JOURNAL OF VIROLOGY, Oct. 1996, p. 7143–7152 Vol. 70, No. 10
0022-538X/96/$04.00+0

N-Butyldeoxynojirimycin-Mediated Inhibition of Human Immunodeficiency Virus Entry Correlates with Changes in Antibody Recognition of the V1/V2 Region of gp120

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Received 18 December 1995/Accepted 18 June 1996

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 NB-DNJ appears to inhibit HIV entry at the level of post-CD4 binding (P. B. Fischer, M. Collin, G. B. Karlsson, W. James, T. D. Butters, S. J. Davis, S. Gordon, R. A. Dwek, and F. M. Platt, J. Virol. 69:5791–5797, 1995), the exact mechanism of action remains to be established. In this study we have examined the effect of NB-DNJ on the structure of recombinant gp120 (rgp120), expressed in CHO cells, by using a panel of 40 monoclonal antibodies. The levels of binding of antibodies to rgp120 produced in the presence [rgp120(+)] and absence [rgp120(−)] of NB-DNJ were compared by enzyme-linked immunosorbent assay and surface plasmon resonance (BIAcore; Pharmacia). The results showed an increase in the binding to rgp120(+) of antibodies directed against the C1 and C2 regions and a decrease in the binding of antibodies directed against the V1/V2 loops compared with antibody binding to rgp120(−). A decrease in the binding to rgp120(+) of antibodies directed against discontinuous epitopes was also observed. No differences were seen in the binding of antibodies directed against the crown of the V3 loop and the C4 region of gp120. Treatment of rgp120 with α-glucosidases I and II had no effect on the differential binding observed, whereas treatment with sialidase abolished the differences seen in the binding of antibodies directed against the C1 and C2 regions of gp120. In addition to these findings, rgp120(+) showed increased sensitivity to proteases released by CHO cells during expression, as well as to exogenous thrombin. Taken together, the data presented in this paper suggest that production of gp120 in the presence of NB-DNJ affects the conformation of the V1/V2 loops of gp120, as well as the overall charge of the C1 and C2 regions. These effects may play a role in the previously described NB-DNJ-mediated inhibition of HIV entry at the level of post-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 (9, 45). α-Glucosidases I and II are responsible for the removal of terminal glucose residues from the precursor N-glycan, which is transferred to the protein as it is being translated 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 (13, 18, 24, 43, 55). In contrast, inhibitors of the later stages of N-glycan processing, such as deoxymannojirimycin (DMJ) and swainsonine, have no effect (18, 39). NB-DNJ is one of the most potent of the α-glucosidase inhibitors. It exhibits very little cytotoxicity and is currently in clinical trials as an HIV therapeutic agent. However, the exact mechanism of action of NB-DNJ as an inhibitor of HIV replication remains to be determined.

Antiviral concentrations of NB-DNJ are known to have either no effect (6, 51) or a relatively small effect (12) on the number of virus particles released from HIV-infected cells. The amount of infectious virus released, however, is greatly reduced (6, 12, 51), indicating that the major mechanism of action for NB-DNJ is a reduction in the relative infectivity of the virions produced. This effect correlates with an impairment of the processing of gp120-associated N-glycans, which results in the retention of predominantly neutral glycosylated high-mannose-type N-glycans (23, 39, 43, 51). This change in glycan composition does not affect the affinity of the gp120-CD4 interaction (12, 42) or the binding of virus particles to CD4-positive cells (12). Virus produced in the presence of NB-DNJ does, however, fail to enter the cells after CD4 binding (12). This demonstrates that the NB-DNJ-mediated impairment of virion infectivity is due to an effect on postbinding events necessary for viral entry (12).

It has previously been shown that treatment of HIV-infected cells with α-glucosidase inhibitors leads to a decrease in the processing of gp160 into gp41 and gp120 (6, 39, 55), without causing a reduction in the amount of gp120 released from the cells (18, 43, 55). The production of recombinant gp120 (rgp120) in the presence of NB-DNJ has also been shown to have an effect on the recognition of gp120 by one monoclonal antibody directed against the V3 loop of gp120 (21). Taken together, these results suggest that the NB-DNJ-mediated impairment of postbinding events may be caused by an effect on the conformation of regions of gp120 involved in entry events without an effect on CD4 binding. We therefore used a panel of monoclonal antibodies directed against epitopes spanning the whole of gp120 in order to identify regions of rgp120

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affected by an NB-DNJ-mediated change in N-glycan composition.

MATERIALS AND METHODS

Reagents. NB-DNJ (SC-48344) was a gift from Searle/Monsanto, St. Louis, Mo.

The following monoclonal antibodies were obtained from the Medical Research Council AIDS Reagent Project (National Institute of Biological Standards and Control, Potters Bar, United Kingdom) and are listed by antibody name {with original hybridoma clone name, source, species, and status (purified [P], culture supernatant [CS], and ascites [A]) in parentheses} ADP360 (4A7C6, R. B. Ferns, mouse, A) (37), ADP332 (187.2.1, C. Thiriart and C. Bruck, mouse, CS) (53), ADP3050 (7E2/4, S. Ranjbar, mouse, A) (37), ADP324 (CRA3, M. Page, rat, A) (38), ADP3035 (11/4C, C. Shotton and C. Dean, rat, CS) (34), ADP325 (CRA4, M. Page, rat, CS) (38), ADP3049 (6C4/5, S. Ranjbar, mouse, A) (38), ADP3041 (11/68b, C. Shotton and C. Dean, rat, CS) (34), ADP334 (213.1, C. Thiriart and C. Bruck, mouse, CS) (53), ADP358 (ABT1001, American Bio-Technologies, mouse, P), ADP3046 (IIIB-V3-01, J. Laman, mouse, P) (28), ADP3047 (IIIB-V3-13, J. Laman, mouse, P) (28), ADP3048 (IIIB-V3-21, J. Laman, mouse, P) (28), ADP3037 (11/75a, C. Shotton and C. Dean, rat, CS) (33), ADP3039 (8/38, C. Shotton and C. Dean, rat, CS) (33), ADP3025 (0.5β, K. Takatsuki, mouse, A) (8), ADP3013 (5F7, A. von Brunn, mouse, CS) (54), ADP328 (60.1.1, C. Thiriart and C. Bruck, mouse, CS) (53), ADP3028 (2D7-V4, B. Vestergaard and E. Hulgaard, mouse, P) (37), ADP388 (ICR 38.1a, J. Cordell and C. Dean, rat, A) (5, 35), ADP323 (CRA1, M. Page, mouse, CS) (37), ADP301 (221, R. Daniels and M. Aymard, mouse, P) (37), ADP3016 (1.5E, J. Robinson, human, P) (20), ADP3017 (2.1H, J. Robinson, human, P) (52), ADP390 (ICR 39.13g, J. Cordell and C. Dean, rat, A) (5, 35), and ADP3040 (8/19b, C. Shotton and C. Dean, rat, CS) (34).

