AN EXPERIMENTAL MODEL FOR STUDY OF SIALOGLYCOPROTEINS OF HUMAN IMMUNODEFICIENCY VIRUS 1 EPITOPE STRUCTURES

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Summary. – Sialic acid (SA) molecules located terminally on retrovirus glycoproteins (gps) play a key role in virus-cell interactions. The specificity of sialylation of Human immunodeficiency virus 1 (HIV-1) gps has not yet been studied. Looking for a convenient and reproducible experimental virus-cell model for studying the problem mentioned above we compared viral sialoglycoprotein (Sgp) patterns in H9/HTLV III B cells chronically infected with laboratory-adapted HIV-1_{LAI} and MT-2 cells acutely infected with the same virus. Cytosols (CSs) and supernatant concentrates (SNs) from these cells and cell cultures, respectively, following N-acetyl-D-[U-^{14}C]-mannosamine ([^{14}C]NAcMan) labeling were subjected to preparative isoelectrofocusing and the obtained fractions were assayed for ^{14}C-incorporation, reverse transcriptase (RT) activity and protein content. Sgp patterns in CSs from the two types of infection were similar. Highly sialylated peaks clustered mainly in the acidic region where the highest ^{14}C-incorporation, RT activity and protein content were found. The ^{14}C-incorporation was higher in CS than in SN. Analysis of CS from MT-2 cells infected with HIV-1 for the markers described above seems to be the experimental approach and model of choice for clinical isolates of HIV-1.

Key words: glycoproteins; HIV-1; isoelectrofocusing; chronic infection; acute infection; sialic acids; sialoglycoproteins; cell culture

Introduction

SAs and Sgps are widely spread in the nature – a fact suggesting their role in a number of biological processes (Fukuda, 1996). There is almost no biological event in mammals in which these ubiquitous compounds are not involved. Therefore, errors in their biosynthesis and degradation or changes in their structure and conformation due to mutations would have dramatic biological consequences (Schauer, 2004). Sialylated glycans as regular constituents of both viral and cellular receptors play a role in HIV-1 infection (Schauer, 2004).

Two viral envelope proteins – the transmembrane glycoprotein 41 (gp41) and membrane-associated surface gp120 are translated as a precursor that migrates through the endoplasmic reticulum where it is glycosylated to form gp160 (Erickson, 2001). A cellular protease in the Golgi apparatus cleaves gp160 to produce gp41 and gp120 (Erickson, 2001). Both gp160 and gp120 are heavily glycosylated and sialylated (Hu *et al.,* 1996; Taverna *et al*., 1999); thus, by chemical structure they are Sgps. It has been reported that sialylation affects physical and biological properties of gp120. For example, gp120 is resolved by isolelectric focusing in a wide acidic pH range despite a basic pI predicted for its polypeptide backbone (Stein *et al*., 1990). A number of data demonstrate activation in infectivity,

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Abbreviations: [^{14}C]NAcMan = N-acetyl-D-[U-^{14}C]mannosamine; CS(s) = cytosol(s); gp(s) = glycoprotein(s); HIV-1 = Human immunodeficiency virus 1; pI = isoelectric point; p24Ag = p24 antigen; RT = reverse transcriptase; SA(s) = sialic acid(s); Sgp(s) = sialoglycoprotein(s); SN(s) = supernatant concentrate(s)

syncytium formation and replication by human and simian lentiviruses after desialylation of the virion surface (Hu *et al*., 1996; Sun *et al*., 2002; Stamatos *et al*., 1997).

The growing interest in biological functions of gps and Sgps of HIV-1 is conferred by their active participation in the masking of number of antigen determinants, thus helping the virus to become unrecognizable and inaccessible to neutralizing antibodies (Weiss, 2003). On the other hand, Sgps are directly involved in the recognition process permitting access to receptors (Wei *et al*., 2003). The study of structure and formation of viral epitopes remains a priority for understanding their role in virus escape from neutralizing antibodies and persistence.

