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EFFECT OF *N*-(3-PHENYL-2-PROPENYL)-1-DEOXYNOJIRIMYCIN ON THE LECTIN BINDING TO HIV-1 GLYCOPROTEINS

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SUMMARY: The effect of *N*-(3-phenyl-2-propenyl)-1-deoxynojirimycin (ppDNM) on the lectin binding to HIV-1 glycoprotein was analyzed by using biotinylated lectins of various sugar specificities as probes. ppDNM potentially inhibited HIV-1-induced syncytium formation and viral infectivity of HIV-1 without cytotoxicity. The lectin binding assay showed that ppDNM treatment reduced Con A binding to gp120 of HIV-1.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), infects and destroys cells bearing CD4 molecule on the surface (1-3). The env gene of HIV-1 encodes an intracellular precursor glycoprotein, gp160, that is normally processed in infected cells by proteolytic cleavage to form gp120, the external viral glycoprotein, and gp41, the viral transmembrane glycoprotein. The interaction between gp120

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and CD4 molecule is required to initiate the infectious cycle (4,5). Gp41 anchors gp120 in the viral envelope and a hydrophobic domain at the amino terminus of gp41 is suggested to be responsible for the membrane fusion events involved in the HIV infection and syncytium formation (6,7). Gp120 is much heavily glycosylated and removal of the carbohydrate chains from gp120 by enzymatic treatment results in a significant reduction of the CD4-binding activity (8). Various N-linked glycoprotein processing inhibitors reduce the HIV-1 infectivity and block the HIV-1-induced syncytium formation and cytopathicity (9-11). Thus, the carbohydrate moieties of HIV-1 envelope glycoproteins appear to be closely associated with the biological activity of the glycoproteins. Analysis of the effect of N-linked oligosaccharides processing inhibitors may be essential in elucidating the role of glycosylation in HIV-1 replication and possibly lead to the development of some anti-HIV agents. The major glycosylation pathway utilized by the viral and cell membrane glycoproteins begins with the transfer of the precursor oligosaccharide Glc₃Man₉ (GlcNAc)₂ from the carrier lipid, dolichol phosphate, to the nascent polypeptide chain of the glycoprotein. The first step of such processing is initiated in the endoplasmic reticulum by the removal of three glucose residues by glucosidase I and II. Subsequently manosidase I in the Golgi complex completes the removal of four mannose residues, followed by the addition of N-acetylglucosamine, the removal of two more mannose residues by mannosidase II, and the addition of peripheral sugar residues such as galactose, N-acetylglucosamine, and sialic acid by specific glycosyltransferases, to form the complex oligosaccharide moieties of the glycoprotein molecule. 1-deoxynojirimycin (DNM), an inhibitor of the trimming enzymes, has been shown to inhibit glucosidase I activity in the endoplasmic reticulum. In our previous study, novel DNM derivatives were found to inhibit HIV-1-induced syncytium formation and to reduce the infectious HIV-1 yields from continuously infected cell lines at lower concentrations than DNM (Shimizu, unpublished data).

Recently, lectin binding assay was developed to analyze carbohydrate structures on gp120 by using carbohydrate-specific lectins (12,13). We applied this technique to study the effect of the DNM derivative on carbohydrate structures on gp120 and gp41 of HIV-1.

MATERIALS AND METHODS

Virus and cells: For syncytium formation inhibition assay, MOLT-4 cells continuously infected with HTLV-III_B (MOLT-4/HTLV-III_B) (2) and uninfected MOLT-4 cells (14) were used. Cell-free supernatants from TALL-1 cells (15) continuously infected with LAV (TALL-1/LAV) (1) were used as a source for lectin binding assay. MT-4 cells (16) were used to titrate infectious HIV. The cells were cultured at 37 C in RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI1640/10% FCS).

Chemicals: A DNM derivative, *N*-(3-phenyl-2-propenyl)-1-deoxynojirimycin (ppDNM), was used as a glucosidase inhibitor. This compound was synthesized from DNM (17) and the purity was confirmed by the 400 MHz NMR spectra being $\geq 98\%$. The structures of DNM and ppDNM are shown in Fig. 1. Biotinylated lectins and avidin-biotinylated alkaline phosphatase (ABC-AP) kits were purchased from Vector Laboratories (Burlingame, CA). *Allomyrina dichotoma* lectin (Allo A) was obtained from Cosmo Bio (Tokyo). *Gerardia savaglia* lectin was a gift of Dr. W. E. G. Müller, Institut für Physiologische Chemie, Federal Republic of Germany (18). Allo A and *Gerardia savaglia* lectin were labeled with *N*-hydroxysuccinimidobiotin (Pierce Chemical Co., Rockford, IL) by the method of Hofmann et al (19).