The following antibodies were obtained from the companies or individuals listed: 9284 (HIV-018-48172, mouse, P) from Capricorn (Scarborough, Maine) (46); B2, B18, C4, C13, B13, C12, B32, and B15 (mouse, CS) from George Lewis (Baltimore, Md.) (1); G3-4, G3-136, and G3-299 (mouse, P) from M. Fung (Tanox Biosystems, Houston, Tex.); and D59 (mouse, P) from L. Åkerblom (Uppsala, Sweden) (2). D7324, a polyclonal sheep anti-gp120 antibody, was obtained from Aalto Bio Reagents (Dublin, Ireland).

Endoglycosidase H (derived from *Streptomyces plicatus*) was purchased from Boehringer Mannheim (Lewes, United Kingdom). Sialidase (derived from *Arthrobacter ureafaciens*) was purchased from Oxford Glycosystems (Oxford, United Kingdom). α1,2-Glucosidase I was purified from porcine liver microsomes by affinity chromatography as described elsewhere (23). α1,3-Glucosidase II was purified from rat liver microsomes by anion-exchange and gel filtration chromatography as described elsewhere (23). Human thrombin was purchased from Sigma (Poole, United Kingdom).

rgp120. rgp120 was produced in Chinese hamster ovary (CHO) cells, as described elsewhere (23), in either the presence or the absence of 2 mM NB-DNJ. The concentrations of the two types of rgp120 were standardized by amino acid composition analysis.

Binding of monoclonal antibodies to rgp120: surface plasmon resonance (BIAcore; Pharmacia). Equal molar quantities of the two types of rgp120 were immobilized onto flow cells (sensor chip CM5, research grade; Pharmacia) as described elsewhere (4). Briefly, flow cells were activated with a 1:1 mixture of 0.1 M *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide and 0.1 M *N*-hydroxysuccimide in water. rgp120 was immobilized at 417 nM in 10 mM sodium acetate, pH 4.5, and the remaining binding sites were blocked with 1 M ethanolamine, pH 8.5 (Pharmacia). Finally, the flow cells were washed with 100 mM phosphoric acid (BDH). The running buffer used was HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (HBS) (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20 [pH 7.4]; Pharmacia), and all experiments were carried out at 25°C. Antibodies were passed over the flow cells at nonsaturating concentrations ranging from 20 to 50 μg/ml in either HBS, culture supernatant, or diluted ascites, depending on the antibodies used.

Binding of monoclonal antibodies to rgp120: 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 then blocked with Tris-buffered saline (TBS) (140 mM NaCl, 10 mM Tris-HCl, 0.1% NaN_3 [pH 7.4]) containing 5 mg of bovine serum albumin (BSA) (Sigma) per ml (TBS-BSA) for 30 min. The plates were then incubated with rgp120 at 2.08 nM in TBS-BSA for 7 h at room temperature; this procedure was followed by an overnight incubation at room temperature with serial dilutions of monoclonal antibodies in TBS-BSA. The plates were developed by incubation with alkaline phosphatase-conjugated goat anti-mouse or anti-rat immunoglobulin G diluted in TBS-BSA for 3 h at room temperature, followed by *p*-nitrophenolphosphate (1 mg/ml) diluted in diethanolamine buffer (10% diethanolamine, 0.5 mM $MgCl_2$, 0.02% NaN_3 [pH 9.8]).

Endoglycosidase H treatment of rgp120. rgp120 (16.7 nmol) was incubated in citrate buffer (50 mM citric acid [pH 5.5]) for 18 h at 37°C in either the presence or the absence of 0.01 U of endoglycosidase H. Samples were then analyzed by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE) under nonreducing conditions as described elsewhere (27) and blotted onto an Immobilon P membrane (Millipore, Bedford, Mass.) at 0.8 mA/cm^2 for 1 h. The membrane was blocked in TBS containing 0.05% Tween 20 (Sigma) overnight and stained with a monospecific polyclonal sheep anti-gp120 antibody (D7324) at 0.5 μg/ml in TBS-BSA for 2 h. The blot was developed with alkaline phosphatase-conjugated donkey anti-sheep immunoglobulins (Sigma) diluted in TBS-BSA for 2 h followed by a solution of 0.1 mg of nitroblue tetrazolium per ml, 0.05 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml, and 4 mM $MgCl_2$ in ethanolamine buffer (0.1 M ethanolamine [pH 9.0]).

α-Glucosidase treatment of rgp120. rgp120 (167 nmol) was incubated in 10 mM HEPES–140 mM NaCl (pH 7.4) containing 0.5% Triton X-100 for 48 h at 37°C in either the presence or the absence of α-glucosidases (4 U of α1,2-glucosidase I and 52 U of α1,3-glucosidase II). One unit is defined as the amount of enzyme necessary to release 10 cpm (approximately 43 fmol) per minute of [^{14}C]glucose from either [^{14}C]glucose-labelled $Glc_3Man_9GlcNAc_2$ (α1,2-glucosidase I) or $Glc_2Man_9GlcNAc_2$ (α1,3-glucosidase II).

Sialidase treatment of rgp120. rgp120 (167 nmol) was incubated in sodium acetate buffer (100 mM sodium acetate, 140 mM NaCl [pH 5.0]) for 24 h at 37°C in either the presence or the absence of 0.2 U of sialidase.

Thrombin cleavage of rgp120. rgp120 (167 nmol) was incubated with 5 U of human thrombin (T-3010; Sigma) overnight at 37°C in citrate buffer (50 mM sodium citrate, 150 mM NaCl [pH 6.5]). Samples were analyzed by SDS–7.5% PAGE as described elsewhere (27).

RESULTS

Glycosylation status of rgp120(+) and rgp120(−). We have previously shown that production of CHO-derived rgp120 in the presence of 2 mM NB-DNJ results in the presence of only glucosylated, high-mannose-type oligosaccharides on purified rgp120 (23). To confirm that this was also the case with the preparation of rgp120 used in this study, and to compare the glycosylation status of rgp120 produced in the presence of 2 mM NB-DNJ [rgp120(+)] with that of CHO-derived rgp120 produced in the absence of NB-DNJ [rgp120(−)], we analyzed the sensitivities of rgp120(+) and rgp120(−) to endoglycosidase H. In the absence of endoglycosidase H, rgp120(+) showed the expected decrease in mobility on SDS-PAGE gels relative to rgp120(−) (23), indicating that glucosidase inhibition had occurred (Fig. 1, lanes 1 and 3). Treatment of rgp120(−) with endoglycosidase H resulted in a broad band migrating at around 90 kDa (Fig. 1, lane 2). This is consistent with the heterogeneous oligosaccharide composition previously reported for rgp120(−) (36). Treatment of rgp120(+) with endoglycosidase H resulted in a well-defined band at approximately 55 kDa (Fig. 1, lane 4), which is consistent with the conversion of all rgp120(+)-derived N-glycans to endoglycosidase H-sensitive glucosylated high-mannose-type oligosaccharides. The intensity of the 55-kDa band compared with a minor band around 96 kDa showed that the majority of the rgp120(+) molecules contained such endoglycosidase H-sensitive N-glycans.

