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N-Butyldeoxynojirimycin-Mediated Inhibition of Human Immunodeficiency Virus Entry Correlates with Impaired gp120 Shedding and gp41 Exposure

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The α-glucosidase inhibitor *N*-butyldeoxynojirimycin (NB-DNJ) is an inhibitor of human immunodeficiency virus (HIV) replication and HIV-induced syncytium formation in vitro. Although an NB-DNJ-mediated change in viral envelope N-glycan composition inhibits HIV entry at the level of post-CD4 binding, the exact mechanism of inhibition remains to be established. In this study we have examined the effects of NB-DNJ on virion envelope composition and CD4-induced gp120 shedding and gp41 exposure. Virion composition analysis revealed an NB-DNJ-mediated reduction of 15% in overall virion envelope glycoprotein content and a reduction of 26% in the proteolytic maturation of virion gp160. Taken together, these two effects resulted in a reduction of approximately 40% in virion gp120 content. CD4-induced shedding of gp120 from the surfaces of envelope-transfected Cos cells was undetectable when gp120 was expressed in the presence of NB-DNJ. Similarly, the shedding of virion-associated gp120 was reduced 7.4-fold. CD4-induced exposure of cryptic gp41 epitopes on the surfaces of HIV-expressing ACH-2 cells was also greatly impaired, and the exposure of virion-associated gp41 epitopes was reduced 4.0-fold. Finally, CD4-induced increases in the binding of antibodies to the V3 loop of ACH-2-cell-expressed envelope glycoproteins were reduced 25-fold when the glycoproteins were expressed in the presence of NB-DNJ. These results suggest that the NB-DNJ-mediated retention of glycosylated N-glycans inhibits HIV entry by a combined effect of a reduction in virion gp120 content and a qualitative defect within the remaining gp120, preventing it from undergoing conformational changes after CD4 binding.

The N-linked oligosaccharide processing inhibitor *N*-butyldeoxynojirimycin (NB-DNJ) is a glucose analog which inhibits α-glucosidases I and II in the endoplasmic reticulum (12, 40). α-Glucosidases I and II are responsible for the removal of the terminal glucose residues from the precursor N-glycan, which is transferred to the protein during its translation into the lumen of the endoplasmic reticulum (25). Inhibition of these enzymes therefore leads to the production of glycoproteins carrying predominantly neutral, glucosylated, high-mannose-type oligosaccharides.

α-Glucosidase inhibitors, such as deoxynojirimycin, NB-DNJ, and castanospermine, are potent inhibitors of human immunodeficiency virus (HIV) replication and HIV-mediated syncytium formation in vitro (15, 20, 24, 36, 48). In contrast, inhibitors of the later stages of N-glycan processing, such as deoxymannojirimycin and swainsonine, have no effect (20, 33). Although these results implicate the retention of glucosylated N-glycans in the antiviral activity of NB-DNJ, the exact mechanism remains to be determined.

Antiviral concentrations of NB-DNJ are known to reduce the relative infectivities of virus particles released from HIV-infected cells (10, 13, 44). This effect correlates with impaired processing of gp120-associated N-glycans, which results in the retention of predominantly neutral glucosylated high-mannose-type N-glycans (22, 33, 36, 44). This change in N-glycan composition does not affect the affinity of the gp120-CD4 interaction (13, 35) or the binding of virus particles to CD4-positive cells (13, 35, 48). The entry of virus produced in the presence of NB-DNJ into CD4-positive cells is, however, greatly impaired (13), indicating that NB-DNJ-mediated inhibition of virion infectivity is due to an effect on postbinding events necessary for viral entry.

It has previously been shown that treatment of HIV-infected cells with α-glucosidase inhibitors leads to a decrease in the intracellular proteolytic maturation of the envelope precursor glycoprotein, gp160, into gp41 and gp120 (10, 33, 48). No effect of the inhibitors on the amount of gp120 released from the cells has, however, been observed (20, 36, 48). NB-DNJ-mediated incorporation of predominantly nonfusogenic gp160 into virions could therefore explain the inhibition of HIV entry at the level of post-CD4 binding. However, the NB-DNJ-mediated retention of glucosylated N-glycans has also been shown to affect the conformation of the V1/V2 loops of gp120 (14), which have been proposed to be involved in postbinding events, such as conformational changes and exposure of gp41, necessary for HIV entry (16, 43). Inhibition of HIV entry could therefore also be explained by an effect of altered N-glycans on the function of gp120. We therefore examined the effects of NB-DNJ on virion envelope composition and CD4-induced gp120 shedding and gp41 exposure in order to determine their relative contributions to NB-DNJ-mediated inhibition of HIV entry.

MATERIALS AND METHODS

Reagents. NB-DNJ (SC-48344) was a gift from Searle/Monsanto (St. Louis, Mo.). Baculovirus-derived recombinant soluble human CD4 (sCD4) was obtained from the Medical Research Council AIDS Reagent Project (National Institute of Biological Standards and Control, Potters Bar, United Kingdom [UK]). The mouse anti-V3 loop monoclonal antibodies (with original clone names and contributors in parentheses) ADP3047 (IIIB-V3-13; J. Laman) and ADP3025 (0.5β; K. Takatsuki) were obtained from the Medical Research Council AIDS Reagent Project; the human anti-gp41 monoclonal antibody 50-69 was obtained from Susan Zolla-Pazner (VA Medical Center, New York, N.Y.), the human anti-gp120 monoclonal antibodies 17b and 48d were obtained from James

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Robinson (University of Connecticut Health Center, Farmington, Conn.), and the sheep polyclonal anti-gp120 antibody D7324 was obtained from Aalto Bio Reagents (Dublin, Ireland).

Recombinant gp120 (rgp120) was produced in Chinese hamster ovary (CHO) cells, as described elsewhere (22), in either the presence [rgp120(+)] or the absence [rgp120(−)] of 2 mM NB-DNJ. The concentrations of the two types of rgp120 were standardized by amino acid composition analysis.

