Experimental model to study co-infection of human immunodeficiency virus-type 1 (HIV-1IIIB) and influenza virus in cell culture

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ABSTRACT
People living with human immunodeficiency virus (HIV) demonstrate highly expressed immune deficiency and are vulnerable to exogenous viral infections (co-infections), including influenza. We were interested in developing an in vitro model to examine HIV-1 and influenza virus (Flu) co-infection based on HIV-1 sialoglycoprotein (Sgp) synthesis and virus replication in double-infected cells. A convenient and reproducible experimental model for studying the sialylation of HIV-1 glycoprotein 120 (Gp120) by direct radioactive precursors of sialic acid synthesis, [14C]N-acetylmannosamine) in cell culture was successfully introduced for investigation of co-infection. The co-infection provoked desialylation of viral Sgps, followed initially by decreased and later on, by increased HIV replication. Monoclonal antibodies (Mab) to the main Gp120 V3 neutralization epitope but not to broadly reactive Mab against Gp120 recognize and neutralize the newly exposed epitopes. A hypothesis was outlined that exogenous neuraminidase (NA) (Flu virus) changes the configuration of HIV-1 Gp120 through desialylation resulting in the exposure of a novel antigen. The proposed model could contribute to better understanding of pathogenesis of Flu co-infection in people living with HIV.

In vivo, Flu co-infection most probably leads to desialylation of both HIV and the cell surface, thus facilitating the escape of HIV from immune control. The findings described here reflect the interaction between components of two viruses – NA of Flu virus and Sgps on HIV-1 surface without considering the cell surface. The hypothesis could be extended to other exogenous co-infections with agents containing NA in HIV-infected people.

Abbreviations:
- Sgps: sialoglycoproteins
- Gp: glycoprotein
- SA: sialic acid, N-acetyl neuraminic acid
- Mab: monoclonal antibodies
- NA: neuraminidase, sialidase
- HA: heamagglutinin
- Flu virus: Influenza virus
- CS: cytosol
- IEF: isoelectrofocusing
- Env: envelope
- MOI: multiplicity of infection
- RT: reverse transcriptase
- RT-qPCR: reverse transcription-quantitative real time PCR

Introduction
People living with human immunodeficiency virus (HIV) demonstrate highly expressed immune deficiency and are vulnerable to exogenous viral infections (co-infections, COI), including influenza. It is well known that after co-infection with influenza or after influenza vaccination of HIV-infected individuals, the HIV RNA viral load increases several times.

The central event in HIV pathogenesis, i.e. the virus escape from immune control, is associated, among others, to epitope masking due to glycosylation/sialylation changes.[1] Being a trimer, the HIV-1 envelope (Env) is formed by glycoprotein 120 (Gp120)/glycoprotein 41 (Gp41) heterodimers exposed on the virion surface and rich in N-linked carbohydrates.[2] N-linked glycosylation patterns in HIV Env differing by strain and source are complicated by the fact that different cell types have different glycan modification.[3] Thus, cell-specific glycosylation profiles may affect immune interactions. Therefore, studies of HIV-1Gp120 sialylation under experimental conditions should be based on the synthesis of HIV-1 sialoglycoproteins (Sgps) – the prominently positioned sialic acid (SA) residues on HIV-1 Gps participating in virus escape from immune control. That is why we were
interested in developing an in vitro model to examine HIV-1 and influenza virus (Flu) co-infection based on studying the HIV-1 Sgps synthesis and virus replication in double-infected cells.

Both Gp160 and Gp120 on the surface of HIV-1 are heavily glycosylated and sialylated.[4,5] Thus, based on their chemical structure, they are Sgps. It has been reported that sialylation/desialylation affects the physical and biological properties of Gp120.[6] The natural target cells also contain highly sialylated glycoconjugates.[4,5,7] In previous studies, the potential implication of SA and/or neuraminidase activity (NA) in the HIV-1 life cycle have been investigated. For example, Hu et al. [4] reported that desialylation of HIV-1 increases the virus infectivity and others demonstrated that desialylation of freshly isolated human peripheral blood mononuclear cells created a cellular environment more suitable for virus growth [8]. Moreover, desialylation of glycoconjugates on the surface of monococytes activates the extracellular signal-related kinases ERK 1/2 and results in enhanced production of specific cytokines.[9]