Binding of antibodies to rgp120(+) and rgp120(−). The binding of monoclonal antibodies to rgp120(+) and rgp120(−) was analyzed by both surface plasmon resonance (BIAcore; Pharmacia) and ELISA.

In duplicate experiments, the BIAcore sensor chips were coated with 5,415 (experiment 1) and 4,328 (experiment 2) response units of rgp120(−) and with 6,566 (experiment 1) and 5,273 (experiment 2) response units of rgp120(+). The differences in the amounts of rgp120(−) and rgp120(+) immobilized on the chips are comparable to the differences in the molecular weights of the two proteins, which indicates that equal molar quantities of the two proteins were immobilized. Binding profiles were recorded for each antibody as illustrated in Fig. 2, which shows both the association and dissociation phases of the interaction. Because of the bivalent nature of the antibody binding, kinetic analysis was not used to compare the levels of binding of antibodies to the two types of rgp120. Instead, the overall level of binding observed was used as a

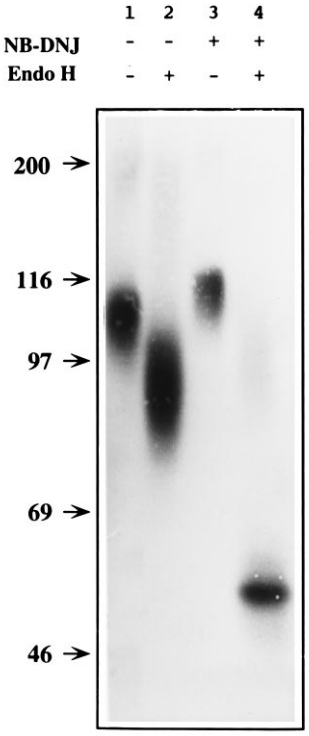

FIG. 1. Sensitivity of rgp120(+) and rgp120(−) to endoglycosidase H (Endo H) digestion. rgp120(−) and rgp120(+) were incubated in either the presence or the absence of endoglycosidase H as described in Materials and Methods. The samples were analyzed by SDS-PAGE under nonreducing conditions followed by Western blotting with an anti-gp120 antibody (D7324). Numbers on the left are molecular masses in kilodaltons.

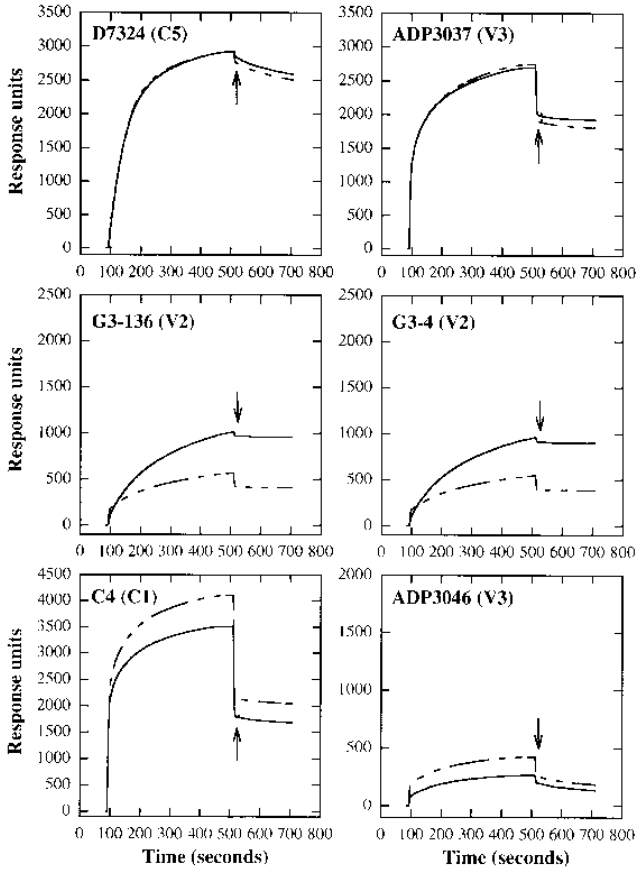

FIG. 2. BIAcore profiles of antibody binding to rgp120(+) and rgp120(−). The binding of monoclonal antibodies to rgp120(−) and rgp120(+) was analyzed by surface plasmon resonance (BIAcore). rgp120(−) and rgp120(+) were immobilized as described in Materials and Methods, and antibodies were passed sequentially over the two sensor chips. Binding profiles containing both association and dissociation phases for six representative antibodies are shown. The region of gp120 recognized by each antibody is shown in parentheses. Solid lines represent the binding of antibody to rgp120(−); dashed lines represent the binding of antibody to rgp120(+).

measure of the interaction. Because of the effect of buffer changes on the refractive index of the sensor chip (and thereby on the number of response units recorded), vertical shifts in the binding curves, not caused by protein-protein interactions, are often seen in the first few seconds of both the association and dissociation phases of the binding profiles (Fig. 2). The time delay between the passing of an antibody over the first and the second of the two rgp120-coated chips led to differences in these shifts and therefore in the number of response units observed at the end of the association phase (Fig. 2). Because of these differences, the levels of antibody binding to the two types of rgp120 were measured immediately after the dissociation phase shift (Fig. 2, arrows). The antibodies tested and their average levels of binding to the two chips seen in two independent experiments are summarized in Table 1. The ratios of the observed binding to rgp120(+) to the observed binding to rgp120(−) for antibodies showing good binding to native rgp120 are shown in Fig. 3.

The results showed substantial differences in the binding to rgp120(+) of antibodies directed against the N-terminal half of gp120. Antibodies directed against the first and second conserved domains (C1 and C2) showed increased binding to rgp120(+), whereas antibodies directed against the first and second variable loops (V1 and V2), and those directed against discontinuous epitopes on gp120, showed decreased binding to rgp120(+). Antibodies directed against the crown of the third variable loop (V3) showed slight variations in binding to the two types of rgp120. Antibodies directed against the fourth conserved domain (C4) and the C-terminal end of the fifth conserved domain (C5) showed no significant difference in binding to the two types of rgp120.

