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The α-Glucosidase Inhibitor 1-Deoxynojirimycin Blocks Human Immunodeficiency Virus Envelope Glycoprotein-Mediated Membrane Fusion at the CXCR4 Binding Step

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ABSTRACT

1-Deoxynojirimycin (DNM) is a saccharide decoy that inhibits cellular α-glucosidase I-II activity. Treatment by DNM of human immunodeficiency virus (HIV)-infected lymphocyte cultures inhibits virus spread. The functional properties of the membrane-associated Env glycoprotein (Env) modified in the presence of DNM remain unclear because previous reports on this subject have essentially used recombinant soluble Envs whose properties differ notably from those of Env anchored on the surface of the virus. To model virus-associated Env synthesized in the presence of DNM, native Env was expressed at the surface of mammalian cells treated with DNM. As expected, its glycosylation pattern was altered in the presence of the inhibitor. Env was found able to bind CD4, whereas its ability to induce membrane fusion was abolished. The immunoreactivity of regions involved in interactions of Env with CXCR4 (V1, V2, C2, and V3) was modified and Env displayed altered interaction with this coreceptor. These results are consistent with the inhibition by DNM of virus entry at the Env/coreceptor interaction step. Finally, preliminary data indicate that suboptimal concentrations of DNM and natural or synthetic CXCR4 ligands used in combination potently inhibit the Env-mediated membrane fusion process. Altogether, our results suggest that DNM and its analogs deserve further investigation as anti-HIV agents in combination with experimental compounds targeting CXCR4 to inhibit each partner of this crucial step of HIV entry.

The mature human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein (Env) originates from the intracellular cleavage of precursor gp160 into outermembrane gp120 and transmembrane gp41 subunits. Both species remain noncovalently linked within oligomeric structures during routing to the cell surface (Einfeld, 1996). After virus budding, gp120 exposed on the viral surface mediates HIV binding to CD4+ lymphocytes through interaction with cell surface antigen CD4. Gp120 domains, and in particular the variable V1, V2, and V3 loops, interact then with virus coreceptors, including chemokine receptors (Wyatt and Sodroski, 1998). In the context of the cell surface, which displays catalytic activities, including disulfide isomerase (Fenouillet et al., 2001), these events induce conformation changes of Env resulting in unmasking of the gp41 fusion peptide and triggering of HIV/cell fusion (Wyatt and Sodroski, 1998).

Env glycans, which are composed of half oligomannose and half complex species, represent about 50% of the molecular mass of the glycoprotein. About 25 structures are distributed on gp120 and a cluster of three to five moieties is present on gp41 (Fenouillet et al., 1994). A number of studies have demonstrated that the sugar padding of Env enables its folding: mutation of clusters of glycosylation sites as well as use of glycosylation inhibitors alter the fusogenicity of Env. In contrast, glycans of mature folded Env expressed on the virus surface are not critical for its functions (for review, see Ratner, 1992; Fenouillet et al., 1994).

Deoxynojirimycin (DNM) and analogs are saccharide decoys that inhibit the cellular α-glucosidase I-II activity. Accordingly, they block at the level of the endoplasmic reticulum the trimming of the glycan precursor (i.e., the cleavage of the three Glc residues from the $Glc_3Man_9GlcNAc_2$ precursor glycan). Glycoproteins presenting large, abnormal, glucosylated oligomannosidic moieties are therefore produced (Elbein, 1987). Glucosidase inhibitors display a potent antiviral

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ABBREVIATIONS: HIV, human immunodeficiency virus; Env, envelope glycoprotein; DNM, 1-deoxynojirimycin; Glc, glucose; Ab, antibody; aa, amino acid; mAb, monoclonal antibody; rVV, recombinant vaccinia virus vector; SDF, stromal cell-derived factor; PBS, phosphate-buffered saline; PBSC, phosphate-buffered saline, 2% casein, 0.05% NaN_3; D7324; anti-gp 120 C terminus antibody; CEM, human lymphoid $CD4^+$ cell; SPC_3, V_3-derived multiple antigen peptide; ELISA, enzyme-linked immunosorbent assay; C, conserved domain; V, variable domain.

effect on the Lai strain of HIV (Gruters et al., 1987; for review, see Ratner, 1992; Fenouillet et al., 1994). This result and the fact that they induce essentially only gastrointestinal side effects in humans prompted their evaluation in anti-HIV clinical trials (ACTG100; Tierney et al., 1995).

