JOURNAL OF VIROLOGY, Nov. 1996, p. 7462–7470 Vol. 70, No. 11
0022-538X/96/$04.00+0

Infectivities of Human and Other Primate Lentiviruses Are Activated by Desialylation of the Virion Surface

HUILING HU,[1] TATSUO SHIODA,[1] CHIKAYA MORIYA,[1] XIAOMI XIN,[1] MOHAMMAD K. HASAN,[1] KOICHI MIYAKE,[2] TAKASHI SHIMADA,[2] AND YOSHIYUKI NAGAI[1]*

Department of Viral Infection, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108,[1] and Second Department of Biochemistry, Nippon Medical School, Bunkyo-ku, Tokyo 113,[2] Japan

Received 16 April 1996/Accepted 21 July 1996

The envelope protein, gp120, of human immunodeficiency virus type 1 (HIV-1) is heavily glycosylated and sialylated. The heavy sialylation greatly affects the physical properties of the protein, as it resolves into a wide acidic pH range despite the basic pI value predicted for its polypeptide backbone (B. S. Stein and E. G. Engleman, J. Biol. Chem. 265:2640–2649, 1990). However, the functional significance of the heavy sialylation remains elusive. Here, we show that desialylation of HIV-1 with neuraminidase greatly augments the initial virus-cell interaction, leading to remarkably enhanced viral replication and cytopathogenicity. This enhancement appeared to be a direct result of the removal of negatively charged sialic acids but not of the exposure of galactose residues or complement activation. Complementing these results, studies with inhibitors of mannosidase I and mannosidase II showed that the processing of HIV-1 oligosaccharides into the complex type to acquire the terminal sialic acid residues impeded the full replication capacity of the virus and that its prevention also enhanced virus replication and cytopathogenicity. Enhancement of infection by desialylation was found widely, with HIV-1 laboratory strains of different cell tropisms and primary isolates as well as HIV-2 and simian immunodeficiency virus. Thus, the sialylation catalyzed by host cell pathways appeared to reduce the infectivity of human and nonhuman primate lentiviruses. Our results further suggested that desialylation would help increase the titers of HIV-based vectors.

One of the striking features of human immunodeficiency virus type 1 (HIV-1) and other lentiviruses is quite heavy glycosylation of the envelope protein. For instance, the 90-kDa polypeptide of the HIV-1 Env gene product is cotranslationally modified in the rough endoplasmic reticulum (ER) by the addition of as many as ~30 *N*-asparagine-linked oligomannosyl carbohydrate residues (for a review, see reference 10). This Env precursor, gp160, is then proteolytically cleaved in the Golgi complex to yield the surface (gp120) and transmembrane (gp41) subunits. While nearly half of the oligosaccharide chains undergo little further processing and remain the high-mannose type, the other half are sequentially modified in both the ER and the Golgi complex to ultimately become the complex type (12, 16, 19, 20, 33). Complex oligosaccharides are generated by a combination of the trimming of oligosaccharides attached in the ER to the protein backbone and the addition of further sugars. This oligosaccharide processing begins in the ER with the removal of the glucose residues from the initial oligosaccharides, consisting of *N*-acetylglucosamine (GlcNAc), mannose, and glucose, followed by the removal of a specific mannose. The remaining steps occur in the Golgi stack, where Golgi mannosidase I first removes three mannoses and GlcNAc transferase I then adds a GlcNAc, which enables mannosidase II to remove two additional mannoses. This yields the core of three mannoses to which GlcNAc, galactose, and sialic acid are sequentially conjugated, finally generating a complex oligosaccharide.

Complex oligosaccharide chains are terminally branched, most often being di- or triantenary and sometimes more divergent, and these multiantenary structures are either completely or partially sialylated. There are additionally some O-linked oligosaccharides which are also terminally sialylated (4). As a result, the mature Env, particularly the surface gp120 subunit possessing most of the oligosaccharide chains, is heavily sialylated. Sialic acid is one of the few sugar residues in glycoproteins that bear a net negative charge. Heavily sialylated HIV-1 gp120 is thus electrofocussed to an acidic pH range, despite the basic pI value (8.0 or more) predicted for its naked polypeptide backbone chain (33).

The role of HIV-1 virion surface sialic acids in virus-cell interactions has been studied by treating the virus with neuraminidase (NA) or by treating infected cells with oligosaccharide-processing inhibitors. Although the authenticity of the action of these reagents under the stated conditions was not sufficiently verified, the data indicated that the desialylated virions activate complement through the alternative pathway and thereby acquire greater binding capacity and infectivity for the cells expressing complement receptors (22, 23). It is also suggested that the exposure of the second terminal galactose by enzymatic desialylation of gp120 augments its interaction via the galactose receptor with human monocytes and dendritic cells (17). This would lead to augmented uptake and presentation of gp120 antigens by those cells.

Here, we also examined the effect of NA and oligosaccharide-processing inhibitors on the replication and cytopathogenicity of HIV-1. The authenticity of the action of each reagent was carefully assessed both biochemically and biologically. The data demonstrated (i) that in vitro enzymatic desialylation of the virions as well as in vivo prevention of complex oligosaccharide formation by inhibitors of Golgi trimming glycosidases remarkably enhanced viral replication and cytopathogenicity, (ii) that this enhancement was caused primarily by the augmentation of the initial virus-cell interaction, (iii) that this augmented virus-cell interaction was due to neither comple-

* Corresponding author. Mailing address: Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan. Phone: 81-3-5449-5285. Fax: 81-3-5449-5409. Electronic mail address: ynagai@hgc.ims.u-tokyo.ac.jp.

ment activation nor the exposure of galactose residues but could be a direct result of the removal or lack of virion surface sialic acids, and (iv) that the enhancement is a phenomenon commonly observed with HIV-1, including laboratory strains of different cell tropisms, primary isolates, HIV-2, simian immunodeficiency virus (SIV), and an HIV-1 Env-based vector. We thus suggest that oligosaccharide processing into the mature complex type could be an impediment rather than necessary or irrelevant for full infectivity of human and other primate lentiviruses. Desialylation would not only facilitate virus isolation from patients but also help increase the titers of HIV vectors.

MATERIALS AND METHODS

Viruses and cells. HIV-1 strains SF2 and SF162 (32) were propagated in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs). HIV-1 strain NL43 (1) and SIVmac strain 239 (24) were grown in the MT4 T-cell line, and HIV-2 strain GH123 (30) was grown in another T-cell line, M8166. PBMCs were obtained from normal seronegative donors and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) and 25 U of interleukin-2 (Genzyme Corp., Cambridge, Mass.) after stimulation with phytohemagglutinin (3 μg/ml) for 3 days. Primary blood monocytes were obtained from PBMCs by the plastic adherence procedure and maintained in RPMI 1640 containing 10% FCS and 5% heat-inactivated human serum to allow their differentiation into macrophages (32). MT4 and M8166 cells were grown in RPMI 1640 supplemented with 10% FCS. Details about the HIV-1 Env-based vector HXN and its packaging and production were described previously (31). Briefly, the HIV-1 vector plasmid pHXN was constructed by inserting the thymidine kinase-driven neomycin resistance gene between the long terminal repeats of HIV-1 (strain BH10). Packaging was done by cotransfection with the plasmid pCGPE expressing Gag, Pol, and Env under the control of the cytomegalovirus promoter.