Proviral DNA constructs and envelope expresser plasmids. The pHXBH10ΔenvCAT plasmid is a derivative of the HXBH10 plasmid with a deletion in the envelope gene (ΔBglII) and a bacterial chloramphenicol acetyltransferase (CAT) gene in place of the *nef* gene (45). The HXBc2 envelope plasmid pSVIIIenv(Kpn) (23) and the BH10-derived cleavage-negative envelope plasmid pIIIexE7(508-511) (5) are described elsewhere. The pSVLtat plasmid was used to provide *tat.*

Cells. HeLa cells were propagated in Dulbecco modified Eagle medium containing 5% fetal calf serum (FCS) (PAA Laboratories Inc., Newport Beach, Calif.) and antibiotics (50 U of penicillin and 50 μg of streptomycin per ml) (Cellgro/Mediatech, Washington, D.C.). Cos-1 cells were cultured in Dulbecco modified Eagle medium containing 10% FCS and antibiotics. Molt 4 clone 8 cells were cultured in RPMI medium containing 10% FCS and antibiotics. H9 and ACH-2 cells were cultured in RPMI medium containing 10% FCS (Sigma, Poole, UK) and antibiotics (Gibco, Paisley, UK). For radioactive labelling of virions, cysteine- and methionine-free RPMI medium containing 5% FCS, antibiotics, and 50 μCi each of [^{35}S]cysteine (1,075 Ci/mmol) and [^{35}S]methionine (1,175 Ci/mmol) (NEN Products, Boston, Mass.) per ml was used. For radioactive labelling of viral envelope glycoproteins, cysteine-free RPMI medium containing 5% FCS, antibiotics, and 75 μCi of [^{35}S]cysteine per ml was used.

Virion composition analysis. Virus was generated by cotransfection of HeLa cells (7×10^5 per 10-cm-diameter plate) with 30 μg of pHXBH10ΔenvCAT and 10 μg of HXBc2 envelope plasmid by the calcium phosphate precipitation technique. Fourteen hours posttransfection, the cells were washed and fresh medium containing either 0 or 2 mM NB-DNJ was added. Forty-eight hours posttransfection, the cells were washed in phosphate-buffered saline (PBS), and virion-labelling medium containing either 0 or 2 mM NB-DNJ was added. Sixty-four hours posttransfection, the medium was removed and the cells were spun at $1,500 \times g$ for 20 min at 4°C and filtered (pore size, 45 μm). The cells were then washed in PBS, lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]), scraped, and kept at 4°C for 30 min. The cell lysate was clarified by centrifugation at $60,000 \times g$ for 30 min at 4°C. The clarified tissue culture medium was layered onto 20% sucrose in PBS and spun at $90,000 \times g$ for 90 min at 4°C. Samples were collected from the supernatant and inactivated with one-fifth the volume of 5× RIPA buffer. The remaining supernatant and sucrose were then removed, and the virus pellet was resuspended in RIPA buffer.

The samples were immunoprecipitated overnight at 4°C with 125 μl of a 50% solution of protein A-Sepharose (Pharmacia, Piscataway, N.J.) and 5 μl of HIV-positive human serum. Bovine serum albumin (BSA) (0.5%) and a nonlabelled clarified HeLa cell lysate were used to prevent nonspecific binding. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography and were quantified by densitometry.

Single-round infectivity assay. Recombinant virus was generated in HeLa cells in the presence or absence of NB-DNJ, as described above, with the pHXBH10ΔenvCAT and HXBc2 envelope plasmids. Virus preparations were normalized according to reverse transcriptase activity and used to infect Molt 4 clone 8 cells. The cells were cultured for 72 h postinfection, harvested, washed once in PBS, and lysed in 250 mM Tris-HCl (pH 7.8). The cell lysates were assayed for CAT activity as described elsewhere (21).

sCD4-induced shedding of gp120 (cell surface). HXBc2 envelope was expressed by cotransfecting Cos-1 cells (10^6 per 10-cm-diameter plate) with 10 μg of HXBc2 envelope plasmid and 0.5 μg of plasmid pSLVtat by the DEAE-dextran technique. Fresh medium containing either 0 or 2 mM NB-DNJ was added immediately after the dimethyl sulfoxide shock. Forty-eight hours posttransfection, the cells were washed in PBS and envelope-labelling medium containing either 0 or 2 mM NB-DNJ was added. Sixty-four hours posttransfection, the cells were washed in PBS containing 2% FCS (PBS-FCS) and then incubated for 1 h at 37°C with various concentrations of sCD4 diluted in PBS-FCS. The supernatant was removed and used for the immunoprecipitation of soluble envelope glycoproteins as described above. As a control for envelope expression, the cells were lysed in Tris-buffered saline (TBS) containing 0.5% Nonidet P-40 and cell-associated envelope glycoproteins were immunoprecipitated. For studies on the effect of the presence of cell surface-associated, uncleaved gp160 on the sCD4-induced shedding of gp120, Cos-1 cells were cotransfected with 5 μg of HXBc2 envelope plasmid, 5 μg of the cleavage-negative envelope plasmid pIIIexE7(508-511), and 0.5 μg of pSVLtat.

sCD4-induced shedding (virions). HIV-1_{IIIB} was generated in H9 cells in either the presence or the absence of 2 mM NB-DNJ as described elsewhere (13). Virus-containing supernatants were incubated for 2 h at either 37 or 4°C, in either the presence or the absence of 50 μg of sCD4 per ml. Soluble and virus-associated envelope glycoproteins were separated by size exclusion chromatography on S-1000 (Pharmacia, Herts, UK) as previously described (13, 31). Following separation, the samples were heat inactivated (56°C, 30 min) in the presence of 0.1% Empigen BB (Calbiochem, La Jolla, Calif.). The gp120 contents of the various fractions were determined by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (MaxiSorb; Nunc, Roskilde, Denmark) were coated with a monospecific polyclonal sheep antibody (D7324) directed against the C terminus of gp120 at 2 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 [pH 9.6]) at room temperature overnight. The plates were blocked with TBS (140 mM NaCl, 10 mM Tris-HCl, 0.1% NaN_3 [pH 7.4]) containing 0.05% Tween 20 (Sigma) (TBS-tw) and 2% milk powder for 30 min. The plates were incubated with the samples diluted in TBS-tw for 7 h at room temperature and then were incubated overnight at room temperature with 0.5 μg of polyclonal rabbit anti-gp120 (Medical Research Council AIDS Reagent Project) per ml diluted in TBS-tw containing 10% lamb serum (Gibco). The plates were developed by incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) diluted in TBS-tw containing 1% lamb serum for 3 h at room temperature, followed by 1 mg of *p*-nitrophenolphosphate per ml diluted in diethanolamine buffer (10% diethanolamine, 0.5 mM $MgCl_2$, 0.02% NaN_3 [pH 9.8]). Dilutions of rgp120(−) were used to generate a standard curve for samples produced in the absence of NB-DNJ, and dilutions of rgp120(+) were used for samples produced in the presence of NB-DNJ.