Nevertheless, the implication of SA and NA in the HIV-1 life cycle is not yet clearly understood. For enveloped viruses like the Flu virus, the principal route of cell entry is via a combination of receptor binding and fusion, which are well characterized events. SA was first identified as being responsible for binding of Flu viruses to SA residues that are present on either Gp or glycolipids. The specific conformation of SA linkage (α 2–3 vs. α 2–6) has also been established to control the species tropism of the virus.[4,8] Actually, the outer surface of the Flu virus bears Gps of two types: haemagglutinin (HA) and NA. HA binds SA on cellular and viral Gps participating in entry process, while NA is required mainly for the release of virus particles.[10] However, it has been shown that the activity of bacterially derived NA could modulate the replication of HIV-1, including the attachment process, by reducing the level of sialylation of glycoconjugates expressed on the surface of viruses and target cells.[11]

In an attempt to find a convenient and reproducible experimental model to study the sialylation patterns of HIV-1, a direct radioactive precursor of SA, [14C]N-acetylmannosamine ([14C]NACMan), was used, providing data on both viral and cellular SA biosynthesis.[6] Labelled virus Sgps were obtained by isoelectric focusing (IEF). A quantitative assay for reverse transcriptase (RT) activity was performed in IEF fractions demonstrating that sialylation of HIV-producing cells was virus-specific. Sgp patterns of the virus grown in chronically infected H9/HTLVIII B cells and those grown in acutely infected MT-2 cells were similar.[6]

Materials and methods

A supernatant from H9/HTLVIII B cell culture served as a source of HIV-1III B (a gift from Dr R. Gallo, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). The supernatants were collected, pooled and clarified by low-speed centrifugation. Each viral pool was characterized by infectivity using the MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium bromide] assay [12] and RT activity as described below. The viral pool used in this study contained 2 × 10^6 infectious virions/mL and 565.3 pg RT/mL. H9/HTLVIII B cells served as chronically infected and HIV-producing cells — a model similar to the human chronic HIV infection.

MDCK-SIAT1 cells (Madin–Darby Canine Kidney cells transfected with cDNA of human α-2,6-sialyltransferase (SIAT1)) were purchased from the European Collection of Cell Cultures. Cells were maintained in serum-free Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and CaCl2 (4 mmol/L) to preserve NA activity. MDCK-SIAT1 cells engineered to express increased levels of α-2,6-linked SA receptors have been proven superior to conventional MDCK cells for isolation of circulating influenza A and B viruses.[13] The passage medium for MDCK-SIAT1 cells was supplemented with 1 mg/mL G418 (Invitrogen). Virus infection with influenza virus type B was performed in the absence of G418.

Influenza virus type B (Flu B/Brisbane/60/2008) was a gift from the Influenza Coordination Centre of the World Health Organization (WHO-CC) in London and was used as a reference control after passaging in fertilized chicken eggs. Virus stocks of Flu B were thereafter prepared by infecting MDCK-SIAT1 cells. The infection of H9/HTLVIII B cells with FluB virus was performed after ultraviolet (UV) inactivation of HA viral activity.[14]

[14C]NACMan (Amersham UK, specific activity 2.17 GBq/ mmol) was added 24 h after passaging or infecting of H9/HTLVIII B cells with UV inactivated FluB/Brisbane/60/2008 at final concentration of 0.05 MBq/mL and the cells were cultivated for another 18–20 h.

Cytosol fractions (CSs) were prepared from cell pellets by chilling in ice-cold water and disruption in Dounce’s homogenizer. Aliquots before and after IEF were taken to measure the [14C] incorporation (cpm), protein content (μg/mL) and RT activity (pg/mL) where applicable. Preparative IEF of CSs was performed in a column model 8100-1 (Pharmacia) and [14C] radioactivity was measured by standard liquid scintillation counting.

RT activity was measured by the non-radioactive Lenti RT Assay HS Kit (Cavidi, Sweden), and expressed in pg RT/mL. The protein concentration (μg/mL) was detected
according to Bradford method.[15] Virus replication was detected by measurement of RT activity.

RT-qPCR for Flu B/Brisbane/60/2008 virus was performed with SuperScript® One-Step RT-PCR with Platinum® Taq (Invitrogen, USA) kit and iScript™ One Step RT-PCR Kit for Probes (BioRad, USA) of Chromo 4 (BioRad system), using specific primers and probes (TaqMan®), a gift from CDC Atlanta (USA) and performed according to the CDC protocol.[16]

HIV-1 Gp120 was measured (pg/mL) in supernatants by Gp120 Antigen Capture Assay (Advanced BioScience, USA) over a concentration range 62.5–1000 ng/mL of monoclonal antibodies (Mab). Mab against Gp120 broadly reactive and Mab against V3 of Gp120 between aminoacids 307 and 320 of HIV-1IIIB were purchased from Advanced BioScience (USA).