Infection experiments and isolation of virus from patients. In infection studies with laboratory strains, 10^5 cells in 1.0 ml of medium were exposed at 37°C for 2 h to, unless otherwise noted, 10 ng of viruses in p24 antigen (HIV-1 strains SF2, NL43, and SF162) or 2×10^5 cpm of reverse transcriptase (RT) activity of viruses (HIV-2 GH123 and SIVmac 239). The cells were then washed twice with RPMI 1640 medium and maintained in the respective culture media described above. In some experiments, cells were cultured with unheated fresh FCS-containing RPMI 1640 or serum-free ASF103 medium (Ajinomoto, Tokyo, Japan). ASF103 medium contained bovine serum albumin (1,000 mg/liter), human transferrin (5 mg/liter), and bovine insulin (5 mg/liter) as the protein constituents. Culture supernatants of infected cells were periodically assayed for RT activity (35) and/or p24 core antigen levels by using an enzyme-linked immunosorbent assay (ELISA) kit (Abbott, Wiesbaden-Delkenheim, Germany). Data are shown as means ± standard deviations for duplicate cultures. For coinfection experiments, MT4 cells were infected with the temperature-sensitive (*ts*) Sendai virus mutant *ts*23 (14) at an input multiplicity of 10 PFU per cell and incubated at 37°C (semipermissive for the virus) for 24 h prior to HIV-1 infection. The HIV-1 vector virus was inoculated onto the monolayers of CD4-expressing HeLa cells, and its infectivity was assayed by counting cell colonies resistant to neomycin as described previously (31). To isolate HIV-1 from patients, about 3×10^5 PBMCs obtained from HIV-1-infected individuals (patient 43 or 77) were cocultivated with 10^6 normal seronegative donor PBMCs with or without 0.03 U of NA. Hexadimethrine bromide (Polybrene [PB]) (2.0 μg/ml) was added to NA-negative cultures. Virus isolation was monitored by periodically assaying RT activity in the culture supernatants.

HIV-1 binding and entry assays and proviral DNA quantitation. One hundred nanograms of p24 of HIV-1 strain NL43 or 10^5 MT4 cells, or both, were treated or not treated with 1.0 U of NA at 37°C for 1 h in RPMI 1640 medium. The virions were then allowed to adsorb to the cells at 4°C for 1 h. After the infected cells were extensively washed at 4°C to remove unbound virus and the enzyme, the cells were lysed in 200 μl of lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl [pH 7.5]), and the amount of virus bound to cells was quantitated by p24 ELISA. For the entry assay, cells bound with the virus were subsequently incubated at 37°C for 1 h, and the virions remaining on the cell surface were digested with trypsin (500 μg/ml) at 37°C for 10 min (5). The cells were lysed and assayed for p24 as described above. To quantify proviral DNA, infected cells were further incubated at 37°C for 24 h, and then the intracellularly copied DNA (of a 177-bp region in the long terminal repeat) was PCR amplified and analyzed by polyacrylamide gel electrophoresis (PAGE) exactly as described previously (6).

Lectin binding assay. NL43 virions were grown in MT4 cells with serum-free ASF103 medium. Culture supernatant (30 ml) containing 1,000 ng of p24 per ml was treated with 1.0 U of NA per ml at 37°C for 1 h or left untreated, and the virions were concentrated by centrifugation at 26,000 rpm for 1 h in a Beckman SW28 rotor. Pelleted virions were then lysed, and the viral proteins were resolved by sodium dodecyl sulfate (SDS)–10% PAGE (15) and then electrically transferred to nitrocellulose membranes. Resolved proteins on the membranes were then probed with a 5-μg/ml concentration of biotin-labeled RCA-1 lectin (EY Laboratories, San Mateo, Calif.), which specifically recognizes the galactose residues of asialoglycoproteins, in 0.05 M Tris-HCl–0.14 M NaCl (pH 7.5) at room temperature for 30 min. The bound lectins were visualized by incubation with 5 mg of horseradish peroxidase-labeled avidin (Organon Teknika, Turnhout, Belgium) per ml at room temperature for 30 min followed by incubation with 3.0 mg of 4-chloro-1-naphthol per ml–0.02% H_2O_2 in 0.02 M Tris-HCl–0.1 M NaCl (pH 7.5). Another set of membranes similarly prepared was probed with pooled anti-HIV-1 serum from infected patients to quantify the virion proteins applied to SDS-PAGE and blotted onto the membranes.

Digestion with endo H. About 2×10^7 MT4 cells infected with 2,000 ng of p24 of HIV-1 strain NL43 as described above were incubated at 37°C for 3 days in the presence or absence of 2.5 mM 1-deoxymannojirimycin (DMM). The cells were then radiolabeled with 500 kBq of EXPRE^{35}S^{35}S protein-labeling mix (>37 TBq/mmol; Du Pont-New England Nuclear, Wilmington, Del.) per ml in methionine-free medium for 16 h in the presence or absence of DMM. Radiolabeled virions were concentrated from the culture supernatant by centrifugation as described above and lysed in 1% Triton X-100–0.5% deoxycholic acid–0.1% SDS–0.15 M NaCl–1 mM phenylmethylsulfonyl fluoride–50 mM Tris-HCl (pH 8.0). HIV-1 proteins were then immunoprecipitated with anti-HIV-1 serum and protein A-agarose (Gibco BRL, Gaithersburg, Md.), digested with 0.1 U of endoglycosidase H (endo H) (Seikagaku Corp., Tokyo, Japan) per ml, and analyzed by SDS–10% PAGE followed by fluorography as described previously (26).

NAs and other reagents. Highly purified NA of *Clostridium perfringens* origin (type X; specific activity, 200 U/mg of protein; proteinase activity, <0.002 U/mg of protein; *N*-acetylneuraminaldolase activity, 0.1 U/mg of protein) and NAs from *Arthrobacter ureafaciens*, *Salmonella typhimurium*, *Streptococcus* sp., and *Vibrio cholerae* were purchased from Sigma, St. Louis, Mo. The specific inhibitor of NA 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA), the mannosidase inhibitors DMM and swainsonine (SW), the monosaccharides D-galactose and *N*-acetyl-D-galactosamine, and mannan from *Saccharomyces cerevisiae* were also from Sigma. The anti-CD4 antibody Leu3A was from Becton Dickinson, San Jose, Calif.

RESULTS

Effect of NA on the replication of T-cell-line-tropic HIV-1 strains SF2 and NL43. Highly purified NA from *C. perfringens* was added to the culture medium of MT4 cells infected with the T-cell-line-tropic strain SF2, and the virus yields were determined on various days postinfection. This strain exhibited relatively slow replication kinetics, and the virus replication was greatly facilitated by NA during the course of prolonged culturing (Fig. 1). The enhancement was dose dependent over a broad range (0.003 to 1.0 U/ml) of the enzyme concentration and was 100-fold or more late in infection under certain conditions. An obvious cytopathic effect (CPE) characterized by syncytium formation developed in the enzyme-treated cells (Fig. 1), although this virus-cell system is essentially poor in this manifestation.

Another T-cell-line-tropic strain, NL43, exhibits much more rapid replication kinetics and much more severe CPE than SF2. When infection was initiated with the highest dose, enhanced replication of this strain caused by NA was obvious around day 3 (Fig. 2). However, because of extensive CPE beginning to develop on the same day, little further virus multiplication occurred. In the absence of NA, the replication kinetics was much slower and the virus titer and CPE peaked 2 days later. As the input doses were reduced, the enhancing effect of NA became much clearer and observable throughout the incubation period (Fig. 2). These results suggested that the effect of NA on a single round of replication might be small but was sufficient to cause a profound enhancement on logarithmic multiple cycles of replication.