To investigate their mode of action against HIV, several investigations have undertaken to study which Env properties were altered by this class of saccharide decoys, but their results proved controversial. Some studies have reported that Env produced in the presence of glucosidase inhibitors does not bind CD4, whereas others have suggested that the glycoprotein was unable to mediate membrane fusion (Dedera et al., 1990; Fenouillet and Gluckman, 1991; Jones and Jacob, 1991; Ratner et al., 1991; Fischer et al., 1995). Immunoreactivity changes were also observed, which suggests that Env conformation was modified when synthesis occurred in the presence of DNM or analogs (Fenouillet and Gluckman, 1991; Jones and Jacob, 1991; Fischer et al., 1996; Papandréou and Fenouillet, 1998). However, the relevance of these conclusions is questionable because the results were essentially obtained in experiments using recombinant soluble Envs whose conformation differs from that of virus-associated oligomeric Env (Moore et al., 1994; Burton, 1997). We therefore undertook to examine the effects of DNM on the properties of an oligomeric membrane-associated Env.

Experimental Procedures

Reagents

Antibodies. Abs (species as indicated; names of contributors indicated under *Acknowledgments*) recognize the following Env_{HXB2} sequences: SR2 (mouse) is directed against amino acids (aa) 31 to 50; 4A7C6 (mouse): aa 81 to 90; 187.2.1 (mouse): aa 101 to 120; SR1 (mouse): aa 162 to 171; 11/4C (rat): aa 162 to 170; 11/68b (rat): V1+V2+C4; CRA3 (mouse): V2+C1; CRA4 (mouse): V2; 213.1 (mouse): aa 252 to 261; IIIB-V3–21 (mouse): aa 294 to 299; IIIB-V3–13 (mouse): aa 309 to 317; 5F7 (mouse): aa 308 to 322; 0.5β (mouse): aa 311 to 324; IIIB-V3–01 (mouse): aa 320 to 328; 60.1.1 (mouse): aa 361 to 381; 8/19b (rat): C1+C3; ICR38.1a (rat): aa 429 to 438; 221 (mouse): aa 471 to 490; 2.1H (human): discontinuous epitope; ICR39.13 g (rat): discontinuous epitope; and monoclonal Ab (mAb) 9305 (mouse): aa 308 to 322 (DuPont de Nemours, Dreieich, Germany). Polyclonal Ab D7324 (sheep): APTKAKRRVVQREKR (C terminus of gp120; Aalto, Dublin, Ireland). Abs directed against human, rat, and mouse IgGs were obtained in goat (Sigma Chemical, St. Louis, MO).

Recombinant Virus Vectors (rVVs). VV9-1 is an rVV encoding native fusogenic HIV_{Lai} Env; VV1163 encodes a soluble form of $gp160_{Lai}$, which does not remain associated with the cell surface (Kieny et al., 1988). $VVEnv_{BH10}$ encodes native fusogenic HIV_{BH10} Env (a generous gift from M. Mackett). VBD3 encodes the native fusogenic envelope of the dual-tropic primary HIV-1 isolate (89.6) that uses both CXCR4 and CKR-5 as entry cofactors (a generous gift from R. Collman and R. Doms). vPE12 encodes uncleaved oligomeric soluble $gp140_{BH8}$ (generous gift from P. Earl and B. Moss).