The specificity of sialylation of HIV-1 gps is so far an open question. Such an investigation would be highly important, because HIV-1 gps form a glycan canopy overlapping potential epitopes only partially and allowing receptor binding. It is believed that this mechanism confers viral ability to escape from the immune response (Wei *et al*., 2003).

In order to better understand the structure of HIV-1 Sgp epitopes and its changes in the course of infection we initiated a research, the first step of which was to create an experimental model as a tool for studying HIV-1 gps under laboratory conditions. For this purpose, we used a direct radioactive precursor of SAs providing data on both viral and cellular SA biosynthesis. Labeled viral Sgps were differentiated from cellular ones by isoelectric focusing. Also highly specific viral markers as RT activity and p24Ag were followed. Sgp patterns of the virus grown in chronically infected H9/HTLV III B cells and that grown in acutely infected MT-2 cells in laboratory conditions were characterized.

Materials and Methods

Cells. Uninfected cells of the CD4-positive H9 lymphocyte line (designated in the text as uninfected H9 cells) and H9 cells (HTLV III B strain) persistently infected with HIV-1 (designated as H9/HTLV III B cells) were obtained from Dr. R. Gallo, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA (Popovich *et al.,* 1984). MT-2 cells, a clone of the Human T-lymphotropic virus type 1 (HTLV-1) – transformed T-lymphoblastoid cell line were used for acute HIV-1 infection. All cell lines were grown in the RPMI 1640 medium supplemented with 10% of fetal calf serum (FCS). MT-2 cells were infected with HIV-1 in suspension 24–36 hrs after splitting at a multiplicity of infection (MOI) of 1 for 60 mins at 37°C and further maintained at 37°C in 5%CO_2.

Virus. As a source of HIV-1 a supernatant from H9/HTLV III B cell culture was used. It was collected, pooled and clarified by a low-speed centrifugation. The stock virus was characterized by infectivity (2 x 10^6 infectious virions/ml), p24Ag content (460.0 pg/ml) and RT activity (565.3 pg RT/ml).

HIV-1 infectivity was evaluated by end-point titration in MT-2 cells using a microtiter assay based on cytopathic effect (Montefiori *et al.,* 1988).

p24Ag content was measured in pg/ml by the ELISA-Murex HIV Antigen MAb VK86 Kit (Murex, Germany).

RT activity was assayed using the non-radioactive Lenti RT Assay HS Kit (Cavidi, Sweden) and was expressed in pg RT/ml.

Proteins were assayed according to Bradford (1976) and expressed in A_{280}.

Radioactive labeling of Sgps. Forty-eight hrs after infecting or passaging (in case of chronic infection) of appropriate cells [^{14}C]NAcMan (Amersham, specific activity 10 GBq/mmol) was added to final concentration of 1.5 μCi/ml and the cells were cultivated for another 18–20 hrs.

Cytosols (CSs) were prepared from cell pellets by chilling in ice water and disruption in Dounce's homogenizer. Aliquots of CSs before electrofocusing and those of fractions after elecrtofocusing were taken to measure the ^{14}C-incorporation, RT activity and protein content.

Supernatant concentrates (SNs) were prepared by centrifuging the culture supernatants at 105,000 x g for 90 mins (MSE ultracentrifuge, UK) on a 20% sucrose cushion in STE buffer pH 7.6 (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl, and 0.001 mol/l EDTA) and by resuspending the pellets in the same buffer to obtain 5-fold concentrate. Aliquot of SNs before electrofocusing and those of fractions after electrofocusing were taken to measure the same parameters as for CSs.

Preparative isoelectrofocusing of SNs and CSs was performed using a LKB model 8100-1.

^{14}C radioactivity was measured by standard liquid scintillation counting.

Results

Chronic infection

The distribution of [^{14}C]NAcMan-labeled Sgps in the CS fom H9/HTLV III B cells chronically infected with HIV-1 and uninfected H9 cells according to their pI, protein content and RT activity is shown in Fig. 1. Highly sialylated proteins were observed in the acidic regions for both infected and uninfected cells; but the regions were much wider for chronically infected compared to uninfected cells (pI peaks 4.47; 4.56; 4.87; 5.28; 5.74; 5.99 vs 3.57; 3.68). Moreover, the rate of incorporation of the labeled precursor in chronically infected cells was higher compared to uninfected cells. It is apparent that the proteins in the CSs from both infected and uninfected cells contained poorly sialylated proteins in the alkaline region.

