two types of flu virus preparation were tested: partially purified and purified preparations, the latter resulting from ultracentrifugation of the supernatant of flu-infected MDCK cells. Most of the NA activity detected in the studied flu virus preparations was likely virion-associated because there was a direct correlation between NA activity and the number of infectious flu virus particles (i.e. PFU) for both virus preparations. The NA activity of both partially purified and purified flu A/H3N2/England/427/88 was found to be highly sensitive to zanamivir (Fig. 4A). For example, a 50% inhibition of NA activity was achieved when using zanamivir at a 10 nm concentration, whereas an inhibition of greater than 95% was obtained with 160 nm (Fig. 4B).

Treatment of 1G5/J1.1 co-cultured cells with UV-inactivated flu A/H3N2/England/427/88 viruses resulted in an increase in cellular aggregation and HIV-1-mediated syncytium formation that was sensitive to zanamivir (Fig. 5A). Measurements of HIV-1 LTR-driven luciferase activity confirmed data obtained by visual observation of co-cultured cells incubated with UV-treated flu virus preparations. Indeed, a linear correlation was seen between HIV-1 LTR-dependent reporter gene activity and increasing doses of UV-inactivated flu A/H3N2/England/427/88 virus (Fig. 5B). Another UV-inactivated flu strain (i.e. B/Hairbin/07/94) was also capable of augmenting HIV-1-mediated syncytium formation, thus suggesting that it is a generalized phenomenon for different flu virus types (Fig. 5C).

The putative modulatory effect of flu virus on the process of infection with cell-free virions was also studied. As depicted in Fig. 6A, a zanamivir-sensitive augmentation of HIV-1 infection is observed following incubation of 1G5 cells with partially and purified flu virus preparations. A similar enhancement of HIV-1 replication is seen with three other flu virus strains (Fig. 6B). The direct involvement of virus-encoded NA glycoprotein is again provided when flu NA-specific inhibitor zanamivir is added to the cell culture.

The effect of flu virus-associated NA on HIV-1 infection was also studied in the context of primary human cells (i.e. phytohemagglutinin/IL-2-stimulated human PBMCs). In agreement with data obtained when PBMCs were pretreated with purified flu NA enzyme (Fig. 2), infection of susceptible primary human cells with X4 and R5 HIV-1 was markedly augmented (up to 6-fold) upon the addition of UV-inactivated flu virus, and this increase was again abrogated by a treatment with zanamivir (Fig. 7). Similar findings were obtained with PBMCs originating from two other healthy subjects and (data not shown).

**DISCUSSION**

The effect of endogenous NA on the process of HIV-1 infection has been difficult to dissect because modification of NA activity is always accompanied by other simultaneous biological processes. Given that NAs from different species share the same substrates (e.g. glycoproteins, glycolipids, and oligosaccharides) (48), exogenous NAs derived from few microorganisms (mostly of bacterial origin) have been used to deduce the role played by NAs with respect to the life cycle of HIV-1. Interestingly, several bacteria and viruses produce NA as virulence factors either on their surface or in a secreted form (19). Some of these microbial pathogens are recognized as opportunistic agents in the course of AIDS (49–52). Considering that secondary lymphoid organs are the preferential sites where microbial agents are concentrated during the normal immune response and where concomitantly high level of HIV-1 replica-
tion is thought to occur (53–55), it is therefore of high importance to delineate the role of pathogen-derived NA in the biology of HIV-1.

In this report, we have initially tested the modulatory role of purified flu NA on HIV-1-mediated syncytium formation and cell-free virus infection. We showed that treating cells with flu-derived NAs remarkably augmented the initial cell-cell interaction and thereby promoted HIV-1-mediated cytopathic effect (i.e., syncytium formation). We have also noticed that desialylation of target cells increased susceptibility of target cells to infection with cell-free HIV-1 particles. Here, we demonstrate for the first time that virion-associated flu NA exhibits a similar enhancing effect on HIV-1-mediated syncytium formation and cell-free virus infection.