Lectin binding assay: HIV in the cell-free supernatant from TALL-1/LAV was purified by a cycle of pelleting, resuspension and sucrose density gradient centrifugation. The virus was solubilized by adding Nonidet P-40 (NP-40) to a final concentration of 5%. The viral antigens were separated by sodium dodecyl

Fig. 1. Structures of DNM and ppDNM. (1) DNM. (2) ppDNM.

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to Immobilon filters (Millipore Ltd., Bedford, MA) by the method of Towbin et al (20). After electrotransfer, the filters were cut and taken for immunodetection with a human serum positive of anti-HIV antibody or mouse monoclonal antibodies against gp120 and gp41 (Epitope Inc., Beaverton, OR), or with biotinylated lectins. For lectin binding assay, the filters were soaked in 0.01 M phosphate buffered saline (PBS, pH 7.4) containing 2% Tween-20 (PBS/2% Tween-20) at room temperature for an hour. The filters were washed in PBS containing 0.05% Tween-20 (PBS/0.05% Tween-20) and incubated at room temperature for an hour with biotinylated lectins. After further washing in PBS/0.05% Tween-20, lectins bound on the filters were stained with ABC-AP kits following the manufacturer's instructions. For immunodetection with a human serum positive of anti-HIV antibody or mouse monoclonal antibodies against gp120 and gp41, the filters were soaked in PBS containing 2% fetal calf serum at room temperature for an hour after electrotransfer. The filters were washed in PBS/0.05% Tween-20 and incubated at room temperature for an hour with a human serum positive of anti-HIV antibody or mouse monoclonal antibodies against gp120 and gp41. After washing, the filters were incubated at room temperature for an hour with biotinylated goat anti-human IgG or goat anti-mouse IgG. After further washing, the filters were stained with ABC-AP kits as described above.

Densitometrical analysis of the bands stained in lectin binding assay and immunodetection were performed by an API electrophoresis image analyzer system TIAS (API Japan, Kanagawa, Japan).

Syncytium formation inhibition assay: MOLT-4/HTLV-III_B cells were precultured overnight at 37 °C in RPMI 1640/10% FCS containing ppDNM of various concentrations. MOLT-4 and pretreated MOLT-4/HTLV-III_B cells were mixed in a ratio of 1:1 (final cell density: 5×10^5 cells/ml). Then the mixed cell suspension was cultured for 20 hr with the same dose of ppDNM as in the pretreatment. Viable cell number was counted by the trypan blue dye exclusion method and the fusion index (FI) was calculated as follows (21): $FI = (\text{cell number in control MOLT-4 well}) / (\text{cell number in test well}) - 1.0$. The FI of the control (MOLT-4 and MOLT-4/HTLV-III_B cells alone) was expressed as zero. Next, the percent inhibition of cell fusion at each concentration of ppDNM was calculated. Assays were carried out in triplicate.

Assay for infectious virus yield: MOLT-4/HTLV-III_B cells were washed five times with RPMI1640 and cultured for 4 days in RPMI1640/10% FCS with or without ppDNM. The infectious virus titer of the cell-free supernatant was measured with MT-4 cells. 6×10^5 MT-4 cells were seeded in each well of a 96-well microplate, mixed with each of serial 10-fold dilutions of the supernatant and cultured for 6 days. Then, the cytopathic effects induced by HIV-1 were observed by light microscopy and the 50% tissue culture infective dose per ml (TCID₅₀/ml) was calculated.

Cytotoxicity: Quantitative data on the effect of ppDNM on noninfected MOLT-4 cells (cytotoxicity) were obtained by comparing their growth rates to those of control cells not exposed to the drug. MOLT-4 cells were cultured for 6 days in RPMI1640/10% FCS containing ppDNM at various concentrations and then the viable cells were counted by the trypan blue dye exclusion method.

RESULTS

Lectin Binding Assay

As shown in Fig. 2, the gp120 band was densely stained with concanavalin A (Con A, lanes 1 and 2) and *Gerardia savaglia* lectin (lanes 10 and 11), and faintly stained with wheat germ agglutinin (WGA, lane 4). No staining of the gp120 band was found with *Ricinus communis* agglutinin I (RCA I, lane 3), peanut agglutinin (PNA, lane 5), *Ulex europaeus* agglutinin I (UEA I, lane 6), *Dolichos biflorus* agglutinin (DBA, lane 7), Soybean agglutinin (SBA, lane 8) or

Fig. 2. Lectin blot assay of HIV-1 glycoproteins. Probes: (1) 2 μ g/ml Con A, (2) 0.2 μ g/ml Con A, (3) 20 μ g/ml RCA I, (4) 20 μ g/ml WGA, (5) 20 μ g/ml PNA, (6) 20 μ g/ml UEA I, (7) 20 μ g/ml DBA, (8) 20 μ g/ml SBA, (9) a human serum positive of anti-HIV-1 antibody, (10) 20 μ g/ml *Gerardia savaglia* lectin, (11) 2 μ g/ml *Gerardia savaglia* lectin, (12) 20 μ g/ml Allo A, (13) 2 μ g/ml Allo A, (14) a human serum positive of anti-HIV-1 antibody.