In order to confirm the results obtained by BIAcore analysis, the binding of antibodies to rgp120(−) and rgp120(+) was also analyzed by ELISA. The fact that there was no differential recognition of rgp120(−) and rgp120(+) by the D7324 antibody directed against the C-terminal end of C5 allowed the use of a capture ELISA configuration similar to that previously described by Moore et al. (37). The binding curves obtained by ELISA confirmed the findings obtained by BIAcore analysis (Fig. 4). Antibodies directed against the crown of the V3 loop showed less differential binding to the two types of rgp120 by ELISA than by BIAcore analysis. However, one antibody (9284) mapping to the N-terminal side of the crown of the V3 loop showed an enhanced difference in binding by ELISA compared with that seen by BIAcore analysis.

Effect of α-glucosidase treatment on the binding of antibodies to rgp120(+) and rgp120(−). To test whether the differential binding was due to the presence of terminal glucose on the rgp120(+)-associated N-glycans, we analyzed the effect of α-glucosidase treatment of rgp120 on antibody binding. rgp120(−) and rgp120(+) were treated with α1,2-glucosidase I and α1,3-glucosidase II as described in Materials and Methods, under conditions previously found to remove terminal glucose residues from rgp120(+) (22). Confirming the removal of ter-

TABLE 1. BIAcore analysis of the binding of antibodies to rgp120(+) and rgp120(−)

Domain	Antibody	Epitope (amino acid)	Antibody binding (RU)[a] to:	
			rgp120(−)	rgp120(+)
C1	ADP3050 (7E2/4)	31–50	550	441
	ADP360 (4A7C6)	81–90	2,024	2,109
	B2	94–97	2,011	1,914
	B18	106–110	1,036	1,213
	C4	109–113	1,742	2,033
	ADP332 (187.2.1)	101–120	1,482	1,681
V1/V2	ADP3035 (11/4C)	152–181	1,803	1,637
	ADP3049 (6C4/5)	160–175	124	142
	ADP3041 (11/68B)	V1/V2 + C4	1,425	876
	ADP325 (CRA4)	V2	969	613
	ADP324 (CRA3)	Base V2 + C1	912	389
	G3-136	Base V2/C2	967	404
	G3-4	Base V2/C2	952	418
C2	C13	252–273	526	697
	B13	257–262	668	859
	ADP334 (213.1)	250–270	898	1,260
V3	ADP3048 (IIIB-V3-21)	294–300	8	9
	9284	302–312	1,880	2,072
	ADP3039 (8/38)	300–315	2,067	1,851
	ADP3047 (IIIB-V3-13)	307–315	2,373	2,542
	ADP358 (ABT1001)		2,512	2,138
	D59	310–316	711	657
	ADP3037 (11/75a)		1,812	1,744
	ADP3025 (0.5β)	307–330	1,469	1,239
	ADP3013 (5F7)	308–322	2,098	1,895
	ADP3046 (IIIB-V3-01)	320–328	154	207
C3	ADP328 (60.1.1)	362–381	238	353
	C12	376–380	88	275
	B32	382–384	115	142
V4	ADP3028 (2D7-V4)	392–400	185	170
	B15	395–400	302	352
C4	ADP388 (ICR 38.1a)	427–436	2,784	2,814
	G3-299		1,109	1,117
V5	ADP323 (CRA1)	461–480	185	252
C5	ADP301 (221)	471–490	29	38
	D7324	497–511	2,509	2,517
Discontinuous epitopes	ADP3016 (1.5e)		972	635
	ADP3017 (2.1h)		517	311
	ADP390 (ICR 39.13g)		1,376	981
	ADP3040 (8/19b)		1,487	1,229

[a] Levels of antibody binding to rgp120(+) and rgp120(−). Each value is the level of binding (expressed as response units [RU]) seen at the beginning of the dissociation phase, immediately after the dissociation phase shift, and is the average of two independent experiments.

minal glucose, rgp120(+) showed an increase in mobility by SDS-PAGE after treatment with glucosidases, whereas the mobility of rgp120(−) was unaltered (data not shown). The effect of the α-glucosidase treatment of rgp120 on antibody binding was analyzed by an ELISA in which the binding of antibodies to untreated rgp120 was compared with their binding to rgp120 treated with α-glucosidases. Eight representative antibodies showing differential binding to rgp120(−) and rgp120(+) by ELISA and BIAcore analysis were analyzed. The antibodies tested were B2, C4, G3-4, G3-136, C13, B13, 9284, and ADP390. The results showed that treatment of the two types of rgp120 with α-glucosidases I and II had no effect on the differential antibody binding observed (Fig. 5).

Effect of sialidase treatment on the binding of antibodies to rgp120(+) and rgp120(−). To test whether the differential binding was due to the absence of terminal sialic acid on the rgp120(+) N-glycans, we analyzed the effect of sialidase treatment on antibody binding. rgp120(−) and rgp120(+) were treated with sialidase as described in Materials and Methods. rgp120(−) showed an increase in mobility by SDS-PAGE after treatment with sialidase, whereas the mobility of rgp120(+) was unaltered (data not shown). The effect of the sialidase treatment on antibody binding was analyzed by an ELISA in which the binding of antibodies to untreated rgp120 was compared with their binding to rgp120 treated with sialidase. Twelve representative antibodies showing differential binding