Inhibitors and Ligands. DNM was donated by R. Gruters and H. Ploegh. Purified recombinant soluble CD4 was a gift from I. M. Jones. SDF1-α was obtained from Peprotech (London, UK). SPC_3 was a gift from J. M. Sabatier.

Cell Infections

Human lymphoid $CD4^+$ cells (CEM; 10^6 cells/ml) and $CD4^-$ baby hamster kidney cells (BHK-21; 10^6 cells/ml) were cultivated as described in Fenouillet et al. (2001).

Env Expression. CEM cells were cultivated for 4 days in the presence or absence of 3 mM DNM and subsequently infected in 24-well plates in the presence or absence of DNM by using VV9-1 (3–5 plaque-forming units/cell); cell aggregates and cell-to-cell fusion events (syncytia) were scored at 24 h postinfection (Barbouche et al., 2000a; Fenouillet et al., 2001). BHK-21 cells were similarly processed and infected with rVVs (5–10 plaque-forming units/cell) for 24 h (Fenouillet et al., 2001).

Determination of IC_{50} Value for DNM, SDF1-α, and SPC3. CEM cells were treated with DNM (2–0.2 mM) for 3 days before infection by using VV9-1, as described above. Alternatively, cells were infected in control conditions and then treated with either SPC3 (10–0.3 μM) or SDF1 (100–3 nM) at 4 h postinfection. Cell-to-cell fusions were assessed at 24 h postinfection as described previously (Barbouche et al., 2000a; Fenouillet et al., 2001). In some experiments, cells were treated with DNM for 3 days before infection and then with either SPC3 or SDF1 at 4 h postinfection; fusions were assessed at 24 h postinfection.

Labeling Procedures. Anti-species Abs (7 μCi/μg) and SDF1-α (150 μCi/μg) were labeled using lactoperoxidase as described in Barbouche et al. (2000a) and Fenouillet et al. (2001), respectively. Soluble CD4 was labeled using iodogen (30 μCi/μg) as described in Fenouillet et al. (2001). After labeling, antigens were purified by Sepharose G_{25} chromatography.

Characterization of gp120 Glycosylation

Gp120 secreted by VV9-1-infected cells was treated overnight at 37°C with either endoglycosidase H (Endo H; Roche Molecular Biochemicals, Mannheim, Germany) or *Clostridium perfringens* sialidase (Sigma Chemical) (Fenouillet et al., 1997). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (10%). After blotting, nitrocellulose filters were saturated with 2% casein and incubated for 2 h with ^{125}I-D7324 (2×10^7 cpm) in PBS, 2% casein, 0.05% NaN_3 (PBSC), 0.5% Tween 20. Strips were scanned (PhosphoImager; Bio-Rad, Les Ullis, France).

Quantification of Membrane and Soluble Env

The amount of Env expressed at the cell surface was semiquantified, as reported (Fenouillet et al., 1997, 2001) with modifications: cells (10^6) expressing surface Env (VV9-1 or $VVEnv_{BH10}$ infection) were incubated for 90 min with a pool of HIV^+ or HIV^- human sera (1/1,500–1/150,000 dilution in PBSC). After washing, cells were incubated for 90 min with ^{125}I-anti-human IgG Abs from goat (10^6 cpm) in PBSC, 3% goat serum. After three washes, cell-bound radioactivity was assessed. Alternatively, cells expressing Env were incubated for 2 h at 25°C with ^{125}I-D7324 (5×10^5 cpm) and unlabeled D7324 (0.01–1 μg/100 μl of PBSC supplemented with 0.5% sheep serum). After three washes, cell-bound radioactivity was assessed. The amounts of secreted gp120 were determined using a dot-blot method (Fenouillet and Gluckman, 1991): cell supernatants diluted in PBS, 0.5% SDS, were blotted onto nitrocellulose filters. After blocking, strips were incubated with a pool of HIV^+ sera (1/500) in PBSC, 0.5% Tween 20 for 2 h and with ^{125}I-anti-human IgG Abs (2×10^7 cpm) for 90 min. Strips were then scanned for quantification (PhosphoImager). Uninfected cell supernatants were used to determine the background signal. Similar experiments were performed using ^{125}I-D7324. Secreted gp120 was also semiquantified in D7324-coated microwell plates (Fenouillet et al., 1997) by using a pool of HIV^+ or HIV^- sera (1/1000) and ^{125}I-anti-IgG Abs.