Allo A (lanes 12 and 13). Gp41 band was densely stained with RCA I (lane 3) and Allo A (lanes 12 and 13), but not with the other lectins.

To observe the effect of ppDNM on lectin binding to gp120 and gp41, the supernatants from MOLT-4/HTLV-III_B cells cultured for 2 days in PMI1640/10% FCS with or without ppDNM were collected and tested by lectin binding assay using 2 μ g/ml Con a or 20 μ g/ml RCA I. Table I summarizes the results of densitometrical analyses of gp120 bands stained in lectin binding assay and immunodetection in Fig. 3. The ppDNM treatment reduced remarkably the Con A binding to gp120 without reduction of the amount of gp120 (Fig. 3 and Table I). Synthesis of gp120 in the presence of ppDNM resulted in an increased molecular mass (Fig. 3-B). In contrast, the ppDNM treatment reduced the amount of gp41 in the culture supernatant but did not affect the molecular mass of gp41, as shown in Fig. 4-B. The density of the band of gp41 stained in lectin binding assay using RCA I was also reduced (Fig. 4-A).

Fig. 3. Effect of ppDNM on Con A binding to HIV-1 gp120. (A) Con A staining of HIV-1 gp120. Virus particles were obtained from TALL-1 cells infected with LAV after treatment with ppDNM at the concentration of 100 μ g/ml (lane 1), 20 μ g/ml (lane 2), or 0 μ g/ml (lane 3). (B) Immunostaining of HIV-1 gp120 with a mouse monoclonal antibody against gp120. Virus particles were obtained from the same cultures as in (A).

Table I. Densitometrical analysis of the lectin binding assay

Probe	ppDNM treatment (concentration: $\mu\text{g/ml}$)	Binding unit ¹	% Binding ²
Con A	100	258	21
	20	844	67
	0	1253	100
anti-gp120 MoAb	100	352	113
	20	476	153
	0	312	100

¹Binding units were determined by densitometric measurement of the gel shown in Fig. 3.

²Binding units without drug were 100% control.

Fig. 4. Effect of ppDNM on RCA I binding to HIV-1 gp41. (A) RCA I staining of HIV-1 gp41. (B) Immunostaining of HIV-1 gp41 with a mouse monoclonal antibody against gp41. Virus particles were obtained from the same cultures as in Fig. 3.

Table II. Effect of ppDNM on HIV-1-induced syncytium formation

Experiment	Concentration (µg/ml)					
	10	5	2.5	1.3	0.63	0
1	0.43	0.55	1.01	1.38	1.47	1.75
2	0.30	0.59	0.89	2.04	1.92	2.68
3	0.10	0.51	0.56	1.00	1.21	1.56
mean	0.28	0.55	0.82	1.47	1.53	2.00
% of inhibition	86	73	59	27	24	–

Table III. Effect of ppDNM on the infectious virus yield from an HIV-1-infected culture

Concentration (µg/ml)	Titer (TCID ₅₀ /ml)
200	10 ^{2.5}
100	10 ^{3.7}
50	10 ^{3.7}
10	10 ^{4.7}
0	10 ^{6.1}

Table IV. Cytotoxicity of ppDNM

Concentration (µg/ml)	Viable cell number (cells/ml)
800	Toxic
400	1.29 × 10 ⁶
200	2.01 × 10 ⁶
100	1.97 × 10 ⁶
No drug	1.96 × 10 ⁶

Syncytium Formation Inhibition Assay

As shown in Table II, ppDNM remarkably inhibited the syncytium formation after coculture of uninfected MOLT-4 and MOLT-4/HTLV-III_B cells. In the control cultures without ppDNM, the syncytium formation became apparent 20 hr after coculture.

Infectious Virus Yield

The ppDNM treatment resulted in the reduction of infectious virus yield from MOLT-4/HTLV-III_B, as shown in Table III.

Cytotoxicity

As shown in Table IV, the treatment with 100 or 200 µg/ml ppDNM did not show any cytotoxicity on MOLT-4 cells. 400 µg/ml ppDNM slightly decreased the viable cell number and 800 µg/ml of this drug was cytotoxic on MOLT-4 cells.