Surface biotinylation. ACH-2 cells (5×10^5/ml) were stimulated in the presence of 10^{-8} M phorbol myristate acetate (PMA) (Sigma) for 20 h in either the presence or the absence of 2 mM NB-DNJ. The cells (4×10^7 cells per sample) were washed three times in PBS, resuspended in 4.5 ml of PBS containing 1.5 mg of the membrane-impermeative biotinylation reagent sulfosuccinimidobiotin (Pierce, Chester, UK), and incubated at 37°C for 30 min. The cells were washed in PBS containing 10% BSA (PBS–10% BSA) followed by PBS containing 0.1% BSA and 20 mM NaN_3 (fluorescence-activated cell sorter [FACS] buffer), incubated with 50 μg of polyclonal rabbit anti-gp120 for 30 min on ice, and washed as described above. The cells were lysed for 30 min on ice in PBS containing 2% Triton X-100 (Sigma) and protease inhibitors (50 μg each of TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone] and TPCK [*N*-tosyl-L-phenylalanine chloromethyl ketone] per ml and 200 μg of phenylmethylsulfonyl fluoride [Sigma] per ml). The cell lysate was clarified by centrifugation, and bound antigen was precipitated by the addition of 150 μl of a 50% solution of protein A-Sepharose (Pharmacia, Herts, UK). The samples were analyzed by SDS-PAGE and Western blotting (immunoblotting). The Western blots were developed with horseradish peroxidase-conjugated extravidin (Sigma) by enhanced chemiluminescence (ECL system; Amersham International, Amersham, UK).

Flow cytometric analysis of sCD4-induced conformational changes in cell surface-expressed viral envelope glycoproteins. ACH-2 cells (5×10^5/ml) were stimulated in the presence of 10^{-8} M PMA for 20 h, in either the presence or the absence of 2 mM NB-DNJ. The cells were washed in fresh medium and incubated (2×10^7 cells per ml) for 2 h either at 37°C or on ice in medium containing PMA, and various concentrations of sCD4, with or without NB-DNJ. The cells were washed first in PBS–10% BSA and then in FACS buffer before 50 μl of cells (5×10^5 cells) was incubated for 30 min on ice with 20 μl of either ADP3047 (10 μg/ml), ADP3025 (10 μg/ml), or 50-69 (15 μg/ml) diluted in FACS buffer. The cells were washed in PBS–10% BSA and FACS buffer and then incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Ortho, Bucks, UK) or sheep anti-human IgG (Serotec, Oxford, UK) diluted in FACS buffer. The degree of staining was analyzed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, Calif.). In order to obtain values which could be quantitatively compared, fluorescein-labelled quantitative microbead standards (Flow Cytometry Standard Corporation, Research Triangle Park, N.C.) were used to generate a standard curve for the conversion of mean channel values to units correlating with the number of antibody binding sites (34). Because of the indirect method of staining, with secondary antibodies, the exact number of antibody binding sites could not be calculated. The number calculated is therefore based on the assumption of a one-to-one interaction between the primary and secondary antibodies and an FITC-to-antibody ratio of 1 for the secondary antibody. FITC-conjugated sCD4 was prepared as described elsewhere (34). The coupling ratio obtained was 3.2 mol of FITC per mol of sCD4.

Antibody binding to virions. HIV-1_{IIIB} was generated in H9 cells in the presence or absence of 2 mM NB-DNJ, as described above. Virus-containing supernatants were incubated for 2 h at 4°C in the presence of 1 μg of anti-gp41 antibody (50-69) per ml and in the presence or absence of 50 μg of sCD4 per ml. Dynabeads (Dynal, Merseyside, UK) (40 μl) coated with sheep anti-human IgG (M-280) were then added, and the samples were incubated for 30 min on ice. The beads were then washed in PBS containing 1 mg of BSA per ml, and bound reverse transcriptase activity was analyzed by using the *Quan-T* reverse transcriptase assay system (Amersham International) directly on the washed Dynabeads. The samples were mixed frequently during incubation to prevent settling of the beads.

RESULTS

Effect of NB-DNJ on virion envelope glycoprotein composition. NB-DNJ exhibits a concentration-dependent inhibition of HIV replication, with 0.5 mM NB-DNJ resulting in a 1-log drop in replicating virus and 2 mM resulting in a 3-log drop in

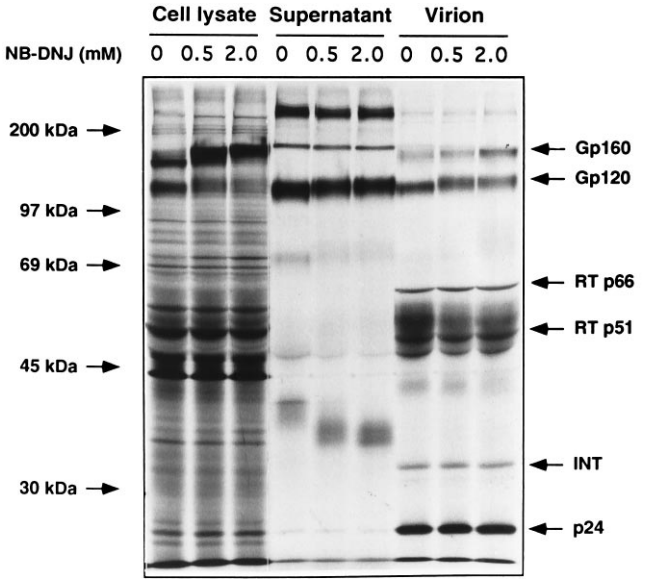

FIG. 1. Effect of NB-DNJ on viral envelope glycoprotein composition (intact virions). Virus was expressed in HeLa cells in the presence of the indicated concentrations of NB-DNJ. Immunoprecipitates of HeLa cell lysates (cell lysate), non-virus-associated soluble protein (supernatant), and virus-associated protein (virion), obtained by using HIV-positive serum, are shown. Identified viral proteins are indicated with arrows. RT, reverse transcriptase; INT, integrase.