The enhancement was clearly NA specific, as it was totally offset by the specific inhibitor DANA (18) (Fig. 3A). The degree of enhancement was similar when infected cells were cultured with medium containing heat-inactivated FCS or fresh FCS or with serum-free ASF101 medium (Fig. 3A). This unequivocally ruled out the involvement of complement activation. The degree of enhancement by NA differed remarkably depending on the time of sampling (Fig. 2). This would explain

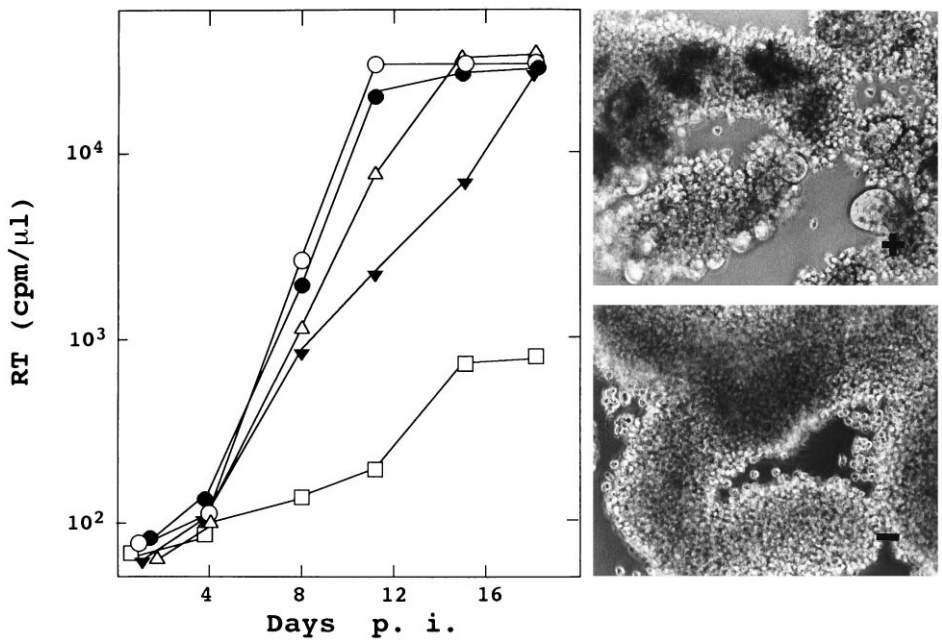

FIG. 1. Effect of NA on the replication and cytopathogenicity of HIV-1 strain SF2 in MT4 cells. Cells infected as described in Materials and Methods were incubated in the presence of 0 (□), 0.003 (▼), 0.03 (△), 0.5 (●), and 1.0 (○) U of highly purified *C. perfringens* NA per ml. Every 3 days, the medium was replaced with fresh medium containing the respective enzyme concentration. Culture supernatants of these infected cells were assayed for RT activity at 3- to 4-day intervals. Syncytia typical of HIV-1 infection developed on day 5 in the presence (+) but not the absence (−) of 0.03 U of NA per ml. p.i., postinfection.

the relatively low level (3.6- to 4.0-fold) of enhancement in these experiments.

There are numerous other bacterial and viral pathogens that express NA, and some of them can coinfect with HIV. We therefore investigated whether various NA species would enhance HIV-1 infection. All four other commercially available bacterial NAs of different origins were similarly effective (Fig. 3B). With respect to viral NAs, no soluble enzyme preparations are available in amounts sufficient for similar studies. In many coinfection studies with HIV-1, the viruses or viral vectors so far used to express NA caused CPE and a loss of cellular viability. This did not allow the unequivocal evaluation of the effect of the viral NA upon coinfecting HIV-1. On the other hand, a *ts* mutant of Sendai virus, which has a defect in RNA synthesis, expresses NA at a semipermissive temperature without serious CPE (14). Thus, although it is not a human pathogen, this Sendai virus mutant appeared to be helpful and was used as a representative of NA-expressing viruses. The coinfecting *ts* mutant enhanced HIV-1 replication, and the actual involvement of the viral NA was clearly verified by the abrogation of enhancement by DANA (Fig. 3B). Thus, NAs of diverse microbial origins appeared to enhance HIV-1 replication.

Virions and the initial virus-cell interaction are the targets of NA action. In order to learn whether virions or cells are targeted by NA and which step in the virus life cycle is affected, we treated NL43 virus and MT4 cells separately with NA and examined the virus replication in various combinations. A 10-times-higher amount (100 ng of p24 antigen) of virus than that for usual infection experiments was used. The enzyme treatment was done at 37°C for 60 min. To minimize the effect of NA in the subsequent process, virus adsorption to cells was done at 4°C for 1 h, and then the enzyme and unbound virions were removed by extensive washing of the cells with ice-cold phosphate-buffered saline. Treatment of virions or both virions and cells with NA remarkably enhanced the virus yield (Fig. 4A). When only the cells were treated, there was always a slight but significant enhancement. Enhanced infection was CD4 dependent, as it was blocked by anti-CD4 antibody (Fig. 4A). Exposing virions to NA promoted virus attachment to cells (Fig. 4B). This led to accordingly increased levels of virus resistant to trypsin and presumably taken up by cells (5) and of intracellular reverse transcripts (Fig. 4C and D). These results strongly suggested that virions and not cells are the principal target of the NA action and that virus binding to cells in the virus life cycle is the step primarily affected. Polycationic PB has often been added to cells to augment virus attachment to cells. However, PB exerted only a slightly positive effect comparable to that of NA treatment of cells (Fig. 4D). The effect of treating virions with NA was always much greater. Neither a synergistic nor an offsetting effect was exerted by NA and PB.

The NA-treated and untreated virion proteins were separated by SDS-PAGE, blotted onto a membrane, and probed with the lectin RCA-1, which preferentially recognizes the galactose residues exposed upon desialylation but not the internal ones and is thus asialoglycoprotein specific. In this lectin binding assay, a serum glycoprotein contaminating the virion preparations and comigrating with gp120 in SDS-PAGE always gave rise to high background levels. It has been very difficult to avoid this contamination. We therefore used the serum-free medium ASF103, as described for Fig. 3A, to grow the virions.

As shown in Fig. 5A, NA remarkably augmented binding of the lectin to gp120. This confirmed substantial removal of the terminal sialic acids from and the exposure of the second terminal galactose residues on gp120 under our experimental conditions. That comparable amounts of NA-treated and untreated samples were initially applied to SDS-PAGE was verified by the similar intensities of gp120 bands in immunoblotting in the two preparations (Fig. 5A). Because of substantial removal of sialic acids, NA-treated gp120 migrated slightly but