CD4 Binding to Env

BHK-21 cells (1, 2, or 3×10^6) expressing similar amounts (see *Results*) of surface Env were washed in PBS and incubated for 2 h at 37°C with ^{125}I-CD4 (3×10^5 cpm/100 μl) in PBSC (Barbouche et al., 2000a; Fenouillet et al., 2001). Cell-bound radioactivity was evaluated. Uninfected cells or cells infected with VV1163 were similarly processed to determine the background signal. Specificity of ^{125}I-CD4

binding was also assessed by preincubation of VV9-1-infected cells with soluble CD4 (1 μg/10^6 cells) for 15 min before addition of ^{125}I-CD4. The ability of gp120 released from VV9-1-infected cells to bind ^{125}I-CD4 was also tested: Env (20, 40, and 80 ng) was incubated in D7324-coated wells and then with ^{125}I-CD4 (3×10^5 cpm/100 μl of PBSC) (Fenouillet et al., 1997). The background signal was determined using unlabeled CD4 (1 μg/100 μl).

SDF1-α Binding Inhibition

Living CEM cells were incubated for 2 h at 37°C in culture medium with 1) secreted gp120 (from VVEnv$_{BH10}$-infected cell supernatant), 2) uncleaved oligomeric soluble gp$^{140}_{BH8}$ (from vPE12B-infected cell supernatant), or 3) uncleaved monomeric soluble gp160$_{Lai}$ (from VV1163-infected cell supernatant). In these experiments, supernatants were concentrated 30 times by using the Ultrafree 15 system (Millipore Corporation, Bedford, MA). Cells were then treated with 0.1% NaN_3 for 10 min and further incubated with ^{125}I-SDF1-α for 1 h at 25°C in RPMI 1640 medium, 10 mM HEPES, 5% bovine serum albumin, and 0.1% NaN_3. Cell-associated and free radioactivity was separated using the dibutyl phthalate/bis(2-ethylhexyl)phthalate two-phase system. To evaluate nonspecific binding, cells were incubated with unlabeled SDF1-α (2×10^{-7} M) for 1 h at 20°C in the presence of NaN_3 in the buffer described above, followed by labeled SDF1-α for 1 h.

Ab Binding to Env

BHK-21 cells (10^6 cells) expressing similar amounts of surface Env$_{BH10}$ (see *Results*) were washed in PBS and incubated for 90 min at 25°C with various dilutions of Abs in PBSC. After one wash, cells were incubated for 90 min with ^{125}I-anti-IgG Abs (10^6 cpm/100 μl of PBSC, 3% goat serum). After three washes, cell-bound radioactivity was assessed. The background signal was determined by 1) similar processing of uninfected cells or of VV1163-infected cells; 2) omitting the incubation with the anti-Env Abs; or 3) addition of 30 μg/ml IgG from nonimmune animals of the corresponding species, instead of anti-Env Abs. Secreted gp120 was also tested after incubation in D7324-coated wells for 2 h at 25°C. After a wash, mAbs diluted in PBSC, 1% sheep serum were added for 2 h at 25°C. After one further wash, antigen-Ab complexes were incubated for 90 min with ^{125}I-anti-IgG Abs (10^6 cpm in PBSC, 3% goat serum, 1% sheep serum). After three washes, bound radioactivity was assessed. The background signal was determined by omitting the incubation with the anti-Env Abs or by the addition of 30 μg/ml Abs from nonimmune animals.