replicating virus (data not shown). Furthermore, 2 mM NB-DNJ is required to achieve a complete conversion of gp120-associated N-glycans to precursor-type oligosaccharides (22) and to achieve efficient inhibition of HIV entry in vitro (13). NB-DNJ was therefore predominantly used at 2 mM throughout this study.

Because of the previously described effect of the NB-DNJ-mediated change in N-glycan composition on the proteolytic maturation of gp160 into gp120 and gp41, we analyzed the effect of NB-DNJ treatment on virion envelope glycoprotein composition.

Immunoprecipitation of the HeLa cell lysates showed the expected NB-DNJ-mediated concentration-dependent decrease in intracellular proteolytic maturation of gp160 (Fig. 1). There was also, as predicted, a decrease in the electrophoretic mobility of the envelope glycoproteins due to the change in N-glycan composition. Purification of released virions revealed that only a minority of the secreted gp120 was virion associated. When the amount of reverse transcriptase precipitated was used as an internal standard (densitometry), an NB-DNJ-mediated reduction of 15% ± 2% (mean ± standard deviation of three independent experiments) in virion envelope glycoprotein content (gp160 and gp120) was observed. This decrease correlated with a decrease in the overall amounts of viral envelope glycoproteins (soluble and virus associated) released from the cells. A 26% reduction in the proteolytic maturation of virus-associated gp160 was also observed, with gp120 contents of 76% ± 2% at 0 mM NB-DNJ and 56% ± 5% at 2 mM NB-DNJ. These changes resulted in a combined reduction of 42% ± 11% in overall virion gp120 content at 2 mM NB-DNJ. No significant change in virion envelope glycoprotein composition was observed at the antiviral concentrations of 0.5 and 2 mM NB-DNJ. However, the entry of virions produced in the presence of 0.5 mM NB-DNJ was greatly impaired, as judged by a single-round infectivity assay (data not shown). A similar result was obtained for virus expressed in Cos-1 cells (data not shown).

Effect of NB-DNJ on CD4-induced gp120 shedding. The effect of altered N-glycan composition on CD4-induced gp120 shedding was analyzed by using envelope-transfected Cos-1 cells and HIV-1_{IIIB} virions generated in H9 cells.

Cos-1 cells transfected with only envelope plasmid showed the expected NB-DNJ-mediated increase in cell-associated gp160 (Fig. 2). Very little cell-associated gp120 was observed in both untreated and treated cells. Incubation of untreated Cos-1 cells with increasing concentrations of sCD4 led to an increase in the amount of gp120 precipitated from the supernatant. However, incubation of NB-DNJ (2 mM)-treated Cos-1 cells with sCD4 led to no increase in the amount of gp120 shed. An identical inhibition of sCD4-induced gp120 shedding was observed at 0.5 mM NB-DNJ (data not shown). A similar result was observed with gp120-expressing HeLa cells (data not shown).

In order to investigate whether the presence of noncleaved, nonsheddable gp160 on the cell surface could adversely affect the shedding of properly cleaved gp120 by virtue of its incorporation into heterooligomers, shedding in cells cotransfected with both a cleavage-competent and a cleavage-incompetent envelope plasmid was analyzed. The result showed that cotransfection of Cos-1 cells with both types of envelope expressers had no effect on the amount of gp120 shed after incubation with sCD4 (data not shown). The envelope glycoprotein expressed from the cleavage-incompetent envelope plasmid is expressed on the cell surface (7) and is incorporated in virions in an uncleaved form (data not shown).

HIV-1_{IIIB} virions generated in H9 cells in the absence of NB-DNJ were found to shed 52% of virion-associated gp120 after incubation with 50 μg of sCD4 per ml (Table 1). However, virions generated in the presence of 2 mM NB-DNJ shed only 7% of virion-associated gp120 under the same conditions. The generation of virions in the presence of 2 mM NB-DNJ therefore led to a 7.4-fold reduction in CD4-induced gp120 shedding. sCD4-induced shedding was calculated as follows: (virion gp120 without sCD4 − virion gp120 with sCD4)/virion gp120 without sCD4. The calculations were therefore based on column-purified virus only, without interference from soluble non-virus-associated gp120. The antibody used for quantification does not recognize gp160 (data not shown), and the overall amount of envelope glycoprotein detected in the treated virion samples was therefore smaller than that detected in

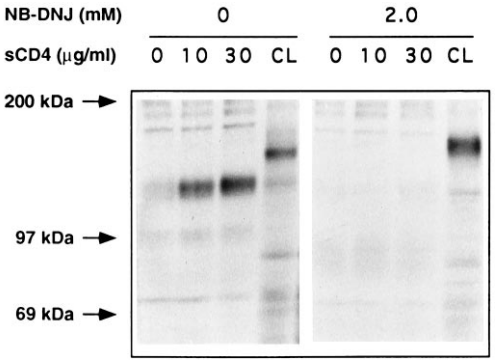

FIG. 2. Effect of NB-DNJ on CD4-induced shedding of Cos-1 cell-expressed gp120. Virus envelope glycoproteins were expressed in Cos-1 cells in the presence or absence of 2 mM NB-DNJ. The cells were incubated with the indicated concentrations of sCD4, and released protein or cell lysates (CL) were immunoprecipitated with HIV-positive serum.