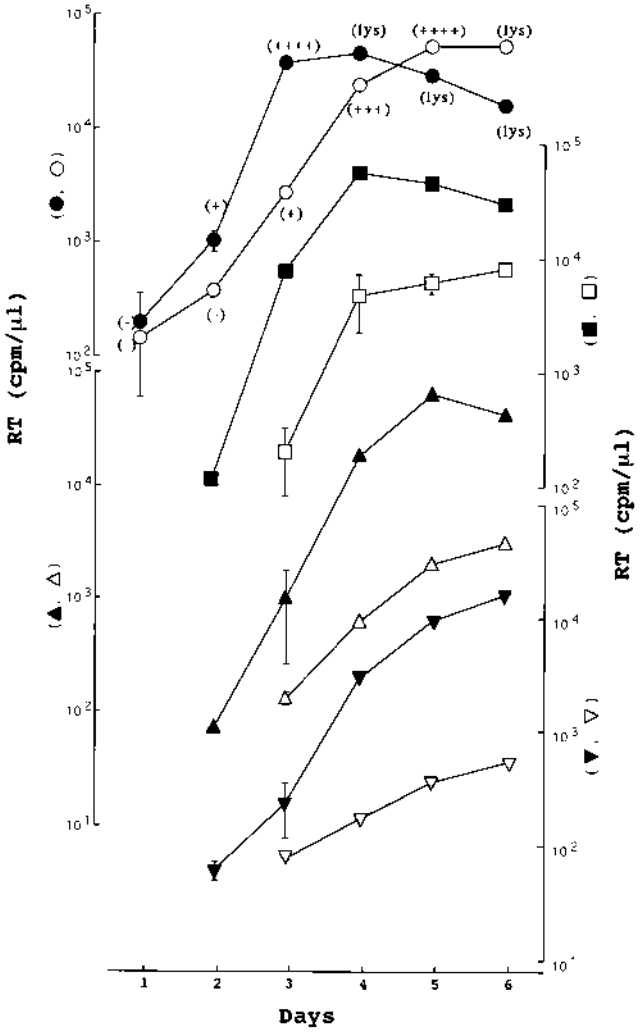

FIG. 2. Effect of NA (0.03 U/ml) on the replication of HIV-1 strain NL43 in MT4 cells. Infection proceeded in the presence (closed symbols) and absence (open symbols) of NA as described for Fig. 1 except that various input virus doses, i.e., 10 (● and ○), 1.0 (■ and □), 0.2 (▲ and △), and 0.04 (▼ and ▽) ng of p24 antigen, were used. The degree of CPE is scored as − (none) to ++++ (maximum) or as cell lysis of the entire culture (lys) for the infection with the highest input dose.

significantly faster than the untreated control in SDS-PAGE (Fig. 5A). Uninfected culture fluids treated with NA or untreated were processed in parallel for centrifugation, SDS-PAGE, and RCA-1 probing. No signal was found at the position corresponding to that of gp120 (control in Fig. 5A), indicating the specificity of the RCA-1 binding assay. Probing with sialic residue-specific lectins of similarly prepared membranes was unsuccessful, possibly because of insufficient affinity or reactivity.

The question arose as to whether the galactose residues exposed upon enzymatic desialylation play a role in the promoted virus replication. To address this, we used the specific competitors D-galactose and *N*-acetyl-D-galactosamine, which specifically compete with NA-treated gp120 for the galactose receptors on human monocytes and dendritic cells (17). At the concentration sufficient for this competition (10 mM) or higher, neither of these saccharides abrogated the enhancing effect of NA (Fig. 5B). It thus appeared most likely that HIV-1 infection enhanced by NA was an immediate result of sialic acid removal from the virion surface rather than an indirect secondary effect mediated by the presumed galactose-galactose receptor interaction.

Further verification of the specific role of virion surface sialylation in HIV-1 infection with inhibitors of trimming glycosidases. The lectin binding and competitor assays (Fig. 5) as well as studies with DANA (Fig. 3) suggested the specific indigenous role of virion surface sialic acids in determining the infection efficiency of HIV-1. Supporting results were obtained by the following studies with oligosaccharide-processing inhibitors.

The generation of complex oligosaccharides with terminal sialic acids is a multistep process involving the stepwise trimming of oligosaccharides attached to the protein backbone and the addition of further sugars. Each step is absolutely dependent on the previous step. Thus, incubating infected cells with DMM or SW, the specific inhibitors of mannosidase I and mannosidase II, respectively, would yield virions possessing only poorly processed oligosaccharides of the high-mannose type without sialic acids. If so and if the high-mannose oligosaccharide predominance in itself has little effect on the virus replication, then the virus replication in cells treated with these inhibitors should be similar to that found with NA. Indeed, DMM similarly promoted infection (Fig. 6A; cf. Fig. 2, top). At 0.5 mM, DMM already greatly enhanced the infection, but coexisting NA exerted a significant additive effect, suggesting an as-yet-insufficient DMM dose. The action of DMM seemed to be almost complete at 2.5 mM, since there was a virtual lack of such an additive effect of NA (Fig. 6A). Enhancement by SW was a bit less striking than that by DMM, and it remained at almost the same level at concentrations of 0.5 to 2.5 mM (data not shown). Little additive effect of NA was seen under these conditions (data not shown). Thus, there was no plausible explanation for the less apparent action of SW.

endo H is known to specifically hydrolyze high-mannose, but not complex, oligosaccharides. To ascertain the authenticity of DMM action, gp120 molecules associated with virions grown in the presence and absence of DMM were exposed to endo H, and their digestion profiles were compared. Previous data have indicated that virion-associated gp120 contains roughly equal amounts of oligomannosidic and complex-type glycans (12) and migrates as a band of about 100 kDa in SDS-PAGE after endo H digestion (33). Exactly the same behavior was found for the gp120 of NL43 virions grown in the absence of DMM (Fig. 6B). This 100-kDa species was totally absent when the virions grown in the presence of DMM were similarly processed for endo H digestion, and instead, a 60-kDa species was generated, which corresponded well in size to the carbohydrate-free polypeptide backbone of gp120 (Fig. 6B). These results strongly suggested that the oligosaccharides on the gp120 of virions produced in the presence of DMM were almost totally sensitive to endo H and hence were exclusively the high-mannose type. DMM thus indeed did not allow the oligosaccharides on gp120 to acquire further sugars characteristic of the complex type. Accordingly, this gp120 species migrated significantly faster in the gel than that from the control virions (Fig. 6B).

High-mannose oligosaccharides on the HIV-1 virion surface are the target of the mannose-binding protein naturally present in human sera. Thus, fresh human serum neutralizes HIV-1 to some extent (9). By this analogy of molecular interaction, if cells express mannose receptors, their interaction with viral high-mannose oligosaccharides would presumably augment the virus-cell interaction. It was thus not ruled out that this type of mechanism is involved in the virus replication