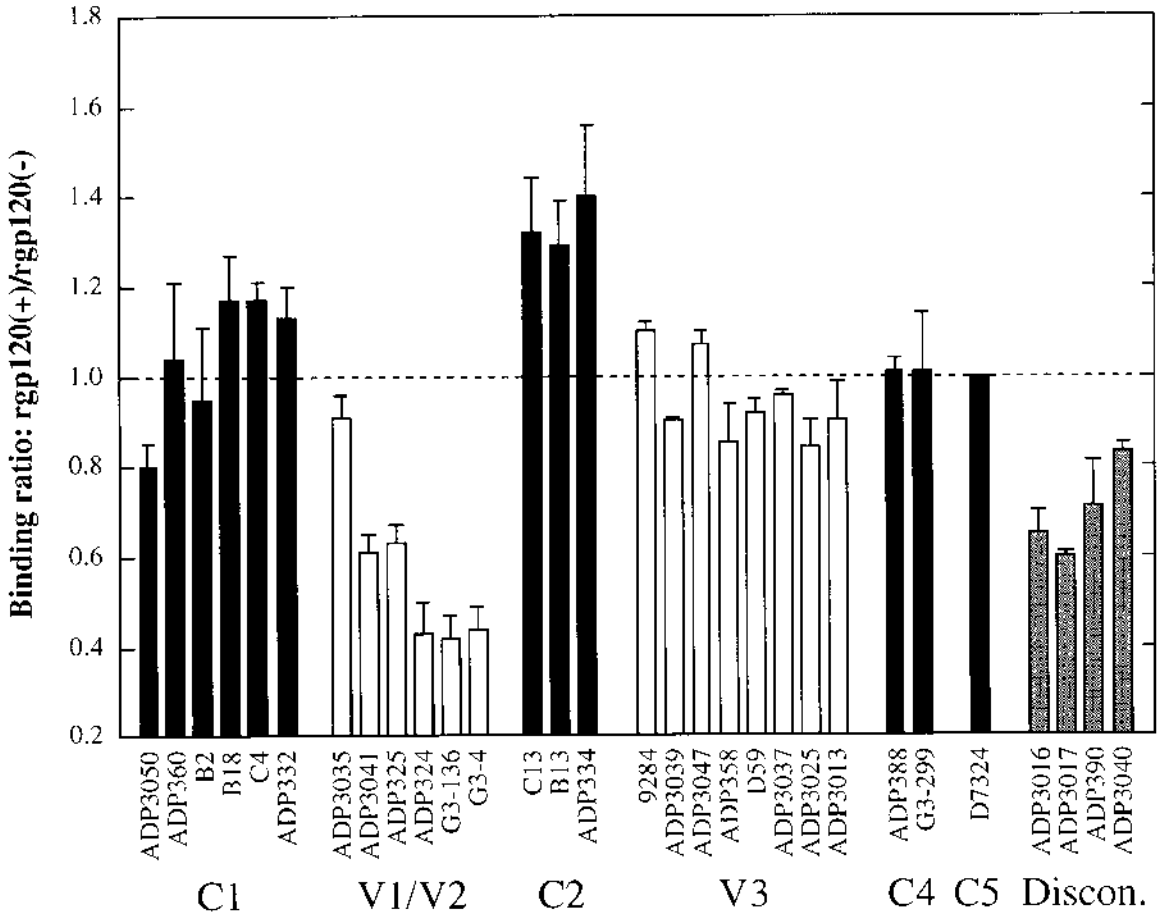

FIG. 3. Antibody binding to rgp120(+) and rgp120(−). (as determined by BIAcore analysis). The ratios of the levels of binding of antibodies to rgp120(+) to the levels of binding of the same antibodies to rgp120(−) are shown. The averages of two independent experiments are given. The dashed line represents equal binding of an antibody to the two types of rgp120. A value below the line indicates preferential binding of an antibody to rgp120(−), whereas a value above the line indicates preferential binding to rgp120(+). Solid and open bars represent antibodies directed against conserved and variable regions of gp120, respectively, and hatched bars represent antibodies directed against discontinuous epitopes (Discon.).

to rgp120(−) and rgp120(+) by ELISA and BIAcore analysis were analyzed. The antibodies tested were B2, C4, G3-4, G3-136, C13, B13, 9284, ADP3047, ADP358, ADP3025, G3-299, and ADP390.

The results showed that treatment of the two types of rgp120 with sialidase completely abolished the differential binding observed for antibodies directed against the C1, C2, and V3 regions of gp120 (Fig. 6). The differences were abolished because of an increase in the binding of antibodies to rgp120(−). In a similar way, the differential binding observed for antibodies directed against the V1/V2 loops and discontinuous epitopes of gp120 was enhanced.

Protease sensitivities of rgp120(+) and rgp120(−). In addition to differences in the binding of antibodies to rgp120(−) and rgp120(+), differences in the sensitivities of rgp120(−) and rgp120(+) to proteases were also observed. SDS-PAGE analysis of the two preparations of rgp120 showed that rgp120(+) had increased sensitivity to proteases released by CHO cells during expression (Fig. 7). This is illustrated by the observation that a fraction of the rgp120(+) molecules analyzed under reducing conditions gave rise to a ladder of protein fragments in addition to the main population of rgp120 molecules, which migrated as intact molecules. Under nonreducing conditions all fragments migrated as full-sized molecules. It was confirmed by Western blotting (immunoblotting) that the fragments were gp120 derived (data not shown).

We analyzed the relative sensitivities of rgp120(−) and rgp120(+) to human thrombin. Treatment of rgp120(−) with thrombin resulted in two molecular species (70 and 50 kDa) under reducing conditions (Fig. 7). Treatment of rgp120(+) with thrombin resulted in a large number of protein fragments, with no single predominant species being observed. All rgp120(+) molecules in the preparation had increased protease sensitivity compared with rgp120(−) molecules, as evidenced by the fact that bands equivalent to the 70- and 50-kDa bands normally found were no longer observed. No difference in the amount of thrombin needed to cleave the two types of rgp120 was observed (data not shown).

DISCUSSION

The outer envelope glycoprotein of HIV, gp120, is highly glycosylated, with about 50% of the molecular weight being contributed by N-linked oligosaccharides (23, 32, 48). The oligosaccharides isolated from gp120 exhibit a very high degree of heterogeneity, with more than 100 different structures distributed over 24 glycosylation sites (HIV-IIIB) (16, 36). The positions of the N-linked glycosylation sites of gp120 are rela-

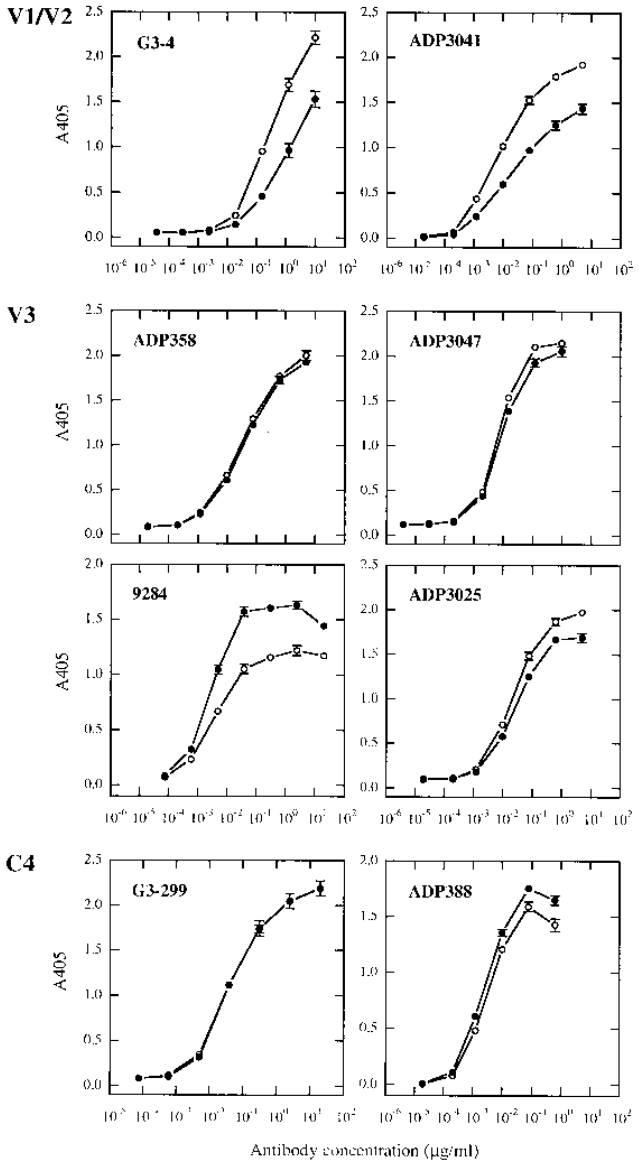

FIG. 4. Antibody binding to rgp120(+) and rgp120(−) (as determined by ELISA). Antibodies at the indicated concentrations were incubated in microtiter plates containing equal amounts of either rgp120(−) (○) or rgp120(+) (●) before the binding to gp120 was assayed. The binding curves for eight representative antibodies, recognizing either the V1/V2, V3, or C4 region of gp120, are shown.