TABLE 1. Effect of an NB-DNJ-mediated change in virion N-glycan composition on sCD4-induced virion gp120 shedding

NB-DNJ	Amt of virion-associated gp120 (arbitrary units)[a]		sCD4-induced shedding (%)
	Without sCD4	With sCD4[b]	
Absent	100	48 ± 0.6	52
Present	100	93 ± 2.4	7

[a] Values are arbitrary units standardized between two independent experiments in order to accommodate batch variations in gp120 content.
[b] Values are means ± standard deviations of two independent experiments.

untreated samples. The data presented are the results of independent experiments using two different preparations of virus. Because of differences in the absolute amounts of gp120 in the two preparations, the conversion of the values to arbitrary units was necessary in order to include standard deviations for the data. The ratio of virion-associated gp120 in the presence of sCD4 to virion-associated gp120 in the absence of sCD4 was calculated for each experiment, and the amount of virion-associated gp120 in the absence of sCD4 was standardized to 100 U.

Effect of NB-DNJ on CD4-induced gp41 exposure. Binding of CD4 to HIV-associated envelope glycoproteins may induce the exposure of gp41 in the absence of gp120 shedding (38, 39). The effect of altered N-glycans on CD4-induced gp41 exposure was analyzed by FACS analysis of HIV-expressing ACH-2 cells and a virion capture assay.

ACH-2 cells are derived from a T-cell clone latently infected with human T-cell lymphotropic virus III/lymphadenopathy-associated virus (9). Stimulation of these cells with PMA induces the expression of viral proteins and the formation of virus particles (9). No significant surface expression of viral envelope glycoproteins (as determined by FACS analysis) or virus particle formation (as determined by reverse transcriptase activity) was observed in the absence of PMA (data not shown). Maximal expression of surface envelope glycoproteins was observed after 20 h of stimulation, and NB-DNJ was found to have no effect on the time course of expression (data not shown). Additionally, ACH-2 cells stimulated to express HIV in the presence or absence of NB-DNJ showed a cell surface viral envelope glycoprotein composition (Fig. 3) similar to that of intact virions generated under the same conditions (Fig. 1). The use of ACH-2 cell surface-expressed viral envelope glycoproteins as a model for virion-associated envelope glycoproteins was therefore valid.

Incubation of untreated HIV-expressing ACH-2 cells with increasing concentrations of sCD4 led to a 14.4-fold increase in the binding of anti-gp41 antibodies to the cells (at 50 μg of sCD4 per ml) (Fig. 4A). However, ACH-2 cells stimulated in the presence of 2 mM NB-DNJ showed only a 3.6-fold increase in anti-gp41 binding upon incubation with sCD4 (Fig. 4B). The change in viral envelope glycoprotein N-glycan composition therefore resulted in a fourfold reduction in CD4-induced gp41 exposure. Identical effects were observed with sCD4 incubations at both 37 and 4°C (Fig. 4).

A similar effect of NB-DNJ on the sCD4-induced exposure of virion-associated gp41 was observed. In the presence of 50 μg of sCD4 per ml, a 123% increase in the number of untreated virions captured onto anti-gp41-coated beads was observed (Table 2). However, when virions generated in the presence of 2 mM NB-DNJ were incubated with sCD4, only a 31% increase was observed. NB-DNJ treatment therefore resulted in a 4.0-fold decrease in the exposure of virion-associated gp41. The values of gp41 exposure were standardized as described above, by calculating the ratio of virions captured in the presence of sCD4 to virions captured in the absence of sCD4 and

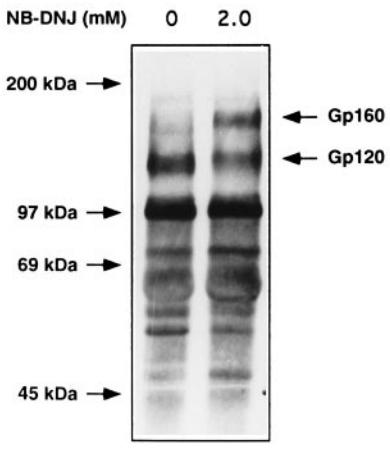

FIG. 3. Effect of NB-DNJ on viral envelope glycoprotein composition (ACH-2 cell surface). ACH-2 cells were stimulated with PMA in the presence or absence of 2 mM NB-DNJ. The cell surface was biotinylated, and cell lysates were immunoprecipitated with polyclonal anti-gp120 antibodies. The labelled viral envelope glycoproteins were visualized by using Western blotting, horseradish peroxidase-conjugated extravidin, and enhanced chemiluminescence.

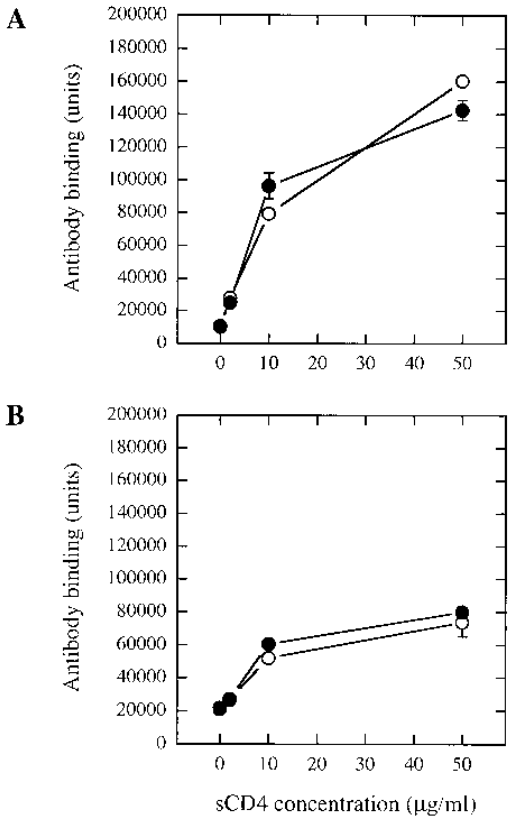

FIG. 4. Effect of NB-DNJ on CD4-induced gp41 exposure (ACH-2 cell surface). ACH-2 cells were stimulated with PMA in the absence of NB-DNJ (A) or in the presence of 2 mM NB-DNJ (B). The cells were then incubated at either 37°C (○) or 4°C (●) in the presence of the indicated concentrations of sCD4. The cells were stained for flow cytometry with an anti-gp41 monoclonal antibody.