In the fractions Nos. 4-32 (pI 4.24–8.72), the RT activity in CS from chronically infected cells correlated well with the sialylation rate of gps. In the fractions Nos. 4 and 5, in which a poor sialylation was observed, no RT activity was detected. On the contrary, although the fractions Nos. 6–32 were RT-positive, the highest RT activity was found in the acidic region with the highest ^{14}C-incorporation. Therefore, the higher sialylation rate, the higher RT activity, i.e. the sialylation in HIV-1-producing cells seems virus-specific.

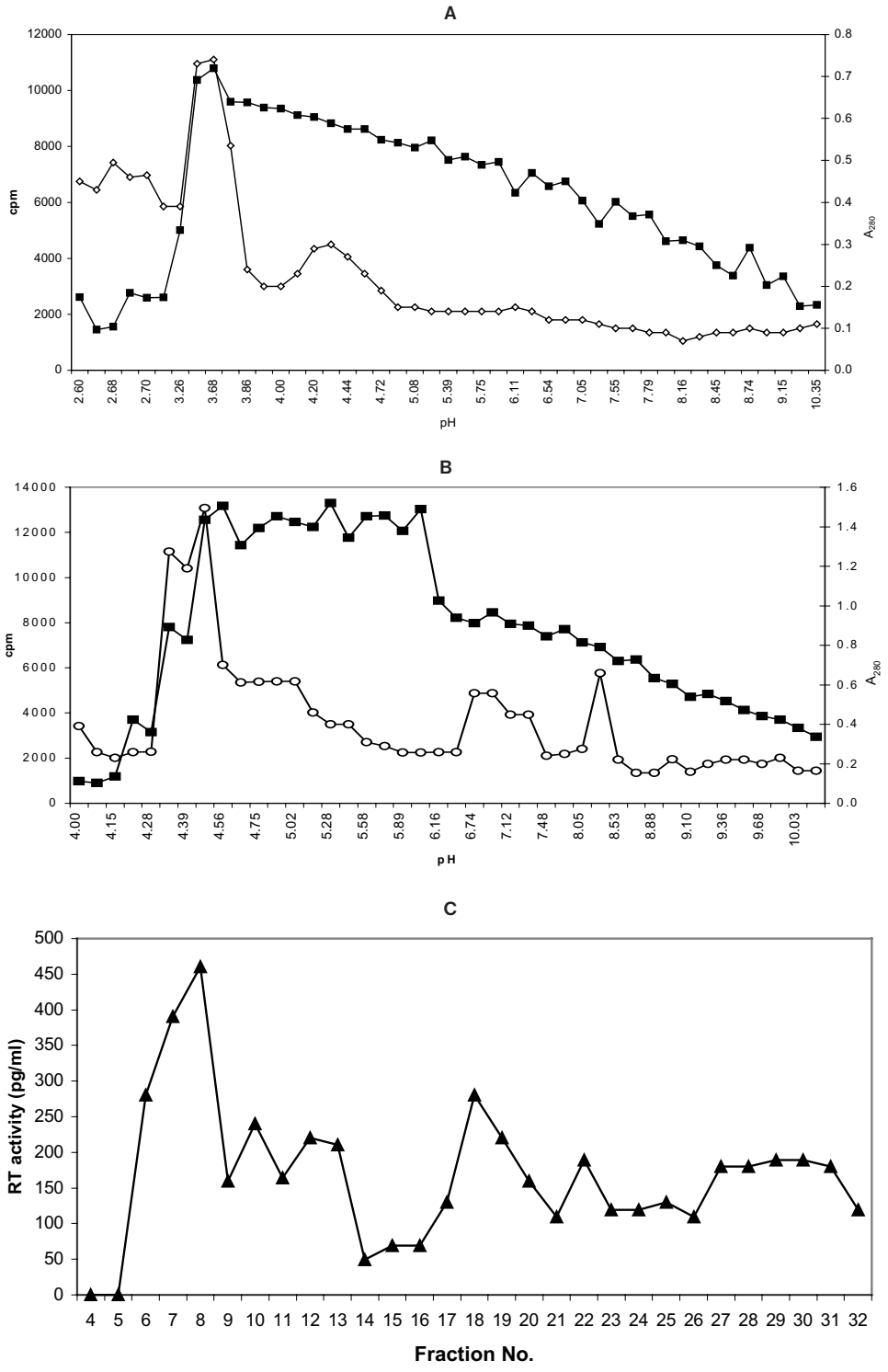

Fig. 1

Isoelectrofocusing patterns of [^{14}C]NAcMan-labeled Sgps in CSs from uninfected H9 cells (A) and chronically HIV-1-infected H9/HTLV III B cells (B) and RT activity of the fractions Nos. 4–32 from the part B (C)

cpm (■), A_{280} (○), pg RT/ml (▲).