Results and discussion

As a first step in our study, a model of co-infection of HIV-1 in H9/HTLVIIIB cells (chronically infected and virus-producing) and FluB/Brisbane/60/2008 virus was experimentally developed. Although it has earlier been shown that Flu virus interacts with and infects human macrophages and lymphocytes,[17] FluB virus replication in H9/HTLVIIIB cells was especially confirmed by RT-qPCR (Figure 1).

Further, the sialylation patterns of HIV-1IIIB (control) and HIV-1IIIB co-infected with FluB were compared by specific sialylation activity (Figure 2). The Sgp patterns for H9/HTLVIIIB cells (control cells) and the same co-infected with FluB showed a well expressed desialylation after co-infection with FluB compared to the HIV control. Sgps from HIV-1Gp120 participate in its binding to cell receptors. Desialylation of Gp120 induced by the NA of Flu virus leads to a decrease of HIV-1 replication. That is why, in our experiments, HIV-1 replication and HIV-1Gp120 concentration were quantified by specific tests (RT test and gp120 Antigen Capture Assay, respectively). As seen in Figure 3, RT activity in fractions after IEF of FluB co-infected H9/HTLVIIIB cells decreased compared to HIV control. After lowering of HIV-1 replication (measured by RT activity) during the first 72 hours after co-infection, a trend to recover and even to outrun the 24th hour values was observed (Table 1). This occurred after desialylation: Gp120 concentration decreased after co-infection with 10° Flu virus compared to Flu non-infected cells and increased in a dose-dependent way (Table 1) and lowering of HIV replication (by RT activity and by microtiter infectivity assay — data not shown) 72–96 h after co-infection. Desialylation clearly increased with diminishing of NA activity from FluB (Table 1). Simultaneously, RT activity of HIV-1 decreased after Flu virus co-infection (Table 1). Probably, similar events happen in vivo during the first hours of co-infection but after 96 hours, the RT showed a trend to recover its activity. The increased RT activity together with higher infectivity and cytopathicity of HIV-1 after co-infection with FluB virus represents a good explanation for the increase in viral titer. At the 96th hour after co-infection, the titer of HIV-1IIIB control (evaluated as tissue culture infectious dose, TCID) was $2 \times 10^6$ TCID/mL and that in the case of co-infection of HIV-1 and undiluted Flu B was $>2 \times 10^7$ TCID/mL (data not shown). This fact coincides well with the increase of viral load observed in HIV-infected persons with Flu co-infection.

It could be suggested that Flu virus co-infection of chronically HIV-infected cells leads to a biochemical interaction (desialylation of HIV-1 Gp120 by the Flu NA) rather than to involvement of other factors. Further, to
study the changes in Gp120 after Flu co-infection, an epitope-specific Mab which binds to the principal neutralizing determinant V3 (between aminoacids 307 and 320) of HIV-1IIIB Gp120 was used (Figure 4). This Mab, but not the broadly reactive one against Gp120, was shown to recognize and neutralize in a concentration-dependent way the epitope newly exposed after FluB infection on HIV-1 virion. Therefore, the effect of Flu NA on HIV-1Gp120 is double: desialylation directly affects HIV-1 configuration and masks the newly exposed epitope, making it invisible for the Mab broadly reactive to gp120, but recognizable for the type-specific Mab binding the principal neutralizing epitope of V3. Moreover, desialylation influenced HIV-1 replication and infectivity and this mechanism of action of Flu virus is quite different from that linked simply to immune deficiency in HIV-infected people.

The proposed experimental laboratory model for the study of co-infection demonstrates another mechanism for HIV-1 to escape from immune control. This model attempts to be closer to naturally occurring conditions of co-infection with Flu virus in people living with HIV. We used the human lymphoblastoid cell line H9/HTLVIIIB chronically producing HIV-1 and further co-infected it with FluB. Co-infection with FluB and its replication were confirmed by RT-qPCR and the HIV-1 infectivity and production by a sensitive microtiter infection assay on MT-2 cells (not shown) [18] and measurement of Gp120 concentration and RT activity as well.

The SA content is one of the key elements regulating cell-to-cell contacts and desialylation caused by Flu NA enzymatic activity results most likely in a higher rate of intercellular interaction, which in turn could increase HIV-1 mediated syncytium formation observed earlier.[14] Since our results demonstrate desialylation, we assume that Flu NA could, among others, also facilitate intercellular interaction. Therefore, the findings described here support the suggestion that the presence of NA-producing micro-organisms in local lymph tissues could promote cell-to-cell HIV-1 transmission.