TABLE 2. Effect of an NB-DNJ-mediated change in virion N-glycan composition on sCD4-induced gp41 exposure

NB-DNJ	No. of virions captured by anti-gp41 antibody (arbitrary units)		sCD4-induced exposure (%)
	Without sCD4[a]	With sCD4[b]	
Absent	100	223 ± 31	123
Present	100	131 ± 14	31

[a] Values are arbitrary units standardized between two independent experiments in order to accommodate batch variations in virus content.
[b] Values are means ± standard deviations of two independent experiments.

then standardizing the number of virions captured in the absence of sCD4 to 100 U.

Effect of NB-DNJ on CD4-induced increases in V3 loop exposure. The effect of altered N-glycans on CD4-induced increases in V3 loop exposure was analyzed by FACS analysis of HIV-expressing ACH-2 cells.

Incubation of stimulated ACH-2 cells with sCD4 at 4°C was found to induce a 3.8-fold increase in the binding of anti-V3-loop antibodies to the cell surface (at 50 μg of sCD4 per ml) (Fig. 5A). At 37°C no such induction was seen (Fig. 5A), a phenomenon which correlated with a decrease in the detection of bound sCD4 by anti-CD4 antibodies (data not shown). When the cells were stimulated in the presence of 2 mM NB-DNJ, only a 1.1-fold increase in the binding of anti-V3-loop antibodies was observed (Fig. 5B). An NB-DNJ-mediated change in N-glycan composition therefore resulted in a 25-fold reduction (275 to 11%) in the CD4-induced increase in V3 loop exposure. Identical results were obtained with two independent anti-V3-loop antibodies: ADP3047 (Fig. 1) and ADP3025 (data not shown).

A 2.1-fold increase in the binding of anti-V3-loop antibodies to NB-DNJ-treated cells incubated in the absence of sCD4 was observed. This apparent increase in the background number of V3 loops on the surfaces of NB-DNJ-treated cells was not correlated with any differences in the binding of FITC-conjugated sCD4 to the two types of cells (data not shown). No binding of FITC-sCD4 to nonstimulated ACH-2 cells was observed (data not shown).

DISCUSSION

The retention of glucosylated N-glycans on the viral envelope glycoproteins is known to inhibit HIV entry at the level of post-CD4 binding (13). However, the exact mechanism of impaired viral entry remains to be established. It has previously been shown that treatment of HIV-infected cells with α-glucosidase inhibitors leads to a decrease in the intracellular proteolytic maturation of the HIV envelope precursor glycoprotein, gp160, into gp41 and gp120 (10, 33, 48). The incorporation of predominantly nonfusogenic gp160 (5, 11, 17, 27) into virions could account for the inhibition of HIV entry at the level of post-CD4 binding. No effects of α-glucosidase inhibitors on the amount of mature gp120 released from HIV-infected cells have, however, been observed; neither have any significant amounts of uncleaved gp160 released from the cells been reported (36, 44, 48). These studies all used crude cell-free culture supernatants as a source of virus-associated envelope glycoproteins. However, the viral envelope glycoproteins of the particular viral isolates used for these assays (HIV-1_{IIIB}, HIV-1_{HXB2}, and HIV-1_{RF}) are known to be relatively unstable, resulting in spontaneous shedding of gp120 from both infected cells and released virions (19, 26, 28). It is conceivable that the presence of such non-virus-associated gp120 may have masked any effects of the compounds on the envelope composition of the virus particles present in the samples. We therefore analyzed the effect of NB-DNJ on the envelope composition of purified virus particles.

Immunoprecipitation of the purified virions showed that retention of glucosylated N-glycans did perturb the envelope glycoprotein composition of the virus. A 15% reduction in overall virion envelope content (gp120 and gp160) and a 26% reduction in the proteolytic maturation of virion gp160 were observed. Taken together, these effects resulted in a reduction of approximately 40% in the overall virion content of potentially functional gp120. As speculated above, the finding that the majority of the envelope glycoproteins released from the cells, under the conditions used, were non-virus-associated gp120 may explain why no effect on viral envelope composition has previously been observed (36, 44, 48). The small decrease in proteolytic maturation of virion-associated gp160 relative to that found for intracellular gp160 may be explained by two observations. Firstly, only a small fraction of intracellular gp160 is believed to be converted into gp120, with most of the remaining gp160 being degraded in the lysosomes and only a small proportion making it to the surfaces of the cells (41, 49). Secondly, an inefficient incorporation of uncleaved gp160 into virions has been described (11). This may also explain the finding that there was no increase in the amount of virion-associated gp160 at 0.5 mM NB-DNJ, despite a significant

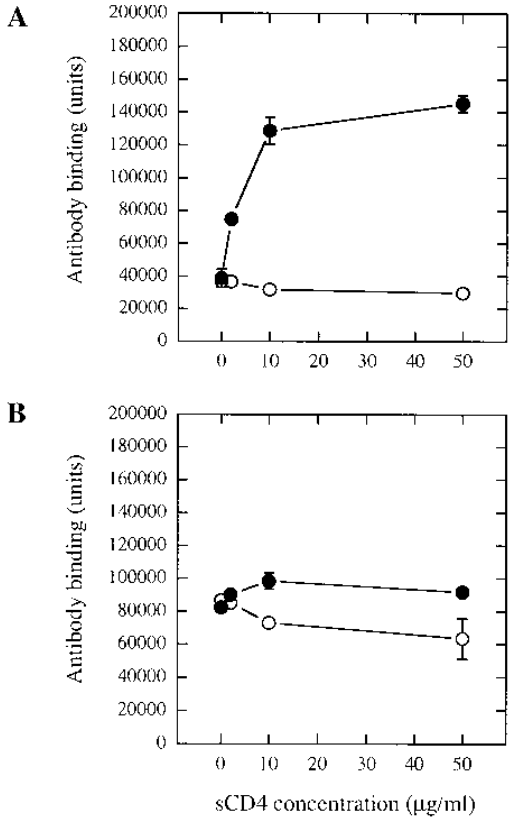

FIG. 5. Effect of NB-DNJ on CD4-induced V3 loop exposure (ACH-2 cell surface). ACH-2 cells were stimulated with PMA in the absence of NB-DNJ (A) or in the presence of 2 mM NB-DNJ (B). The cells were then incubated at either 37°C (○) or 4°C (●) in the presence of the indicated concentrations of sCD4. The cells were stained for flow cytometry with an anti-V3-loop monoclonal antibody (ADP3047).

reduction in the intracellular maturation of gp160 at this concentration.