DISCUSSION

Previous studies suggested that the full pathogenic potential of HIV-1 might only be realized in vitro if viral envelope proteins were N-glycosylated and the carbohydrate moieties were subsequently processed correctly. The envelope glycoprotein gp120 of HIV-1 is highly glycosylated and approximately 50% of the molecular mass of gp120 appear to be N-linked carbohydrates. Previous studies indicated that mature gp120 is a differentially glycosylated molecule containing high-mannose as well as hybrid and complex oligosaccharide structures (8,9).

Recently, an enzyme-linked lectin binding assay using biotinylated lectins as probes was developed to characterize HIV-1 envelope glycoproteins (12,13). Our preliminary study indicated that 2% Tween-20 in PBS and 0.05% Tween-20 in PBS are appropriate for the blocking buffer and the incubation buffer, respectively, in order to obtain a clear band of gp120 stained with Con A (data not shown). Under such conditions, the results of our lectin binding assay using Con A, SBA, DBA, UEA I and PNA showed good agreement with the result of Hammar et al (12). In our lectin binding assay, gp120 band was densely stained with Con A and *Gerardia* lectin, and faintly with WGA. No staining of gp120 band was found with RCA I, PNA, UEA I, DBA, or Allo A. Con A binding could

be due to oligosaccharides of either a high mannose-type or a biantennary complex-type. In the study by Hansen et al. (13), pretreatment of gp120 with endoglucosidase H, which selectively cleaves high mannose-type oligosaccharides from the glycoprotein, resulted in the loss of its Con A staining. The data indicated a substantial content of high mannose-type oligosaccharides in gp120. Binding of gp120 with WGA indicated that gp120 contains terminal N-acetylglucosamine. Negative binding of gp120 with SBA, DBA, PNA and UEA-I indicated that O-linked glycosylation and α -fucosylation might represent only a minor portion of the total glycosylation of gp120, if present. In our study, the gp41 band was densely stained with RCA I and Allo A, but not with the other lectins. This suggests that a major portion of oligosaccharides of gp41 is a complex-type or a hybrid-type oligosaccharides containing terminal β -galactose, in agreement with the study by Pal et al. (22) in which the carbohydrate residues of gp41 were largely resistant to endoglucosidase H digestion.

Several inhibitors of specific reactions in the N-linked glycoprotein processing pathway have recently become available, including castanospermine (CAST) and DNM as inhibitors of glucosidase I, bromocondritolin (BCU) as an inhibitor of glucosidase II, 1-deoxymannojirimycin (DMM) as an inhibitor of mannosidase I and swainsonine (SW) as an inhibitor of mannosidase II. In the study by Gruters et al. (19), DNM and CAST markedly decreased the infectious virus yield and inhibition of syncytium formation, but DMM did not. In the study by Montefiori et al. (23), however, DNM, CAST, and DMM attenuated HIV-1 infectivity and blocked HIV-1-induced syncytium formation and cytopathicity, whereas BCU and SW failed to elicit such effects. They concluded that the carbohydrate moieties of HIV-1 envelope proteins must be processed by actions including the activities of glycosidase I and mannosidase I to elicit a full pathogenic response. In contrast, the processing activities of glucosidase II or mannosidase II appear to be not essential. As shown in Figs. 3 and 4, the ppDNM treatment induced marked changes in the lectin binding to the glycoproteins. To determine the carbohydrate structures of some glycoprotein, oligosaccharides have to be released from the peptide and fractionated by chromatography. However, it needs a special technique and is time-consuming work. It is difficult to analyze the modification of the oligosaccharides in HIV-1 glycoproteins by the inhibitors in the N-linked glycoprotein processing pathway.

In previous studies using inhibitors in the N-linked oligosaccharide processing pathway, only a slight alteration of the electrophoretic mobility of HIV-1 gp120 was observed (11,22-24). In our present study, synthesis of gp120 in

the presence of ppDNM resulted in an increased molecular mass. In addition to this, remarkable reduction of lectin binding to gp120 and gp41 could suggest a change in the oligosaccharide structures in the glycoproteins by the treatment. The results suggest that the inhibitors are working on the oligosaccharide structures in the glycoproteins. Thus, the modification of the oligosaccharides in HIV-1 glycoproteins by ppDNM shown in lectin binding assay could be closely associated with the remarkable inhibition of HIV-1-induced syncytium formation and marked reduction of the infectious virus yield from an HIV-1-infected culture. Lectin binding assay using lectins with various sugar specificities is a convenient and useful method to analyze the oligosaccharide structures of HIV-1 glycoproteins and the effect of the inhibitors in the N-linked glycoprotein-processing pathway.

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