tively well conserved among different viral isolates (44), and the presence of the oligosaccharides is necessary for the correct folding of gp120 in order to obtain a CD4-binding conformation (31). Site-directed mutagenesis has suggested that several glycosylation sites, in both gp120 (29, 56) and gp41 (7, 11), are important for the infectivity of HIV. It is, however, unclear from these mutagenesis studies whether impairment of infectivity is due to an effect on the overall folding of the envelope glycoproteins or the absence of the glycan moiety itself.

The use of inhibitors specific for the enzymes in the N-glycan processing pathway has allowed closer analysis of the influence of the types of glycans present on the functions of the envelope glycoproteins. Experiments using inhibitors of α-glucosidases I and II such as deoxynojirimycin, NB-DNJ, and castanospermine have shown that the presence of only glucosylated high-mannose-type oligosaccharides on the envelope glycoproteins leads to an impairment of viral infectivity and reduced processing of gp160 into gp41 and gp120 (18, 39). On the other hand, the presence of only high-mannose-type and hybrid-type oligosaccharides has no effect on HIV infectivity and envelope processing (18, 39).

The recent finding that the major mechanism of action of NB-DNJ as an inhibitor of HIV replication is an impairment of post-CD4-binding events necessary for virus entry (12) has raised the question of whether the presence of glucosylated

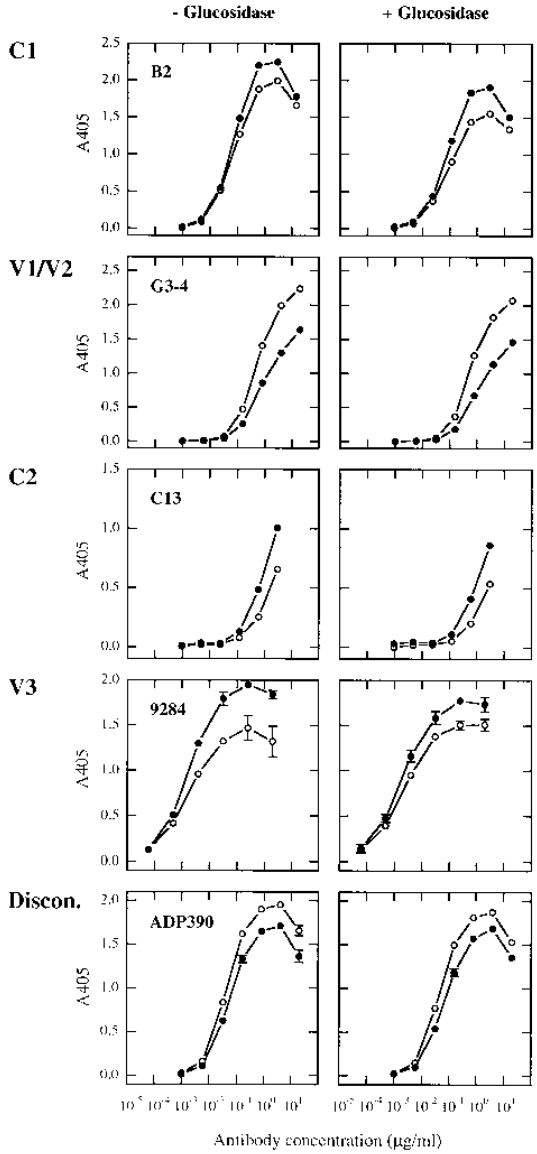

FIG. 5. Effect of glucosidase treatment on the binding of antibodies to rgp120(+) and rgp120(−) (as determined by ELISA). rgp120 was either left untreated or treated with glucosidases as described in Materials and Methods and was captured in microtiter plates. Antibodies at the concentrations indicated were then incubated in the plates before their binding to gp120 was assayed. Symbols: ○, binding of antibody to rgp120(−); ●, binding to rgp120(+). The binding curves for five representative antibodies, recognizing either the C1, (B2), V1/V2 (G3-4), C2 (C13), or V3 (9284) region of gp120 or a discontinuous epitope (Discon.) (ADP390) within gp120, are shown.

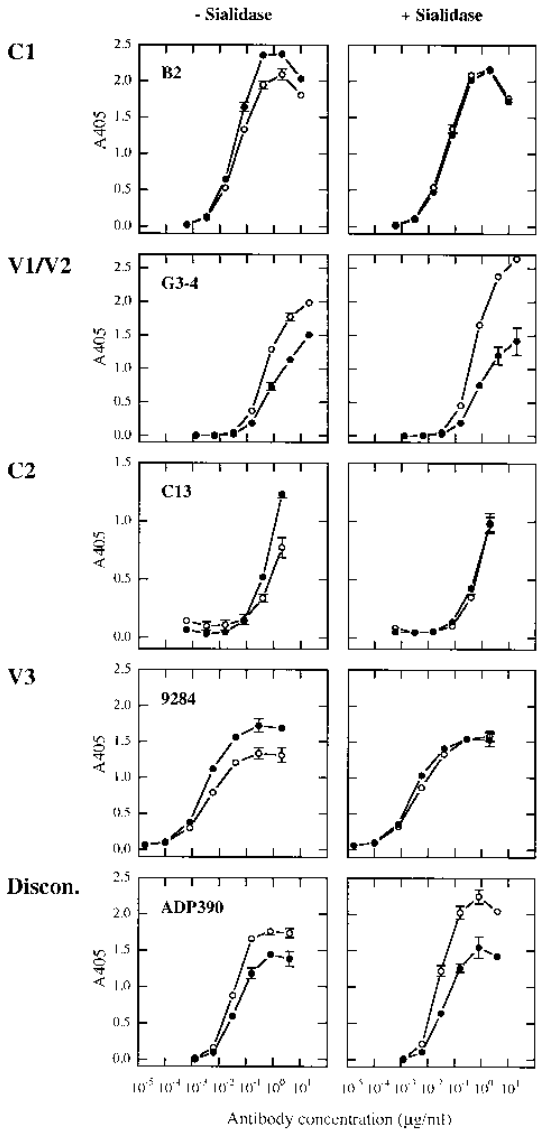

FIG. 6. Effect of sialidase treatment on the binding of antibodies to rgp120(+) and rgp120(−) as determined by ELISA. rgp120 was either left untreated or treated with sialidase as described in Materials and Methods and was captured in microtiter plates. Antibodies at the indicated concentrations were then incubated in the plates before the binding to gp120 was assayed. Symbols: ○, binding of antibody to rgp120(−); ●, binding to rgp120(+). The binding curves for five representative antibodies, recognizing either the C1 (B2), V1/V2 (G3-4), C2 (C13), or V3 (9284) region of gp120 or a discontinuous epitope (Discon.) (ADP390) within gp120, are shown.