Susceptibility of V3 to Thrombin

BHK-21 cells (10^6) expressing cell surface Env were treated overnight at 37°C with bovine thrombin (0.05–1.5 U/100 μl; Roche Molecular Biochemicals) in culture medium, 0.1% NaN_3 (Papandréou and Fenouillet, 1998). Uninfected cells were similarly processed. After one wash, cells were incubated for 90 min at 25°C with either mAb 9305 or an irrelevant mouse mAb (1/100) in PBSC. After one more wash, incubation with ^{125}I-anti-IgG Abs (10^6 cpm) was performed for 90 min. Cell-bound radioactivity was assessed. Cells were incubated in parallel with a pool of either HIV$^+$ or HIV$^-$ sera (1/1500) and with ^{125}I-anti-human IgG Abs to evaluate the quantity of surface Env after thrombin treatment. Secreted gp120 was also studied (Papandréou and Fenouillet, 1998): gp120 was incubated in D7324-coated wells for 2 h at 25°C, followed by thrombin (0.2–200 mU/100 μl) overnight at 37°C. Either 9305 Ab (1/300) or a pool of HIV$^+$ sera (1/1,000) was then added for 90 min. An irrelevant mouse Ab and a pool of HIV$^-$ sera were used as controls. After incubation for 90 min with ^{125}I-anti-species Abs (10^6 cpm in PBSC, 3% goat serum, 1% sheep serum), radioactivity bound to wells was assessed.

Results

Glycosylation and Production of Env in Presence of DNM. To assess the effect of DNM on the glycosylation of cell surface Env, we examined at steady state the glycosidase sensitivity of secreted gp120, assuming that it was identical to that of its surface-associated counterpart. Endo H sensitivity parallels the amount of oligomannosidic structures and sialidase sensitivity correlates with the presence of sialic acid on complex glycans. Gp120 synthesized by BHK-21 cells in the presence (D+) and in the absence (D−) of DNM migrated as 140- and 120-kDa bands, respectively (Fig. 1). Endo H sensitivity of D+ gp120 was superior to that of D− gp120 because their molecular weights decreased after treatment by about 50 and 30%, respectively. This is consistent with the presence on D+ gp120 of high molecular weight-glucosylated oligomannose glycans. The molecular weight of both glycoproteins decreased after sialidase treatment by 10 to 15%. Thus, D+ gp120 displays complex glycans.

We then examined the amounts of Env expressed at the surface of, and released from, BHK-21 cells treated by DNM. Cell growth decreased by about 30% after a 4-day DNM treatment. In contrast, DNM did not alter the amount of Env expressed at the cell surface: samples containing a similar number of VVEnv-infected cells treated or not with DNM generated an equivalent signal when a pool of HIV$^+$ human sera was used to detect surface Env under both saturating (1/1,500–1/5,000) and nonsaturating (1/15,000–1/150,000) conditions (Fig. 2A). The lack of a detectable effect of DNM on Env production may be linked to the fact that its synthesis was carried out for only 1 day, whereas cell culture was performed for 4 days in the presence of DNM. D7324 recognizes Env irrespective of its glycosylation state (Fischer et al., 1996) and its capacity to bind to D+ Env and D− Env was found similar (Fig. 2B). Using these assays, we normalized thereafter the amounts of surface Env in D+ or D− cell samples.