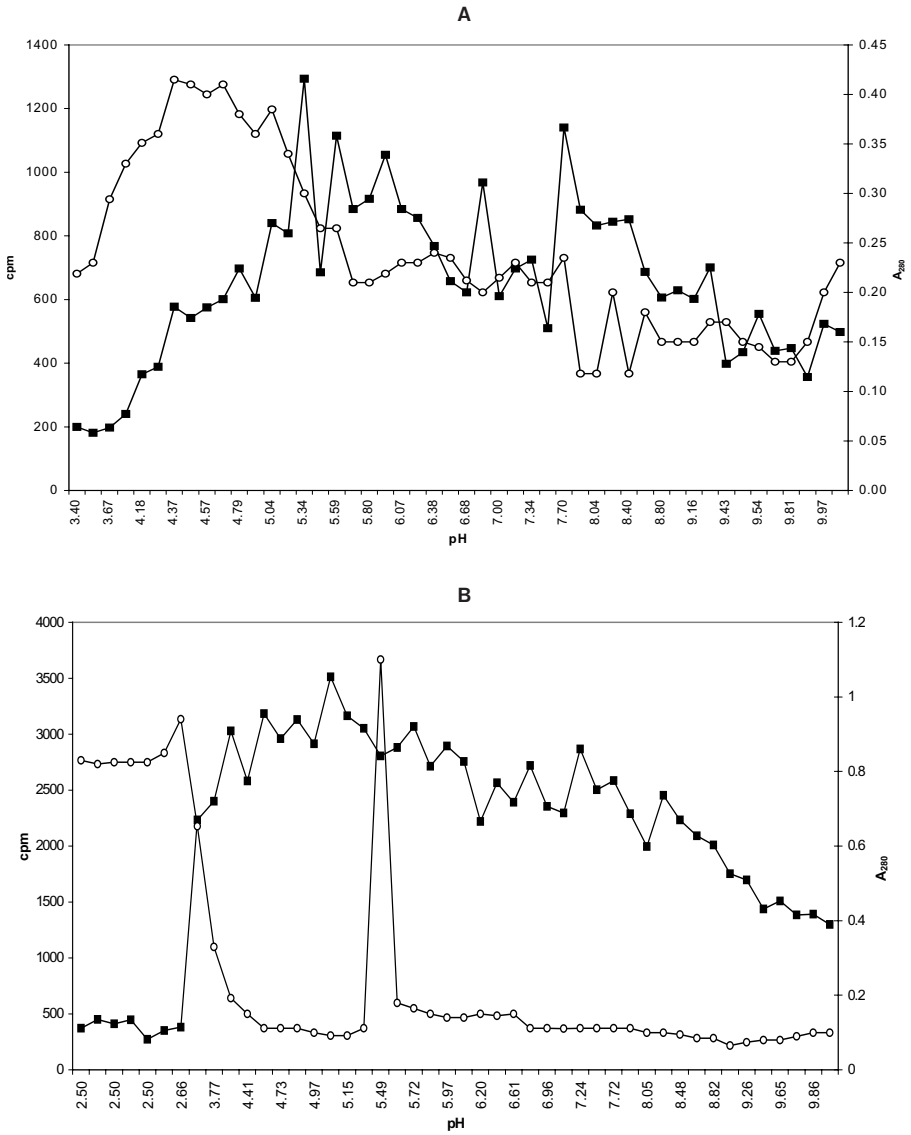

Fig. 2

Isoelectrofocusing patterns of [^{14}C]NAcMan-labeled Sgps in SNs from uninfected H9 cells (A) and chronically HIV-1-infected H9/HTLV III B cells (B)

cpm (■), A_{280} (○).

Aliquotes of CSs from uninfected H9 cells before and after isoelectrofocusing were negative for RT activity (data not shown). Fractions Nos. 6–32 were collected, dialyzed and stored at -70°C for further experiments.

Further, we compared distribution of [^{14}C]NAcMan-labeled Sgps in the SN concentrates from H9/HTLV III B cells chronically infected with HIV-1 and uninfected H9 cells according to their pI and protein content (Fig. 2). Four peaks at pI 4.22; 4.55; 4.82 and 5.03 were observed in ^{14}C-incorporation for SN from chronically infected cells (Fig. 2B) similarly to that for CS from the same cells (Fig. 1B). Such a peaks were not found with uninfected H9 cells (Fig. 2A). Again, a higher ^{14}C-incorporation rate was observed with SN from infected cells compared to uninfected ones.

Summing up, the data for incorporation of [^{14}C]NAcMan into Sgps of H9/HTLV III B cells chronically infected with HIV-1