In our in vitro experimental systems, studied target cells, including freshly isolated PBMCs, were more prone to HIV-1-

**Fig. 5.** UV-inactivated flu virus up-regulates HIV-1-mediated syncytium formation. A, 1G5 (2 × 10⁵) and J1.1 (10⁵) cells were treated with partially purified UV-inactivated flu virus (strain A/H3N2/England/427/88/m.o.i: 0.1) for 30 min at 37 °C in the presence or absence of zanamivir (0.1 μM). Cells were then washed and incubated in culture medium supplemented with zanamivir when appropriate. After 16 h of coincubation, cells were observed and photographed by light microscopy. Original magnification, ×100. B, in some experiments, cocultured 1G5 and J1.1 cells were incubated for 16 h along with increasing amounts of partially purified UV-inactivated flu virus (strain A/H3N2/England/427/88/m.o.i. ranging from 0.1 to 10) either in the absence of the presence of zanamivir (0.1 μM). Cells were then washed and incubated in culture medium supplemented with zanamivir when appropriate. C, cocultured 1G5 and J1.1 cells were incubated for 16 h along with partially purified UV-inactivated flu virus (strain B/Harbin/07/94 at a m.o.i. of 5) either in the absence of the presence of increasing concentrations of zanamivir (0.1 μM). Cells were then washed and incubated in culture medium supplemented with zanamivir when appropriate. For panels B and C, the results are shown as the mean ± S.D of quadruplicate samples and are representative of three independent experiments. RLU, relative light units.
mediated syncytium formation in the presence of either purified flu NA enzyme or different strains of UV-inactivated flu virus. The flu NA-dependent up-regulating effect on HIV-1-induced syncytium formation is likely to occur through a mechanism involving the removal of sialic acids from the cell surface as zanamivir, a specific flu NA inhibitor, suppressed the observed up-regulation. Sialic acid content is one of the key elements regulating cell-to-cell contact (56) and desialylation caused by flu NA enzymatic activity results most likely in a higher rate of intercellular interaction, which eventually increases HIV-1-mediated syncytium formation.

Besides HIV-1-mediated syncytium formation, purified flu NA was also found to affect the process of cell-free HIV-1 infection in several different cell source, an increase in infection which was zanamivir-sensitive. Because transcriptional activity of HIV-1 LTR region in 1G5 cells was not modulated by purified NA, it can be postulated that NA is primarily affecting the early steps of the HIV-1 replication cycle. Experiments conducted with single-cycle luciferase reporter viruses sup-

**FIG. 6.** The process of infection with cell-free HIV-1 particles is also increased when target cells are incubated with UV-inactivated complete flu virus. 1G5 cells (10⁵) were first incubated with UV-inactivated flu A/H3N2/England/427/88 virus (both partially purified and purified) (panel A) or several flu virus strains (A/H1N1/Beijing/262/95, A/H3N2/Sydney/05/97, and B/Harbin/07/94) (panel B) for 30 min at 37 °C in the presence or absence of zanamivir (0.1 μM). Cells were then washed and inoculated with fully infectious HIV-1NL4-3 (10 ng of p24). After 48 h of incubation, cells were lysed and luciferase activity was read by a Dynex luminometer apparatus. Data shown represent the mean ± S.D of quadruplicate samples and are representative of three independent experiments. RLU, relative light units.

**FIG. 7.** UV-inactivated flu virus augments HIV-1 replication in primary human cells. PHA/IL-2 stimulated human PBMCs (10⁵) were either left untreated or treated with UV-inactivated flu virus A/H3N2/England/427/88 strain (mo.i.: 10) for 30 min in the absence or presence of 0.1 μM zanamivir. Cells were next washed with serum-free DMEM and resuspended in culture medium containing recombinant luciferase-encoding X4 (HXB-LUC, panel A) and R5 (Ada-M, panel B) virions (10 ng of p24). Cells were then washed and incubated in culture medium supplemented with zanamivir when appropriate. After 72 h of incubation, cells were lysed and luciferase activity was read by a Dynex luminometer apparatus. Data shown represent the mean ± S.D of quadruplicate samples and are representative of three independent experiments. RLU, relative light units.
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ports the idea that flu NA is most likely affecting the initial events in HIV-1 life cycle. It should be noted that desialylation by bacterial-derived NA has also been observed to promote HIV-1 attachment and entry (15, 17). Such a modified interaction between target cells and HIV-1 particles in the presence of secreted NA by surrounding NA-producing pathogens might have a profound impact on HIV-1 spreading and infection. For example, a more rapid and stable binding of virions to susceptible cells under in vivo conditions will likely positively affect the HIV-1 attachment process.