Using D7324-coated wells and detection by HIV$^+$ sera, we determined that the amounts of gp120 recovered from D+ or

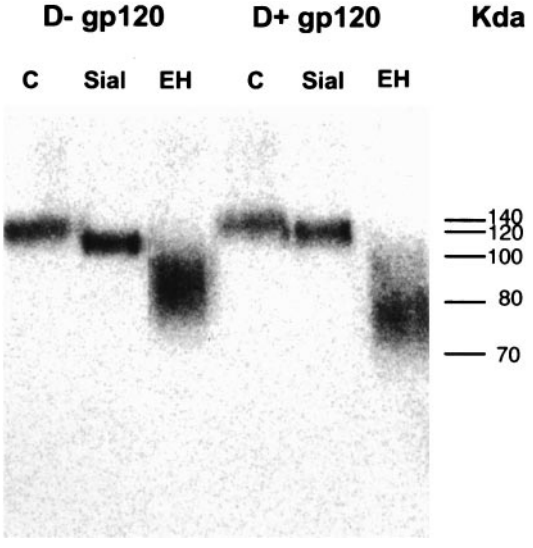

Fig. 1. Glycosylation pattern of Env. Gp120 was incubated with sialidase (Sial) or endoglycosidase H (EH), or mock-treated (C). After SDS-polyacrylamide gel electrophoresis analysis and blotting, gp120 was detected using ^{125}I-D7324 Ab.

D− cell supernatant were similar (about 1.5 $\mu g/10^6$ cells). Because ELISA allows conformation-dependent Ab binding, which may bias the estimation of the protein concentrations, we semiquantified gp120 shedding by using a dot blot assay performed under conditions that suppress such artifact (Fenouillet and Gluckman, 1991). A pool of HIV$^+$ sera and ^{125}I-labeled anti-species Abs or ^{125}I-D7324 was used for staining. We confirmed that glycosylation induced by DNM did not alter gp120 shedding. Semiquantitative Western blot analysis with either a pool of HIV$^+$ sera or ^{125}I-D7324 to detect Env led to similar conclusions (data not shown).

These results indicate that DNM treatment of BHK-21 cells modulates Env glycosylation but not the amount of cell surface Env and its subsequent shedding. Identical results were obtained using CEM cells. Because BHK-21 cells expressed about 2 to 3 μg of Env/10^6 cells, whereas CEM cells expressed only 0.5 to 1 $\mu g/10^6$ cells, we have used the BHK-21 cell expression system for the binding experiments described below.

Immunoreactivity of Env Expressed in Presence of DNM. To identify the regions of Env exhibiting immunoreactivity changes after DNM treatment, we analyzed the reactivity of cell surface Env with a panel of Abs. This was assessed after normalization of the quantity of surface Env in each cell sample series, as described above. Using an excess of anti-species ^{125}I-labeled Abs for detection, we observed that anti-Env Abs bound in a dose-dependent manner to cells expressing Env at their surface (data not shown). For each Ab, in the linear portion of the dose-effect response of the