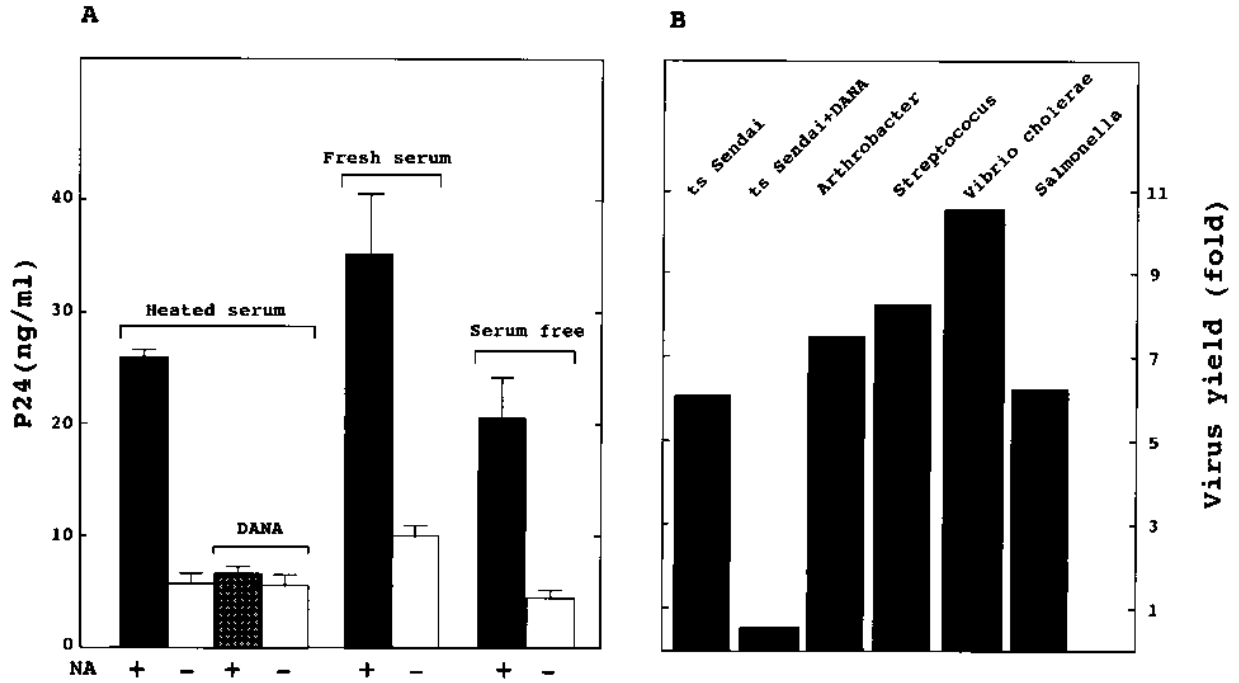

FIG. 3. (A) Enhancement of HIV-1 replication by NA with various media and its abrogation by the NA-specific inhibitor DANA. Infection initiated with strain NL43 (approximately 20 ng of p24 antigen) was studied in the presence (+) or absence (−) of NA in medium supplemented with heat-inactivated (56°C, 60 min) or fresh serum or in serum-free ASF103 medium. The virus yields are shown as p24 antigen amounts on day 4. DANA was used at a concentration of 100 μM. (B) Effects of NAs of other bacterial origin and of Sendai virus *ts* mutant coinfection on NL43 virus yields in MT4 cells. The effect of bacterial NAs (0.03 U/ml) was studied as described for panel A. The *ts* Sendai virus was inoculated at a multiplicity of 10 PFU per cell 24 h before infection with HIV-1. After incubation for 4 days at 37°C, which is semipermissive for the *ts* mutant, the HIV-1 yields were assayed and compared with that of cells singly infected with HIV-1 as well as those exposed to DANA. Enhancement is shown as the fold increase relative to results for the controls without NA or Sendai virus. The amount of p24 antigen in the control samples was 5.7 ± 0.3 ng/ml.

enhanced by DMM or SW. Mannan blocks the mannose-binding-protein-mediated neutralization capacity of human serum (9). A large excess of mannan over the dose sufficient to exert this action (1 mM) (9) did not appreciably offset the effect of DMM (Fig. 6A). This suggested that the prevalence of high-mannose oligosaccharides on the virion surface, in itself, has nothing to do with the virus replication promoted by DMM.

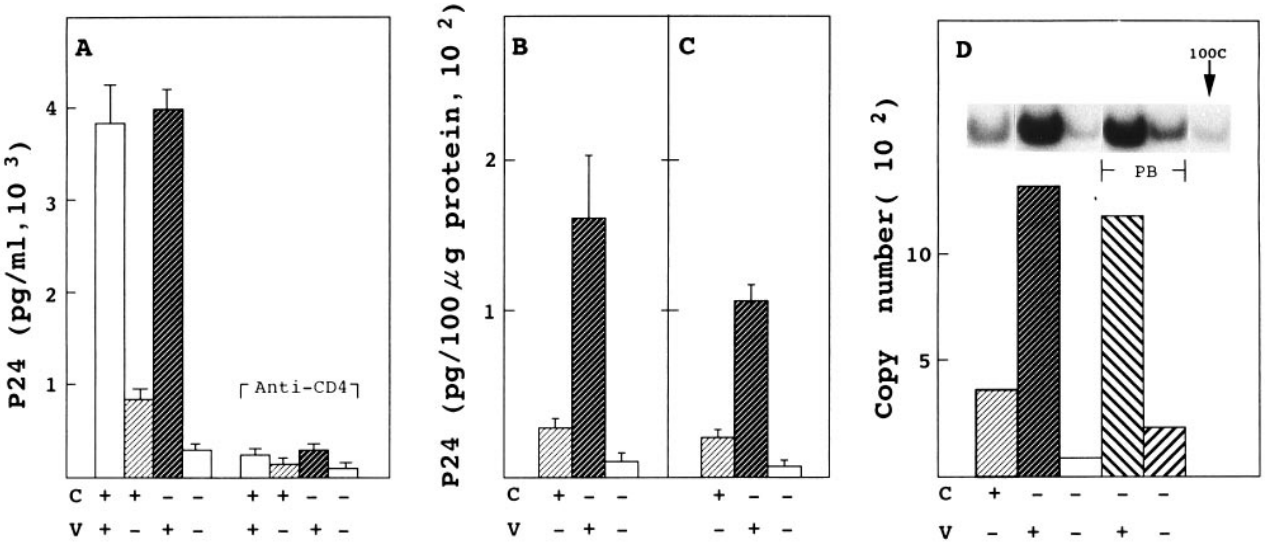

FIG. 4. Effect of NA on the life cycle of HIV-1 strain NL43 in MT4 cells. (A) Cells (C) or virions (V) (100 ng of p24 antigen) or both were treated (+) or not treated (−) with 1.0 U of NA from *C. perfringens* per ml for 1 h at 37°C in RPMI 1640 culture medium. The virions were then allowed to adsorb to the cells at 4°C for 1 h. After extensive washing of the infected cells to remove unbound virus and the enzyme, the cells were incubated for 3 days and the RT activity in the culture fluids was assayed. The anti-CD4 antibody Leu3A (10 μg/ml) was added to a companion set of cultures. (B) Virions or cells were treated with NA, and infections were initiated exactly as described above. The amount of virions bound to cells during 1 h at 4°C was quantitated by the p24 antigen assay. (C) Cells infected as described above were subsequently incubated at 37°C for 1 h to allow the bound viruses to enter the cells, and intracellular p24 antigen was quantitated after trypsinizing the cells to digest the viruses remaining on the cell surface. (D) Cells were incubated for additional 23 h at 37°C, and the relative levels of intracellularly copied viral DNA were determined by PCR followed by electrophoresis of the PCR products in 8% polyacrylamide gels. The effect of PB (2.0 μg/ml) on the cells was analyzed in parallel. 100C, 100 copies of the marker DNA.