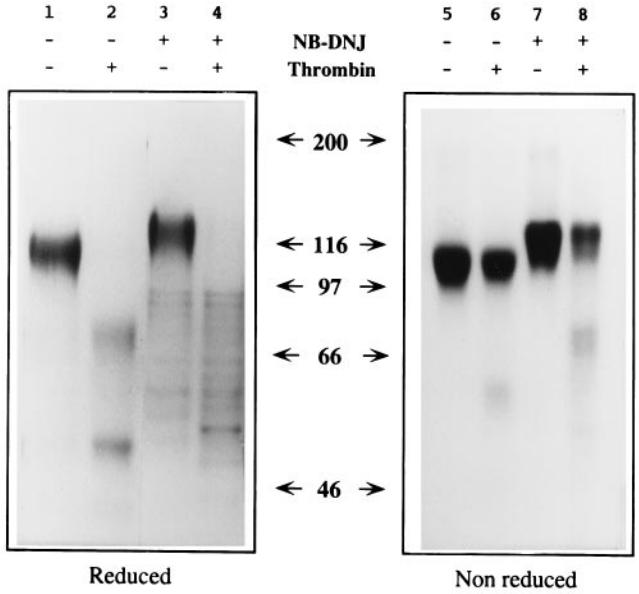

FIG. 7. Increased protease sensitivity of rgp120(+) compared with rgp120(−). The sensitivities of rgp120(−) and rgp120(+) to cleavage by human thrombin were analyzed by SDS-PAGE. Numbers between gels are molecular masses in kilodaltons.

high-mannose-type oligosaccharides adversely affects the conformation of specific regions of gp120 involved in viral entry. We have therefore used a panel of monoclonal antibodies directed against epitopes spanning the whole of gp120 in order to identify regions of rgp120 affected by NB-DNJ-mediated changes in N-glycan composition.

In agreement with previously published data (37), the degree of antibody binding showed good exposure of regions within the C1, V1/V2, V3, and C4 regions, parts of the C5 region, and to a lesser extent parts of the C2 region of rgp120(−). Antibodies directed against the rest of the molecule showed poor binding to native rgp120(−). When the levels of binding of antibodies to rgp120(−) and rgp120(+) were compared, four regions showed significant differences in binding. Antibodies directed against the C1 and C2 regions, plus an epitope on the N-terminal flanking region of the V3 loop, showed an increase in binding to rgp120(+). Antibodies directed against the V1/V2 loops, as well as discontinuous epitopes on gp120, showed a decrease in binding to rgp120(+). In contrast, antibodies directed against the crown of the V3 loop and the C4 region showed no differences in binding to the two types of rgp120.

Treatment of rgp120(−) and rgp120(+) with α-glucosidases I and II showed that the presence of terminal glucose residues on rgp120(+) oligosaccharides played no role in the differential antibody binding observed. Treatment with sialidase did, however, completely abolish the observed differential binding to the C1 and C2 regions and to the N-terminal flanking region of the V3 loop. The loss of differential binding was due to a sialidase-mediated increase in the binding of antibodies to rgp120(−). Sialidase treatment had no effect on the binding of antibodies to rgp120(+). In a similar way, the difference in the observed binding to the V1/V2 loops and discontinuous epitopes was enhanced. This result shows that terminal sialic acid residues on rgp120(−) prevent the binding of a number of antibodies directed against the N-terminal half of the molecule. Similar effects have been observed with other antibodies (3). The modulation of antibody binding by terminal sialic acid is likely to be due to a charge effect. This indicates that the differential binding seen with antibodies directed against the C1 and C2 regions and with antibodies against the N-terminal flanking region of the V3 loop is due to a decrease in the negative charge of these regions after treatment with NB-DNJ. In this context it is important to note that the 9284 antibody, which exhibited differential binding to the N-terminal flanking region of the V3 loop, maps (amino acids 302 to 312) adjacent to a glycosylation site (amino acid 301) which has previously been found to contain a complex-type oligosaccharide which is likely to be sialylated (30).

As mentioned above, the presence or absence of either ter-

minal glucose or sialic acid did not explain the differential binding seen with antibodies directed against the V1/V2 loops and discontinuous epitopes. This indicated that arresting N-glycan processing at the stage of glucosylated high-mannose-type oligosaccharides had an effect on the conformation of regions recognized by these antibodies. Glycosylation has previously been found to govern the binding of antibodies to the V1/V2 loops (15, 17, 57), and the glycosylation of one particular glycosylation site in the center of the V1/V2 loops seems necessary for the formation of complex antibody epitopes (57). However, production of rgp120 in the presence of DMJ, a compound which allows the formation of only high-mannose-type or hybrid-type oligosaccharides, does not have any effect on the binding of three of the anti-V1/V2-loop antibodies used in our study (57). This suggests that the NB-DNJ-mediated retention of terminal glucose residues affects the conformation of the V1/V2 loops. The cause of the conformational change is unknown. It may result from the prevention of the interaction with molecular-folding chaperones, such as calnexin and calreticulin, in the endoplasmic reticulum, which recognize monoglucosylated precursor-type oligosaccharides (19, 47). Alternatively, it may simply be due to an effect on the type of N-glycan present at the highly conserved glycosylation site in the center of the V1/V2 loops, which was previously found to govern the binding of conformationally sensitive antibodies (57).