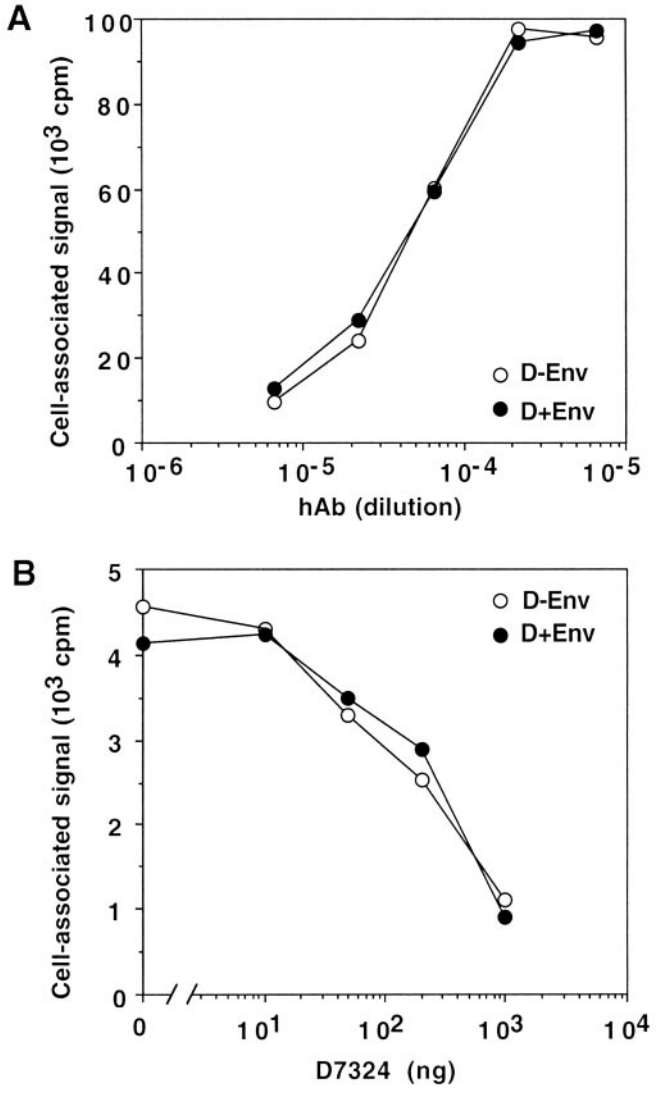

Fig. 2. Expression of Env at the cell surface. A, detection with a pool of human HIV+ sera. BHK-21 cells (10^6) expressing Env at their surface were incubated with various dilutions of a pool of HIV$^+$ sera and then with an anti-human ^{125}I-Abs. Cell-associated radioactivity was assessed and the background signal (incubation with a pool of HIV$^-$ sera) was subtracted [means of double determinations are presented; one experiment (n = 4) is shown]. B, detection with Ab D7324. Cell samples were also incubated with ^{125}I-D7324 and various concentrations of unlabeled D7324 [means of double determinations are presented; one experiment (n = 2) is shown].