The reason for the effect of NB-DNJ on gp160 maturation is unclear. Amino acid changes within the V1/V2 loops may result in impairment of gp160 maturation (16, 43). The fact that retention of glucosylated N-glycans affects the conformation of the V1/V2 loops, as reported recently (14), may contribute to this effect.

Amino acid changes within the V1/V2 loops may also affect post-CD4-binding events independently of any effect on gp160 maturation (16, 43, 52–54). We therefore analyzed the effect of NB-DNJ on the ability of the remaining gp120 to take part in post-CD4-binding events. Possible markers for these events include sCD4-induced dissociation of gp120 (31), exposure of otherwise-cryptic epitopes on gp41 (37), and an increase in the exposure of V3 loop epitopes (37).

The effect of NB-DNJ on CD4-induced shedding of gp120 was analyzed by two independent assays. Both assays showed a dramatic effect of NB-DNJ on CD4-induced shedding. The shedding of cell surface-associated gp120 became undetectable, and the shedding of virion-associated gp120 was reduced 7.4-fold. The extent of shedding seen in the absence of NB-DNJ is in accordance with previously published data (4, 30, 31). The effect of NB-DNJ on CD4-induced exposure of cryptic gp41 epitopes was also analyzed by two independent assays. Both assays showed identical levels of NB-DNJ-mediated inhibition of CD4-induced exposure, with the exposure of both cell surface-associated and virion-associated gp41 being reduced fourfold. Identical degrees of CD4-induced exposure of cell surface-associated gp41 were observed at 4 and 37°C. The fact that gp41 exposure does take place at 4°C in the absence of any gp120 shedding (see below) is in accordance with previously published data, although differences in the degree of exposure were observed at the two temperatures (37, 38).

Finally, the effect of NB-DNJ on the CD4-induced increase in the exposure of V3 loop epitopes was examined. NB-DNJ was found to severely impair V3 loop exposure, with the CD4-induced increase in V3 loop epitopes on cell surface-expressed gp120 being reduced 25-fold. The CD4-induced increases in the exposure of V3 loop epitopes could be observed only at 4°C because of the dissociation of gp120 at 37°C. However, in the absence of sCD4, an increase in the binding of anti-V3-loop antibodies to cells stimulated in the presence of NB-DNJ was observed. This increase in binding may simply represent an increase in the amount of envelope glycoproteins present on the cell surface after treatment with NB-DNJ; a similar increase was observed with antibodies directed against other regions of gp120 (data not shown). No difference in the binding of FITC-labelled sCD4 to the surface was, however, observed, and surface labelling of the cells showed no indication of an increase in envelope glycoprotein expression. This result may therefore also reflect differences in the presentation of the V3 loops on the surfaces of untreated and treated cells, possibly involving the organization of individual gp120 molecules within oligomeric envelope glycoprotein complexes. Finally, the increased binding of anti-V3-loop antibodies to NB-DNJ-treated cells may be explained by the possibility that the conformational changes normally induced by sCD4 have already taken place as a result of the conversion of the gp120-associated N-glycans. Incubation with sCD4 would therefore not increase the exposure any further. However, no evidence for an NB-DNJ-mediated conformational change within the V3 loop has been observed (14).

Differences in CD4-induced effects on the viral envelope between primary and laboratory viral isolates of HIV have previously been correlated with differences in the affinity between sCD4 and virus- or cell surface-associated gp120 (29, 51). Controversy over this correlation does, however, exist (6, 32, 46), and the fact that the retention of glucosylated N-glycans has no effect on the affinity of rgp120 for sCD4 (13), the binding of virus particles to CD4-positive cells (13, 35, 48), or the binding of sCD4 to HIV-expressing cells (data not shown) implies that the effect on CD4-induced events observed in this study may not be explained simply by differences in sCD4 binding.

Another explanation for the NB-DNJ-mediated impairment of CD4-induced conformational changes might be the presence of increasing amounts of gp160 in the viral envelope. gp160 is nonfusogenic (5, 11, 17, 27) and presumably incapable of undergoing the CD4-induced conformational changes observed within the mature envelope. The gp160 in the virion envelope may be present in either homooligomers of gp160 or heterooligomers with properly cleaved gp120/gp41. The presence of gp160 in homooligomers would not be expected to interfere with the function of gp120/gp41 homooligomers. Increasing amounts of gp160 homooligomers would, however, decrease the density of potential functional gp120/gp41 homooligomers and could therefore contribute to the decrease in viral infectivity observed in the presence of NB-DNJ. If uncleaved gp160 is present in heterooligomers with gp120/gp41, the presence of one or more gp160 molecules within the oligomeric structure could disrupt CD4-induced conformational changes within the remaining gp120/gp41 molecules, rendering them nonfunctional. However, the threshold value for the number of gp160 molecules required within an oligomer before CD4-induced conformational changes are affected is not known. The question remains whether a 26% (1.4-fold) reduction in gp160 cleavage is sufficient to explain the almost complete block in gp120 shedding, a 4-fold reduction in exposure of gp41, and a 25-fold reduction in V3 loop exposure. Several points argue against this. Firstly, no significant increase in virion-associated gp160 was observed at 0.5 mM NB-DNJ, a concentration which shows moderate antiviral activity and completely blocks the shedding of gp120 from transfected Cos-1 cells. Secondly, cotransfection of Cos-1 cells with both a cleavage-competent and a cleavage-incompetent envelope glycoprotein had no effect on the CD4-induced shedding of gp120 from the surfaces of the cells (data not shown). Thirdly, expression of HIV envelope glycoproteins in Cos-1 cells by cotransfection with a *tat* expresser, which does not prevent CD4-induced shedding and envelope-mediated syncytium formation, does increase the amount of gp160 found on the surfaces of the cells by approximately 50% (7, 11). This level of surface-associated gp160 is similar to that found in the virus envelope and on the surfaces of ACH-2 cells in the presence of 2 mM NB-DNJ. In conclusion, it is likely that the effect of NB-DNJ on CD4-induced conformational changes may not be fully explained by the observed reduction in gp160 cleavage. The results therefore show that the retention of glucosylated N-glycans independently affects the capacity of the remaining gp120 to undergo conformational changes after CD4 binding.