Most studies, which were aimed at defining the effect of NA on HIV-1 biology, were using purified soluble NA derived from various pathogens of bacterial origin. It can therefore be questioned whether the amount of NA released by or associated with such microorganisms are in the same order of magnitude as the concentrations of purified NA used in these experimental studies. Besides, NAs of several pathogens, including human flu virus A and B, are membrane-associated. Thus, the validity of the data obtained with purified soluble NA remains questionable and might not be representative enough to deduce the exact role played by membrane-bound NA on the biology of HIV-1 in patients dually infected with HIV-1 and NA-bearing pathogens. We therefore assessed whether flu-anchored NA would exhibit a similar positive effect on HIV-1 replication. This specific issue was addressed by using several flu virus isolates that were inactivated by UV treatment to eliminate possible expression of flu-encoded protein(s) within studied cells. This is founded on a previous report showing that expression of flu virus hemagglutinin in mammalian cells induces activation of NF-κB (57), a transcription factor recognized as a powerful activator of HIV-1 transcription (38). Whole UV-inactivated flu viruses were first confirmed to harbor NA enzymatic activity on their surface but importantly were also capable of potentiating HIV-1-dependent giant cell formation and HIV-1 replication in both T cell lines and PBMCs. Both of these events were furthermore positively modulated by flu viruses in a zanamivir-sensitive fashion.

On the basis of these latter results, it could be postulated that an effective treatment against flu infection in HIV-1-positive individuals might be beneficial for such patients. However, previous observations have reported that flu infection in HIV-1-positive individuals did not alter HIV-1 viral load or clinical progression (37). In fact, because the flu virus is mainly localized in the upper respiratory tract, such interaction between this virus and HIV-1 target cells might not be sufficiently predominant. However, one important related issue concerns the safety and risk-benefit ratio of flu vaccination of HIV-1-infected adults, which is still a matter of debate because of the controversy surrounding putative changes in plasma levels of HIV-1 RNA following vaccination of HIV-1-infected patients against flu (35, 36, 58–61). It is plausible that this risk could be even higher with the use of live-attenuated flu vaccines in light of our results.

Our results thus offer a model by which the interactions of NA-bearing pathogens with HIV-1 can be studied. Although in vivo, such interactions between flu viruses and HIV-1 are less likely to occur, other pathogens, which represent opportunistic infectious agents and which are NA-positive, could be tested in our cell lines model for their effect on HIV-1 replication and virus-mediated syncytium formation. In addition, the in vitro activity of zanamivir against flu NA-mediated positive modulation of the HIV-1 life cycle in our system calls for discovery and potential use of NA inhibitors of other NA-producing pathogens known to be frequently detected in HIV-1-infected individuals.

In summary, our findings indicate that human flu virus through the enzymatic activity of NA, one of the two surface glycoproteins of this virus, accentuates syncytium formation and infection by HIV-1. A specific inhibitor of flu NA (i.e. zanamivir) was used to successfully block flu NA-mediated enhancing effect on HIV-1 life cycle. These findings should provide a new model, which has direct physiological relevance because microbial pathogens that produce NAs as virulence factors may affect HIV-1 pathogenesis via desialylating effect of these enzymes. In addition, we are presently studying the use of the ex vivo tonsil fragment model to study the impact of NA-bearing pathogens on HIV-1 replication. Through the results from the presented flu virus model, potent specific inhibitors of NA might be considered for the treatment of patients suffering from infection with HIV-1 and NA-encoding pathogens.

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