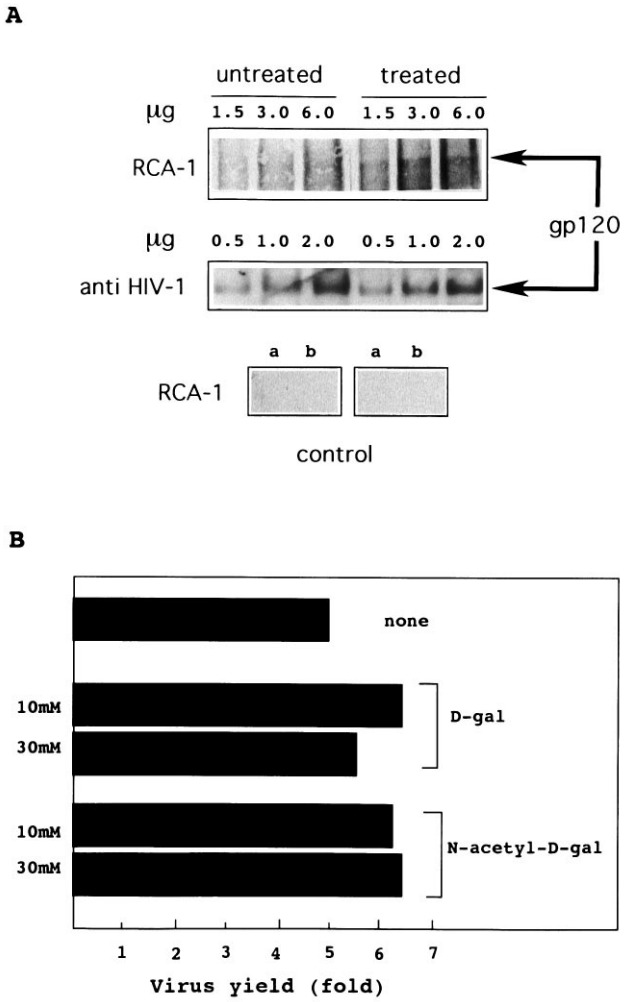

FIG. 5. Exposure of galactose residues on gp120 upon desialylation by NA (A) and the lack of abrogation by galactose receptor-binding saccharides of the enhanced HIV-1 replication by NA (B). (A) HIV-1 NL43 virions grown in serum-free ASF103 medium were treated (+) or not treated (−) with NA (1.0 μg/ml) for 1 h at 37°C, pelleted by ultracentrifugation, resolved by SDS-PAGE, and then electroblotted as described in Materials and Methods. The resolved proteins on the membranes were probed with the asialoglycoprotein-specific lectin RCA-1 or anti-HIV-1 serum. For better quantitation, three different protein amounts were applied to the gels. The uninfected culture medium was parallelly processed for the enzyme treatment and centrifugation. The pelleted fractions equivalent in volume to 3.0 μg (a) and 6.0 μg (b) of virion preparations were then processed for SDS-PAGE, blotting, and RCA binding. No specific signal was present at the positions corresponding to that of gp120 (control). (B) D-Galactose and *N*-acetyl-D-galactosamine, the galactose receptor-binding saccharides (17), were added to MT4 cells infected with the NL43 strain (1.0 ng of p24 antigen) and treated with NA (0.03 U/ml) as described for Fig. 2. The virus yields on day 4 were compared with those in the absence of these saccharides or NA by fold increase relative to the result for the NA-negative control (15.5 ± 2.4 ng/ml).

These data obtained with trimming-glycosidase inhibitors and by enzymatic desialylation appeared to complement each other very well, indicating that virion surface sialylation down-regulates the infectivity potential of HIV-1 and therefore probably represents an important factor in determining the infection efficiency of the virus. Full processing of HIV-1 oligosaccharides into the complex type could be neither necessary nor irrelevant but rather an impediment to full viral infectivity.

Infection-enhancing effect of NA on other human and nonhuman lentiviruses, including the primary isolates of HIV-1, and on an HIV-1 Env-based vector. Although the effect was not as striking as that seen for HIV-1 strain SF2, NA also promoted the replication of a macrophage-tropic HIV-1 strain, SF162, in the primary blood macrophages, of HIV-2 strain GH123 in the M8166 T-cell line, and of SIVmac strain 239 in MT4 cells (Fig. 7A and B). Furthermore, when NA was applied to HIV-1 isolation from two patients, the virus was recovered much earlier with a much higher peak titer in the presence of NA than in the absence, or isolation was successful only with NA (Fig. 7C). To isolate virus without NA, cells were exposed to PB by the routine protocol. We extended this attempt to samples from 24 other patients and obtained similar results (data not shown). The nucleotide sequences of two hypervariable regions, V2 and V3, of gp120 were identical for the isolates obtained by using NA and PB from patient 77 and were

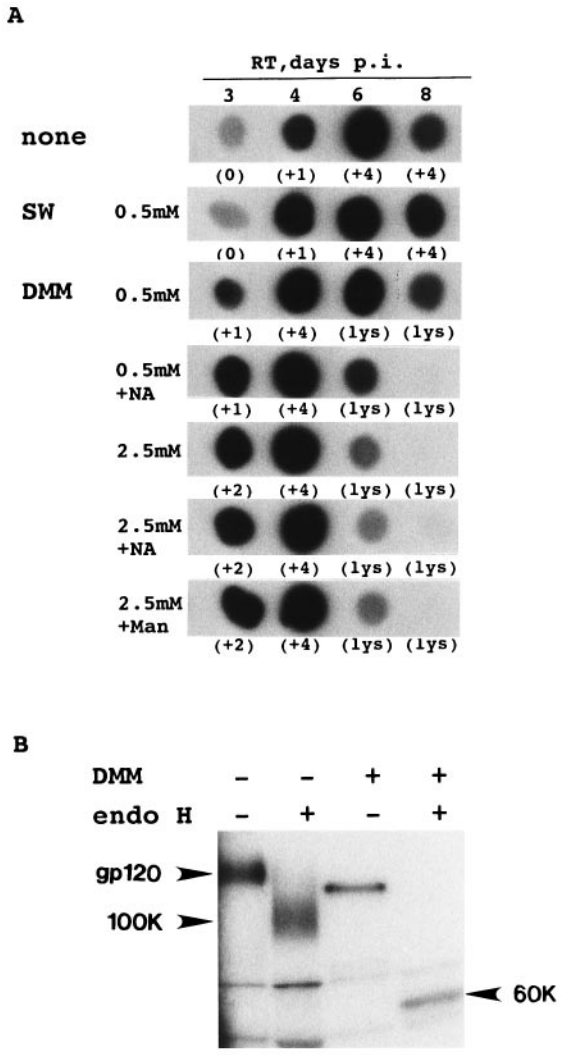

FIG. 6. Enhancement of HIV-1 replication by DMM and SW (A) and specific modification of the glycosylation of virion-associated gp120 by DMM (B). (A) MT4 cells infected with the NL43 strain (10 ng of p24 antigen) as described for Fig. 2 were incubated with DMM or SW at the indicated concentrations, and the virus yields on various days postinfection (p.i.) are shown by autoradiograms of RT assays. CPE is expressed as in Fig. 2. Some DMM-treated cultures received NA (0.03 U/ml) or mannan (5 mg/ml). (B) Virions grown in the presence (+) or absence (−) of DMM (2.5 mM) and metabolically labeled with [^{35}S]methionine were pelleted. The viral proteins were then immunoprecipitated, digested with endo H, and analyzed by SDS-PAGE as described in Materials and Methods.