Structural heterogeneity based on antibody recognition has previously been observed within the V1/V2 loops, with conformation-sensitive antibodies recognizing only a fraction of the gp120 molecules present (57). The differential binding observed with antibodies directed against the V1/V2 loops in the presence or absence of NB-DNJ could therefore also result from an increase in the fraction of misfolded gp120 molecules, which are not recognized by the antibodies, in the presence of NB-DNJ. If this was the case, the lower levels of binding seen by BIAcore analysis would have resulted from a decrease in the amount of correctly folded gp120 molecules present on the chip. Kinetic analysis of BIAcore binding profiles is independent of the amount of protein on the chip. A decrease in the amount of correctly folded gp120 on the chip would therefore give rise to a decrease in the overall binding observed but have no effect on the kinetic analysis of the binding profile. Our analysis showed that the decrease in binding of antibodies to the V1/V2 loops correlated with a substantial decrease in the off-rates of the anti-V1/V2-loop antibodies (data not shown). This therefore suggests that the differential binding observed in this study is due to a decrease in antibody affinity, not to an increase in the fraction of misfolded molecules. The decrease in the level of saturation binding observed by ELISA is compatible with a decrease in the off-rates of the relevant antibodies. The suggestion that all rgp120(+) molecules are affected by the conformational change is further supported by the finding that all rgp120(+) molecules showed increased sensitivity to human thrombin compared with rgp120(−) molecules (Fig. 7).

The V1/V2 loops of gp120 have been suggested to be involved in HIV entry. Deletion of the V1/V2 loops severely impairs HIV infectivity and HIV-induced syncytium formation (58–60), and mutations within the V1/V2 loops may cause inhibition of HIV entry at the level of post-CD4 binding (14, 50). Mutations within the V1/V2 loops may also affect the proteolytic processing of gp160 (14) but do not, in most cases, affect CD4 binding (14, 41, 50, 59). Mutations within the V1/V2 loops therefore create viruses that are phenotypically very similar to those generated in the presence of NB-DNJ, which strongly suggests that changes in N-glycan composition affecting the conformation of the V1/V2 loops may be involved in the NB-DNJ-mediated impairment of HIV entry.

An NB-DNJ-mediated conformational change within the V3 loop of baculovirus-derived rgp120, as detected by the antibody ADP358, has previously been proposed to be involved in the inhibition of HIV infectivity by NB-DNJ (21). However, using CHO-cell-derived rgp120, as well as baculovirus-derived rgp120 (data not shown), we failed to see any differential binding with this antibody. A closer analysis of this discrepancy revealed that the ADP358 antibody exists in two different preparations, with one preparation binding with a sixfold-higher affinity than the other (data not shown). No differential binding to rgp120(−) and rgp120(+) could be found with the high-affinity preparation, whereas the differential binding previously described could be reproduced in the CHO-cell system with the low-affinity preparation (data not shown). The reason for the difference between the two preparations is unclear, as is the relevance of the differential binding seen with the low-affinity preparation. The fact that this antibody, along with other antibodies directed against the crown of the V3 loop, showed no significant differences in binding to the two types of rgp120 indicates that the conformation of this region is unaffected by changes in N-glycan composition.

As mentioned above, no differences in the binding to rgp120(−) and rgp120(+) of antibodies directed against the CD4-binding C4 region of gp120 were observed. This is in accordance with previous findings showing no effect of NB-DNJ treatment on the affinity of the interaction between rgp120 and soluble CD4 (12). In addition, rgp120-derived N-glycans can be enzymatically removed without affecting CD4 binding (10, 31), and in most cases, mutations affecting the conformation of the V1/V2 loops have no effect on CD4 binding (14, 26, 40, 50). Three antibodies (ADP3016/1.5e, ADP3017/2.1h, and ADP390/ICR39.13g) recognizing discontinuous epitopes on gp120 involving the CD4 binding site (5, 52) did, however, show differential binding to rgp120(−) and rgp120(+). This apparent discrepancy may be explained by an effect of NB-DNJ on other regions of gp120 that involves a change in the binding of the antibodies but not in the binding of gp120 to CD4 (52).

The finding that rgp120(+) is more sensitive to CHO-cell-derived proteases and human thrombin supports the hypothesis that production of rgp120 in the presence of NB-DNJ affects the overall properties of the molecule. DMJ, which affects the overall charge of rgp120 in the same way as NB-DNJ, was found to have no effect on the protease sensitivity of rgp120 (data not shown). This suggests that the NB-DNJ-mediated increase in protease sensitivity cannot be attributed to a mechanism whereby a decrease in negative charges of the N-terminal part of the molecule allows greater access to the proteases (as illustrated by the influence of sialic acid residues on antibody binding). Instead, the increased protease sensitivity may be explained by a conformational change affecting the V1/V2 loops or by the possible failure of glucosylated N-glycans to mask potential cleavage sites. Previously published data have shown a decrease in the sensitivity of rgp120(+) to human thrombin compared with that of rgp120(−) (42). The conclusion in that paper was based on a reduction in the appearance of the 70-kDa band, which, in addition to a band at 50 kDa, is normally seen after thrombin cleavage within the V3 loop of gp120 (49). The reduction in the appearance of the 70-kDa band could, however, also be explained by the disappearance of the 70-kDa species due to the proteolytic cleavage observed in Fig. 7. The production of rgp120 in the presence of either DMJ or swainsonine does not lead to an increase in its sensitivity to CHO-cell-derived proteases (data not shown), further

illustrating the specific correlation between an NB-DNJ-mediated presence of terminal glucose residues and an effect on the properties of this molecule.

In conclusion, this study demonstrated that generation of rgp120 in the presence of NB-DNJ affects the conformation of the V1/V2 loops. In addition, NB-DNJ has an effect on the overall charge of the molecule because of the lack of addition of sialic acid. Both effects may contribute to NB-DNJ-mediated inhibition of post-CD4-binding events necessary for HIV entry. However, DMJ, which also reduces the number of sialic acid residues on the protein, does not have any effect on antibody binding to the V1/V2 loops (57). Nor does DMJ affect the sensitivity of rgp120 to CHO-cell-derived proteases (data not shown) or have any effect on HIV infectivity and envelope functions (18, 39). This argues against a role for the reduction of the negative charge of gp120 in NB-DNJ-mediated inhibition of HIV entry. These findings, together with the finding that mutations within the V1/V2 loops create viruses that are phenotypically very similar to those produced in the presence of NB-DNJ (14, 26, 50), strongly suggest a role for conformational change affecting the exposure of the V1/V2 loops in NB-DNJ-mediated inhibition of HIV entry.

ACKNOWLEDGMENTS

We thank Searle/Monsanto for NB-DNJ, Anton van der Merwe for help and advice on BIAcore analysis, Ian Jones for helpful discussion, Khairina Tajul-Arifin for technical assistance, and Colin Beesley for photography.

P. B. Fischer is funded by a Carlsberg-Wellcome Traveling Research Fellowship and supported by E. Danielsens Fond, Leo Nielsens Legat, Jacob Madsens Fond, Beckett Fonden, Wedell-Wedellsborgs Fond, Hørslev-Fonden, L. F. Foghts Fond, and Krista and Viggo Petersens Fond. The antiviral program in the Glycobiology Institute is supported by the Searle/Monsanto Company. The HIV laboratory at the Sir William Dunn School of Pathology is funded by the Medical Research Council AIDS Directed Program.

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