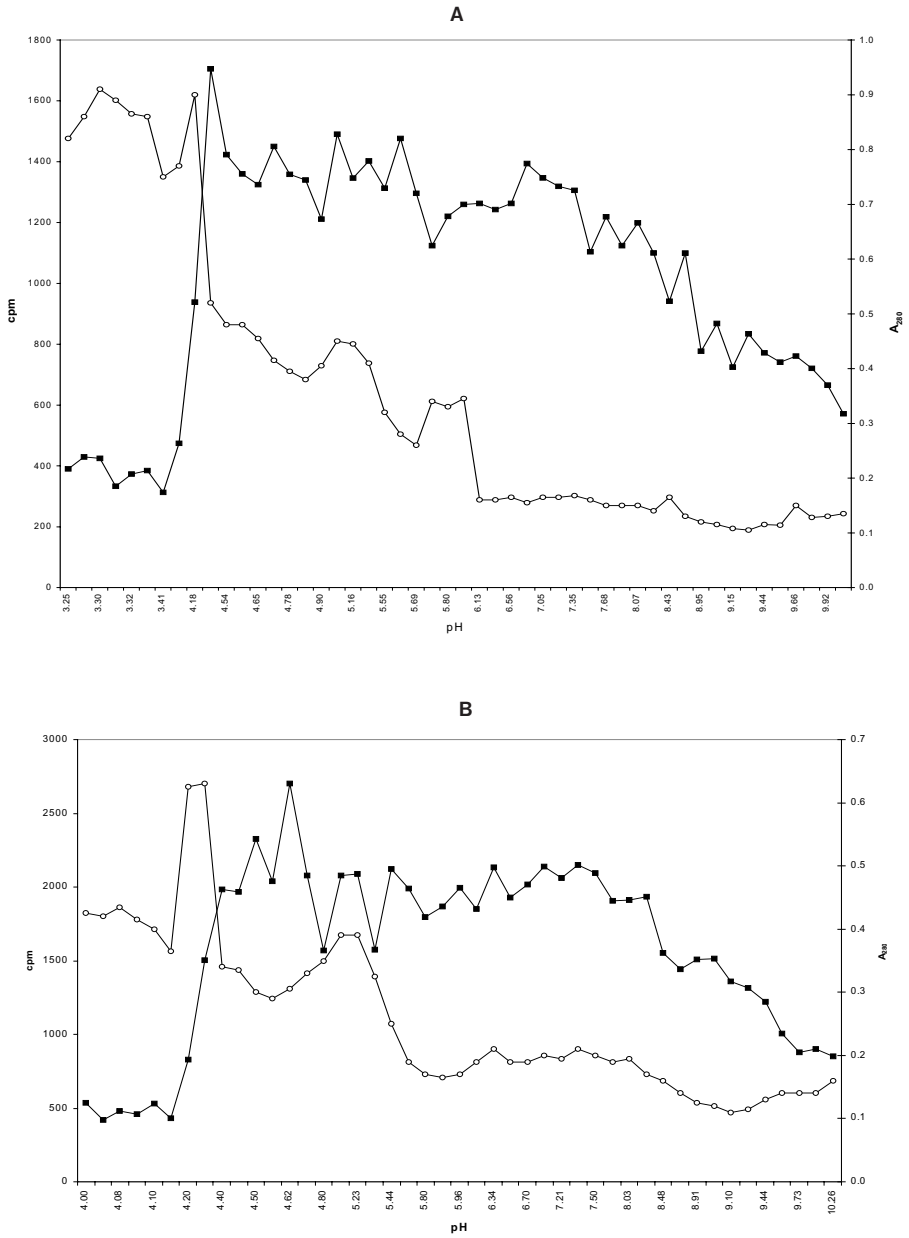

Fig. 3

Isoelectrofocusing patterns of [^{14}C]NAcMan-labeled Sgps in CSs from uninfected MT-2 cells (A) and acutely HIV-1-infected MT-2 cells (B)
cpm (■), A_{280} (○).

showed similar pI peaks for CS and SN but a higher rate for CS compared to SN. On the contrary, no similar pI peaks were seen for both CS and SN from uninfected H9 cells, indicating the presence of different Sgps of non-viral, i.e. cellular origin. Concerning the pI peaks found in the pH region of 7.72–7.79 for CS and SN from both uninfected and chronically infected cells we believe that they belong to cellular Sgps that occur unchanged in physical and chemical properties also in virus-infected cells.

Acute infection

The distribution of [^{14}C]NAcMan-labeled Sgps in the CSs from MT-2 cells acutely infected with HIV-1 and uninfected ones according to their pI and protein content is shown in Fig. 3. Similarly to chronic infection, the ^{14}C-incorporation was higher for infected cells compared to non-infected ones. Similar results were obtained with SNs (data not shown).

Table 1. Similarity of pI peaks of Sgps of HIV-1 from acute and chronic infection