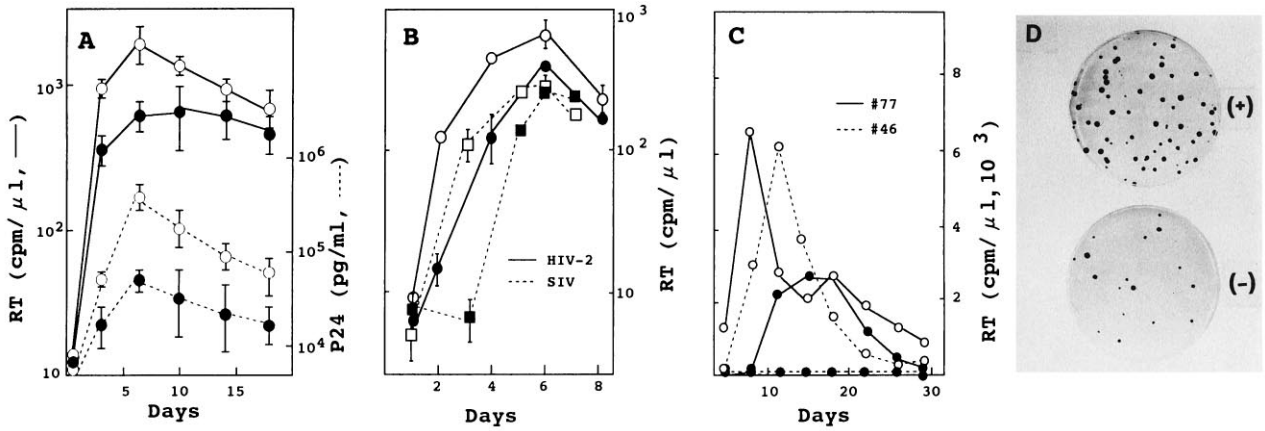

FIG. 7. Effect of NA on the replication of the macrophage-tropic HIV-1 strain SF162 (A), on HIV-2 strain GH123 and SIVmac strain 239 (B), on the isolation of HIV-1 from two patients (77 and 46) (C), and on the infectivity of HIV-1 vector HXN (D). The replication of those laboratory strains and the virus isolation were compared in the presence (open symbols) or absence (closed symbols) of NA. The NA concentrations were 0.03 U/ml for HIV-1 SF162, SIVmac 239, and virus isolation and 0.5 U/ml for HIV-2. PB (2.0 μg/ml) was added to NA-negative cultures for virus isolation. The vector virus containing the thymidine kinase promoter-driven neomycin resistance gene between HIV-1 long terminal repeats was obtained as described in Materials and Methods. The virus was incubated with (+) or without (−) NA (0.5 U/ml) for 1 h at 37°C and then inoculated onto CD4-expressing HeLa cells. Neomycin-resistant colonies, which developed 3 days postinfection, are shown. There is a sixfold difference between the number of colonies in panels (+) and (−).

very similar for those from other patients analyzed so far (data not shown). This suggested that the NA method does not select any particular population from in vivo quasispecies, compared with the routine PB method, and is therefore worthy of recommendation as a novel device for HIV isolation. Finally, we examined the effect of NA on the HIV-1 Env-based vector HXN, the Env protein of which was derived from the T-cell line-tropic BH10 strain (31). A 1-h exposure of this vector virus to NA enhanced its infectivity in CD4-expressing HeLa cells by sixfold (Fig. 7D). This increase should be a faithful reflection of a single round of virus-cell interaction augmented by NA, since the virus vector is replication incompetent (31).

These results demonstrated that enhancement of infection by desialylation is a feature widely shared by human and nonhuman lentiviruses, including the primary isolates of HIV-1. Desialylation may facilitate HIV-1 isolation and enhance the infectivity of HIV vectors.

DISCUSSION

We have demonstrated that treating HIV-1 with NA remarkably augments the initial virus-cell interaction and thereby promotes viral replication and cytopathogenicity. That this enhancement was due to the specific action of NA was verified by its abrogation by the NA-specific inhibitor DANA (Fig. 3A) and by the exposure of the second terminal galactose residues of complex oligosaccharides on gp120 (Fig. 5A). The results obtained by virus binding and other assays (Fig. 3) and the infectivity assay with the HIV vector (Fig. 7D) suggested that the enhancement during a single cycle of replication could be 6- to 10-fold. This appeared to be sufficient to cause a profound effect on logarithmic multiple rounds of replication (Fig. 1 and 2). Enhancement of HIV-1 infection was also achieved by incubating infected cells with the oligosaccharide-processing inhibitor DMM. That this enhancement was indeed due to the specific action of DMM was also verified by endo H digestion analysis (Fig. 6B). The mechanism responsible for augmented virus-cell interaction by desialylation remains to be elucidated. An earlier report has shown that NA did not affect the ability of gp120 to bind CD4 (11). Thus, a less specific mechanism, such as electrostatic reinforcement of viral interaction with the entire cell surface also negatively charged, is a possibility. Alternatively, heavy sialylation may rather be an obstacle in maintaining a stable molecular association between gp120 and gp41 in the absence of putative covalent bonds. However, the amounts of virion-associated gp120 in the NA-treated and untreated samples or in the virions grown in the presence and absence of DMM did not appear to differ in SDS-PAGE.

Desialylation-based augmentation of HIV-1-cell interactions has also been reported (17, 22, 23). However, in contrast to the case for those studies, neither the galactose-galactose receptor-mediated pathway (17) nor complement activation-mediated mechanisms (22, 23) appeared to be involved in our study (Fig. 3A and 5B). The prevalence of high-mannose oligosaccharides resulting from DMM treatment of infected cells also did not appear to be responsible (Fig. 6A). The augmentation seemed to be only a direct result of the removal or lack of virion surface sialic acids, to which the similarly enhanced HIV-1 replication seen in our two independent approaches (with NA and DMM) is commonly attributed. We thus demonstrated, for the first time, the control of infectivity, replication, and cytopathogenicity as a role indigenous to the host enzyme-catalyzed sialylation of the HIV-1 virion surface.

A number of groups have investigated the effect of trimming glycosidases on the replication and cytopathogenicity of HIV-1 (for a review, see reference 10). Although the results appear to be too variable to be generalized, it is likely that the inhibitors targeting the glycosidases in the Golgi apparatus are less effective or are ineffective in preventing virus replication and syncytium induction compared with those targeting the ER enzymes. This concept is represented by one of the earliest reports (13). Others have shown that virus grown in DMM-treated cultures had reduced infectivity (21, 27). To our knowledge, however, no evidence of enhancement by DMM or other inhibitors of Golgi trimming glycosidases has been reported. In this study with the NL43 strain, in which infection was initiated with a high input dose, DMM enhancement was obvious early in infection but not late. In the late stage, the virus titer was even higher in the absence of DMM than in its presence, because cells were already totally destroyed in the latter case (Fig. 6A). Thus, a comparison at an inappropriate single point during the infection would not reveal enhancement or could

lead to a wrong conclusion. This may partly explain the above-described discrepancy regarding the effects of DMM and some other inhibitors on HIV-1 replication and cytopathogenicity. The discrepancy could also be due to differences in sensitivity to the specific action, as well as side effects of the inhibitors, that may exist among various cell types. At any rate, this study indicated that a solid conclusion requires a detailed time-sequential kinetic analysis of virus replication, and it suggested that processing of HIV-1 oligosaccharides beyond the DMM-sensitive mannosidase I step is probably neither necessary nor irrelevant but rather an impediment to full viral infectivity and cytopathogenicity.

The augmented infection by NA was widely found with the laboratory strains of HIV-1, HIV-2, and SIV and primary isolates of HIV-1 (Fig. 7). However, NA might have caused enhancement by different mechanisms depending on the virus-cell systems. For instance, the galactose-galactose receptor-mediated mechanism might be involved in the macrophage system. In any event, it will be important to learn whether desialylation plays a role in HIV spreading in the body and disease progression, because NA is probably widespread in human tissues and body fluids as well as in various bacterial and viral microbes that can coinfect with HIV (7, 28, 29, 34) and because, as suggested here, a variety of microbial NA species are likely able to activate HIV. We also suggest that the use of NA (and probably certain trimming-glycosidase inhibitors) greatly facilitated isolation of HIV-1 from patients. Desialylation will thus be worthy of a trial for virus isolation, especially from asymptomatic carriers and long-term nonprogressors, from whom isolation is often difficult because of generally low virus loads. How to increase the titer is a major concern of those studying HIV vectors but does not appear to be readily solvable by a single technical improvement. Rather, a combination of techniques is likely to be helpful, even if each is not so intensive. Thus, the sixfold titer increase for an HIV-1 vector induced by NA already seems to be remarkable, and desialylation should be applied.