We hypothesize that exogenous NA (Flu virus) changes the configuration of HIV-1 Gp120 through desialylation, resulting in exposure of a novel antigen recognizable by Mab against the principal neutralizing determinant on V3 of Gp120. The co-infection of chronically producing HIV-1 cells with FluB provoked desialylation of cellular and viral Sgps. The Mab against V3 does not interfere with the binding of Gp120 to CD4C, or with the subsequent step of CD4-induced shedding of Gp120 from the viral Env. [19] According to the glycan shield model,[4] the receptor-binding site should not be impaired, thus explaining the HIV activated replication and infectivity.

Table 1. Gp120 and RT concentrations in H9/HTLV IIIB supernatants of cells infected with FluB/Brisbane/60/2008 at different hours after co-infection.

| Supernatant | Hours post passaging of H9/HTLV IIIB cells for HIV control or after co-infection with FluB virus with different MOI | Gp120 (pg/mL) | RT (pg/mL) |
|-------------|---------------------------------------------------------------------------------------------------------------|---------------|------------|
| HIV control | 24                                                                                                           | 2000          | 281        |
|             | 48                                                                                                           | 2650          | 281        |
|             | 72                                                                                                           | 2100          | 271        |
|             | 96                                                                                                           | 2450          | 271        |
| HIV + FluB (10^6) |                                                                                   | 1400          | 110        |
|             | 24                                                                                                           | 1550          | 170        |
|             | 48                                                                                                           | 850           | 135        |
|             | 72                                                                                                           | 800           | 175        |
|             | 96                                                                                                           | 800           | 175        |
| HIV + FluB (10^-1) |                                                                                     | 1900          | 180        |
|             | 24                                                                                                           | 1200          | 165        |
|             | 48                                                                                                           | 1000          | 140        |
|             | 72                                                                                                           | 1100          | 140        |
|             | 96                                                                                                           | 1100          | 140        |
| HIV + FluB (10^-2) |                                                                                     | 2000          | 130        |
|             | 24                                                                                                           | 1650          | 130        |
|             | 48                                                                                                           | 550           | 130        |
|             | 72                                                                                                           | 400           | 180        |

Note: Results are average values of duplicates for each MOI of FluB. Infection with Flu virus was performed after UV inactivation of HA according to [14].
The findings described here concern the interaction between components of two viruses: NA of Flu virus and Sgps on HIV-1 surface without considering the cell surface. Our hypothesis and results could be extended over other exogenous co-infections in HIV-infected people with agents containing NA; for example, *Streptococcus pneumoniae* [19,20] or *Trypanosoma cruzi*.[21] Moreover, without any regard to HIV infection, it has been reported that a number of sialylated Gps and glycolipids can be desialylated in vivo by interaction with endogenous cellular NAs, thus changing their function.[22–24]

As known, a variety of molecules, including Gps, glycolipids and glycosaminoglycans can serve as viral receptors. The domain of the receptor that binds the virus may be a carbohydrate moiety (except a polypeptide sequence) often located at the external tip of the receptor molecule.[10] In these cases, the sugar residue is responsible for binding the viral attachment protein and any changes in glycosylation/sialylation can change — restrict or augment — viral replication.

**Conclusions**

Here, we propose an experimental model contributing to a better understanding of the pathogenesis of co-infection with Flu virus in people living with HIV. The model is convenient and reproducible, based on the use of direct radioactive precursors of SA biosynthesis in chronically HIV-1 IIIB-infected cells. We hypothesize that co-infection with Flu provokes desialylation of viral Sgps (Gp120), which, in turn, directly affects HIV replication and infectivity. Mab against the V3 region of Gp120, but not the broadly reactive Mab against Gp120, recognize and neutralize the novel epitope formed by Flu NA on HIV-1 virions. Thus, the desialylated Gp120 becomes masked — unrecognizable by broadly reactive Mab against HIV-1 Gp120. In vivo, most probably, Flu co-infection/influenza vaccination leads to HIV desialylation responsible for higher HIV replication expressed by increased HIV viral load. This mechanism of action of Flu virus is quite different from that associated simply with immune deficiency in HIV-infected people. The model could be successfully adapted and utilized for investigation of virus—cell interactions, using not only laboratory, but also clinical isolates, clarification of targets/mechanisms of action of new inhibitors, masking/demasking of epitopes, etc. The hypothesis is partially confirmed by observations of other NA-containing micro-organisms which could infect not only people living with HIV. Additional studies are needed to explain all effects of the provoked desialylation on cell/tissue antigenic structure and function.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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