To further test this conclusion, CD4-induced conformational changes within rgp120 were probed by ELISA with two monoclonal antibodies (17b and 48d) known to detect such events (47). The results showed that the retention of glucosylated N-glycans had no effect on CD4-induced changes in the binding of these antibodies (data not shown). Both antibodies did, however, show a 20% reduction in the overall binding to rgp120(+) compared with the binding to rgp120(−). These data indicate that the small sCD4-induced changes in the binding of these antibodies to rgp120 may not be used as a marker for sCD4-induced conformational changes within an oligo-

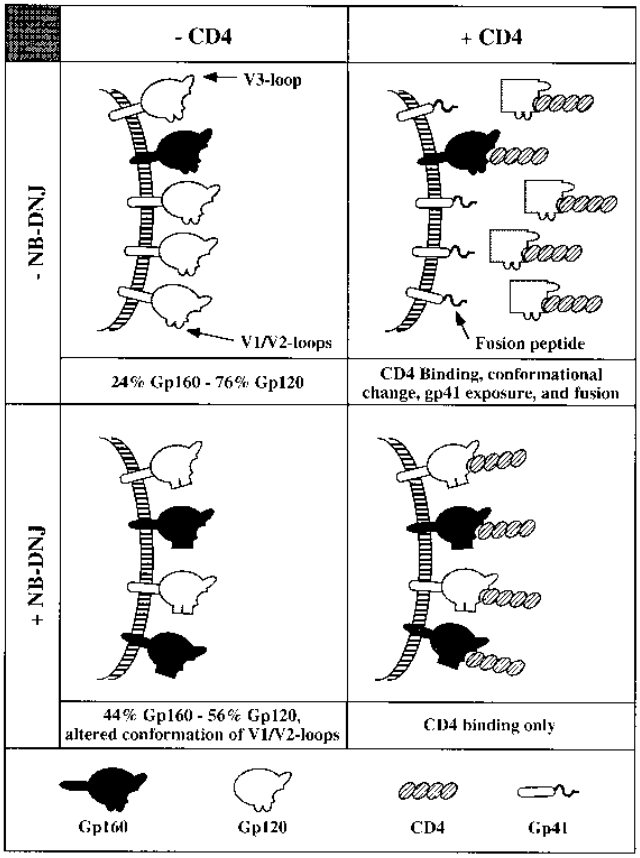

FIG. 6. Proposed model for the mechanism of action of NB-DNJ as an inhibitor of HIV replication. The generation of virus particles in the presence of NB-DNJ results in an altered conformation of the V1/V2 loops, a decrease in the proteolytic maturation of virion-associated gp160, and an impairment of the ability of the remaining gp120 to undergo CD4-induced conformational changes. The envelope glycoproteins shown represent oligomers.

meric envelope complex that result in the shedding of gp120 and the exposure of gp41. In relation to the effect of NB-DNJ on the conformation of the V1/V2 loops (14), previously published data have shown that some amino acid changes within the V2 loop, which reduce the binding of 17b to rgp120, have no effect on sCD4-induced increases in the recognition of rgp120 by this antibody (47).

Controversy about the relationship between CD4-induced conformational changes and HIV entry exists (46, 50). CD4-induced shedding of gp120 is unlikely to be required for HIV entry, and it is a poor marker for the ability of different viral isolates to enter CD4-positive cells (1, 29, 46). However, the interaction between CD4 and viral envelope glycoproteins may result in the formation of an activated complex, accelerating viral entry (2, 3, 8, 18, 42). No attempts to correlate preshedding events, such as V3 loop and gp41 exposure, with HIV entry have been made, but it seems likely that the critical event for HIV entry is the exposure of gp41 in the correct context, i.e., close to the host cell plasma membrane. The failure of a virus to undergo gp41 exposure is therefore much more likely to be a valid marker for its ability to enter the host cell. However, despite its poor reliability in predicting the ability of genetically different viral isolates to enter CD4-positive cells, the use of CD4-induced shedding as a marker for gp120 function after a change in N-glycan composition is likely to be valid because of the identical amino acid background. We therefore believe that the effect of a retention of glucosylated N-glycans on the sCD4-induced V3 loop exposure, gp41 exposure, and gp120 shedding described in this paper may help explain NB-DNJ-mediated inhibition of HIV entry.

In conclusion, this study has demonstrated that NB-DNJ-mediated retention of glucosylated N-glycans on the HIV envelope glycoproteins severely affects both the composition and the function of the viral envelope (Fig. 6). Firstly, NB-DNJ treatment increases the amount of gp160 found in the virus particles. Secondly, it impairs the ability of the remaining gp120 to undergo conformational changes after CD4 binding. The relative contributions of the two effects are unknown. Some inhibition of post-CD4-binding events, such as gp120 shedding, is observed at antiviral concentrations of NB-DNJ (0.5 mM) that do not affect gp160 incorporation. At this concentration a 1-log reduction in viral replication is observed (data not shown). At 2 mM NB-DNJ, a concentration at which both post-CD4-binding events and gp160 incorporation are affected, a 3-log reduction in viral replication is observed (data not shown). Both effects are therefore likely to contribute to NB-DNJ-mediated inhibition of HIV entry at the level of post-CD4 binding.

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