pI region	H9/HTLVIII B cells, chronic infection		HIV-1-infected MT-2 cells, acute infection	
	CS	SN	CS	SN
4.5–4.7	4.56	4.55	4.50, 4.62	4.53
4.8–5.0	4.87	4.82		
5.7–6.0	5.99	5.72, 5.97	5.96	5.98
7.7–7.9	7.70	7.72		

Accordingly, a higher p24Ag content (540 pg/ml) and RT activity (251.26 pg/ml) were found for CS compared to SN (370 pg/ml p24Ag and 149.36 pg RT/ml, respectively) from acutely infected cells. The fractions from CS with the highest sialylation rate and RT/p24Ag contents were collected, dialyzed and stored at -70°C as a [^{14}C]NAcMan-labeled HIV-1 from acutely infected cells. No p24Ag and RT-activity were detected in CS and SN from uninfected MT-2 cells.

Comparison of Sgp patterns of CSs from uninfected H9 and MT-2 cell lines showed no common pI peaks (Figs. 1A and 3A).

According to the data obtained, pI peaks for CSs and SNs from both chronically and acutely infected cells were located in the pH regions of 4.5–4.7 and 5.7–6.0. Peaks in these regions were not observed with uninfected H9 or MT-2 cells. Table 1 summarizes pI peaks of Sgps for CSs and SNs from acute and chronic infections.