In summary, our data suggested that heavy sialylation of the virion surface restricts the infectivities of HIV-1 and other lentiviruses. At the same time, heavy glycosylation and sialylation appear to be an ingenious device with which to mask potential epitopes to evade humoral and cellular immune responses (2, 3, 8, 25). Thus, the glycosylation pattern unique to these viruses appears to be of dual importance for ultimately determining their unique infection profile as featured by lenti.

ACKNOWLEDGMENTS

We greatly appreciate the help of S. Takasaki and T. Kawano in performing lectin binding assays. We also thank T. Mizuochi and R. Inagi for helpful discussions, S. Oka and A. Iwamoto for the supply of patients' blood samples, R. Harukuni and A. Kurotani for technical assistance, and K. Kakuta and A. Mitsuzawa for preparing the manuscript.

This work was supported by the Ministry of Education, Science, Sports and Culture and by the Human Science Promotion Foundation, Japan.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Back, N. K. T., L. Smit, J.-J. De Jong, W. Keulen, M. Schutten, J. Goudsmit, and M. Tersmette.** 1994. An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. Virology **199:**431–438.
3. **Benjouad, A., K. Mabrouk, J. C. Gluckman, and E. Fenouillet.** 1994. Effect of sialic acid removal on the antibody response to the third variable domain of human immunodeficiency virus type-1 envelope glycoprotein. FEBS Lett. **341:**244–250.
4. **Bernstein, H. B., S. P. Tucker, E. Hunter, J. S. Schutzbach, and P. W. Compans.** 1994. Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. J. Virol. **68:**463–468.
5. **Callebaut, C., B. Krust, E. Jacotot, and A. G. Hovanessian.** 1993. T cell activation antigen CD26 as a cofactor for entry of HIV in $CD4^+$ cells. Science **262:**2045–2050.
6. **Connor, R. I., and D. D. Ho.** 1994. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. J. Virol. **68:**4400–4408.
7. **Corfield, T.** 1992. Bacterial sialidases—roles in pathogenicity and nutrition. Glycobiology **2:**509–521.
8. **Doe, B., K. S. Steimer, and C. M. Walker.** 1994. Induction of HIV-1 envelope (gp120)-specific cytotoxic T lymphocyte responses in mice by recombinant CHO cell-derived gp120 is enhanced by enzymatic removal of N-linked glycans. Eur. J. Immunol. **24:**2369–2376.
9. **Ezekowitz, R. A. B., M. Kuhlman, J. E. Groopman, and R. A. Byrn.** 1989. A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus. J. Exp. Med. **169:**185–196.
10. **Feizi, T., and M. Larkin.** 1990. AIDS and glycosylation. Glycobiology **1:**17–23.
11. **Fennie, C., and L. A. Lasky.** 1989. Model for intracellular folding of the human immunodeficiency virus type 1 gp120. J. Virol. **63:**639–646.
12. **Geyer, H., C. Holschbach, G. Hunsmann, and J. Schneider.** 1988. Carbohydrates of human immunodeficiency virus. J. Biol. Chem. **263:**11760–11767.
13. **Gruters, R. A., J. J. Neefjes, M. Y. Tersmette, R. E. de Goede, A. Tulp, H. G. Huisman, F. Miedema, and H. L. Ploegh.** 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature (London) **330:**74–77.
14. **Kanda, T., and H. Shibuta.** 1981. A temperature-sensitive mutant of Sendai virus which establishes persistent infection in Vero cells without cell crisis. Virology **108:**318–324.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**10373–10382.
17. **Manca, F.** 1992. Galactose receptors and presentation of HIV envelope glycoprotein to specific human T cells. J. Immunol. **148:**2278–2282.
18. **Meindl, P., G. Bodo, P. Palese, J. Schulman, and H. Tuppy.** 1974. Inhibition of neuraminidase activity by derivatives of 2-deoxy-2,3-dehydro-N-acetyineuraminic acid. Virology **58:**457–463.
19. **Mizuochi, T., M. W. Spellman, M. Larkin, J. Solomon, L. J. Basa, and T. Feizi.** 1988. Carbohydrate structures of the human-immunodeficiency-virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese-hamster ovary cells. Biochem. J. **254:**599–603.
20. **Mizuochi, T., M. W. Spellman, M. Larkin, J. Solomon, L. J. Basa, and T. Feizi.** 1988. Structural characterization by chromatographic profiling of the oligosaccharides of human immunodeficiency virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese hamster ovary cells. Biomed. Chromatogr. **2:**260–270.
21. **Montefiori, D. C., W. E. Robinson, Jr., and W. M. Mitchell.** 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **85:**9248–9252.
22. **Montefiori, D. C., W. E. Robinson, Jr., and W. M. Mitchell.** 1989. Antibody-independent, complement-mediated enhancement of HIV-1 infection by mannosidase I and II inhibitors. Antiviral Res. **11:**137–146.
23. **Montefiori, D. C., K. Stewart, J. M. Ahearn, J. Zhou, and J. Zhou.** 1993. Complement-mediated binding of naturally glycosylated and glycosylation-modified human immunodeficiency virus type 1 to human CR2 (CD21). J. Virol. **67:**2699–2706.
24. **Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. Daniel, and R. C. Desrosiers.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIV_{MAC}) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV_{MAC}. J. Virol. **62:**4691–4696.
25. **Narayan, O., and J. E. Clements.** 1990. Lentiviruses, p. 1571–1589. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, Ltd., New York.
26. **Nishikawa, K., T. Morishima, T. Toyoda, T. Miyadai, T. Yokochi, T. Yoshida, and Y. Nagai.** 1986. Topological and operational delineation of antigenic sites on the HN glycoprotein of Newcastle disease virus and their structural requirements. J. Virol. **60:**987–993.
27. **Pal, R., G. M. Hoke, and M. G. Sarngadharan.** 1989. Role of oligosaccharides in the processing and maturation of envelope glycoproteins of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**3384–3388.
28. **Ray, P. K.** 1977. Bacterial neuraminidase and altered immunological behavior of treated mammalian cells. Adv. Appl. Microbiol. **21:**227–267.
29. **Roggentin, P., R. Schauer, L. L. Hoyer, and E. R. Vimr.** 1993. The sialidase

superfamily and its spread by horizontal gene transfer. Mol. Microbiol. **9:**915–921.
30. **Shibata, R., T. Miura, M. Hayami, K. Ogawa, H. Sakai, T. Kiyomasu, A. Ishimoto, and A. Adachi.** 1990. Mutational analysis of the human immunodeficiency virus type 2 (HIV-2) genome in relation to HIV-1 and simian immunodeficiency virus SIVagm. J. Virol. **64:**742–747.
31. **Shimada, T., H. Fujii, H. Mitsuya, and A. W. Nienhuis.** 1991. Targeted and highly efficient gene transfer into $CD4^+$ cells by a recombinant human immunodeficiency virus retroviral vector. J. Clin. Invest. **88:**1043–1047.
32. **Shioda, T., J. A. Levy, and C. Cheng-Mayer.** 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) **349:**167–169.
33. **Stein, B. S., and E. G. Engleman.** 1990. Intracellular processing of the gp160 HIV-1 envelope precursor. J. Biol. Chem. **265:**2640–2649.
34. **Vimr, E. R.** 1994. Microbial sialidases: does bigger always mean better? Trends Microbiol. **2:**271–277.
35. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.