Discussion

SAs and Sgps are key elements regulating cell-to-cell contacts and virus-cell interactions (Kelm and Schauer, 1997). Both HIV-1 and permissive human cells contain SAs and Sgps involved in the virus attachment to cell (Sun *et al*., 2002). Following the idea about changes in envelope conformation and glycan packing (Wei *et al*., 2003) we believe that an experimental approach and model demonstrating Sgp profiles in HIV-1 infection are needed. The model should be focused on HIV-1 Sgp characteristics under different conditions, e.g. laboratory strains or clinical isolates, acute or chronic infection. This study attempted to develop a different, so far unexploited approach to describe Sgp profiles of the virus, first of all under standard laboratory conditions.

It is well known that primary HIV-1 isolates from infected persons differ substantially from laboratory adapted strains (Moore *et al*., 1995). Nevertheless, laboratory-adapted strains remain valuable virus models (Poignard *et al.,* 1996).

Looking for a convenient and reproducible experimental model for studying Sgp patterns of clinical HIV-1 isolates we used a labeled precursor of SA biosynthesis and obtained highly purified and well characterized Sgp pattern of [^{14}C]NAcMan-labeled HIV-1 with CSs and SNs from MT-2 cells acutely infected with a laboratory-adapted virus (HIV-1_{LAI}). The same profile was derived for chronically HIV-1-infected H9/HTLV III B cells. Sgp patterns for acutely infected cells did not substantially differ from those for chronically infected cells. These data show that CSs are more convenient than SNs for analysis of acute or chronic HIV-infection because of higher precursor incorporation into Sgps together with similarity of pI peak distribution. The lower precursor incorporation with SNs from chronically or acutely infected cells probably expresses both the Sgps of the virus released and gp120 subunits shed. The higher incorporation with CSs reflects probably highly glycosylated virion spikes that remain intact (McMichael and Hanke, 2003).

These findings encouraged us to recommend to analyze CSs from MT-2 cells infected with HIV-1 for, e.g. Sgp patterns of clinical virus isolates from different patients, treatment, tropism, co-receptor usage, the effect of putative glycosylation inhibitors, neutralization, etc. Highly sialylated [^{14}C]NAcMan-labeled HIV-1 preparations derived from CSs from chronically and acutely infected cells investigated in this study could be compared to similar preparations of clinical HIV-1 isolates.

It was of special interest to compare Sgps of HIV-1 with those of another retrovirus, namely Mc-29 virus. This virus was isolated in Sofia (Ivanov *et al*., 1964) and years long was used at the Institute of Experimental Pathology and Parasitology as experimental model of avian retroviral infection, including its Sgp pattern (Karaivanova *et al*., 1991). The study presented here provides data demonstrating similarity of pI peaks of Sgps of HIV-1 (under both acute and chronic infection) and Mc-29 virus. Three common pI peak regions could be found, namely 4.53–4.56, 5.82–5.99, and 7.70–7.85. As all these peak regions were absent in uninfected chicken liver cells we believe that they represented retrovirus-specific Sgps. It is worth emphasizing that the Sgp profile of Mc-29 virus was strikingly similar to that of HIV-1, although these two viruses belong to different subfamilies and have different hosts (Karaivanova *et al.,* 1991).

Virus-associated Sgps clustered mainly in the acidic region, thus corresponding well to HIV-1 gp120, known to occupy a similar position (Stein *et al*., 1990). The data obtained here may be interpreted by hypersialylation of Sgps, whose pI is shifted to acidic but not to basic region as predicted by their polypeptide chain (Stein *et al*., 1990; Hu *et al*., 1996).

Here, it should be mentioned that the envelope of another lentivirus, Caprine arthritis encephalitis virus (the *Caprine*

arthritis encephalitis virus species, the *Lentivirus* genus) is also hypersialylated. This fact probably confers the virus resistance to neutralization with sera of infected animals (Huso *et al*., 1988).

Taken together, our findings extend the knowledge on how to study prominently positioned SAs on HIV-1 gps participating in virus escape from the immune control – a function mediated by alterations in sialylation, glycosylation and conformation of gps (Wei *et al*., 2003).

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