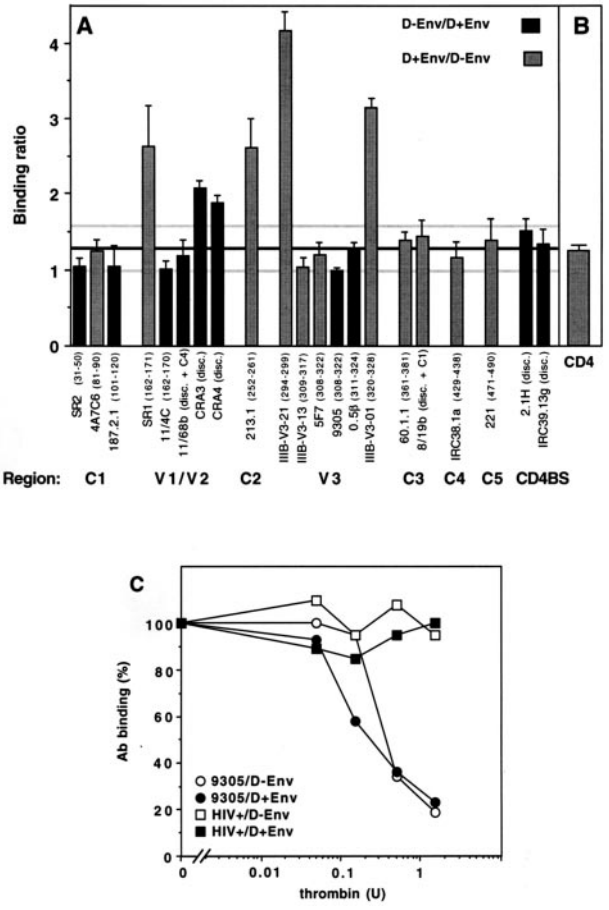

Fig. 3. Properties of membrane Env. A, recognition by mAbs. Cells (10^6) expressing equivalent amounts of Env were incubated with dilutions of Abs and then with anti-species ^{125}I-Abs. Cell-associated radioactivity was counted. For appropriate (see *Results*) Ab dilutions (n = 3–5; for each Ab dilution, duplicates were performed in at least two independent experiments), we determined the binding ratio of D− Env versus D+ Env when DNM treatment resulted in a decreased binding (black columns correspond to a mean of these ratios; standard deviation is indicated). In the opposite way, the binding ratio of D+ Env versus D− Env was determined (gray columns). (The black line corresponds to the mean of the ratios calculated as indicated above; gray line, S.D.) B, CD4 binding. Cells expressing Env were incubated with ^{125}I-CD4. Cell-bound radioactivity was assessed. The binding ratio of D+ Env versus D− Env was determined (n = 2 experiments; the mean is presented). C, thrombin cleavage within V3. Cells expressing Env were treated with various doses of thrombin. V3 cleavage was monitored using incubation with mAb 9305; a pool of HIV$^+$ sera was used to control the effect of thrombin digestion on the amount of Env associated to the cell surface. Ab binding was detected using anti-species ^{125}I-Abs. Cell-associated radioactivity was assessed (n = 2).

binding curve, we determined the binding ratio of D− Env versus D+ Env when DNM treatment resulted in decreased Ab binding (Fig. 3A). The inverse ratio was used in the opposite situation. Because the mean of the ratios + standard deviation = 1.6, we considered that binding of an Ab to D+ Env and D− Env was different when the ratio was above 1.6. (The absence of overlap between the binding ratios obtained with SR1, CRA3, CRA4, 213.1, IIIB-V3–21, IIIB-V3–01, and the other Abs renders statistical analysis irrelevant.) According to these criteria, epitopes situated in the V1/V2 region, at the base of V3 and in the C2 domain, exhibited immunoreactivity changes when synthesis occurred in the presence of DNM. The binding of two anti-V1/V2 Abs mapped to discontinuous epitopes showed a decreased binding to D+ gp120, whereas an Ab mapped to the N-terminal flanking region of V2, an Ab mapped to linear epitopes of C2, as well as two Abs raised against sequences located upstream and downstream from the crown of V3 bound preferentially D+ Env. The reactivity of Abs directed against the CD4 binding region, the apex of V3 and against the C1, C3, or C5 regions was not significantly modified.

The reactivity of soluble gp120 captured on D7324-coated wells was studied (as reported above, D7324 reacts similarly with D+ and D− Env). Most of the Abs were found to bind similarly secreted D+ and D− gp120 (Fig. 4A). Some mAbs (4A7C6, III-V3–21, and 60.1.1) reacted with membrane (Fig. 3A) but not with captured soluble gp120. This confirms that reactivity of soluble gp120 differs from that of membrane gp120.

V3 accessibility on D+ Env was also assessed through the analysis of its susceptibility to thrombin cleavage. Thrombin specifically cleaves gp120 within the GPGR//AF sequence present at the crown of V3 (Clements et al., 1991). Gp120 was either expressed at the cell surface (Fig. 3C) or adsorbed onto D7324-coated wells (Fig. 4C) before thrombin treatment. V3 cleavage was monitored by reduction of binding of mAb 9305 (recognizing a thrombin-sensitive epitope). Using both assays, mAb 9305 binding to Env decreased after thrombin treatment in a dose-dependent manner, as previously reported (Papandréou and Fenouillet, 1998). D+ and D− Env exhibited a similar sensitivity to thrombin. Reactivity of a pool of HIV$^+$ sera remained unchanged in both assays, irrespective of the dose of thrombin used for treatment: this indicates that the reduction in mAb 9305 binding after thrombin digestion resulted from the specific disruption of the epitope by the enzyme, and not from a decrease of the quantity of Env associated to cell membranes or to plastic wells. This result further shows that the accessibility of the crown of V3 on D+ Env was unchanged.

DNM, CD4 Binding, and Env-Mediated Membrane Fusion. The impact of DNM treatment on both the CD4 binding capacity and the fusogenicity of Env was assessed. D+ and D− Env were expressed on cell surface and fusion partners lymphoid cells were added (Barbouche et al., 2000a; Fenouillet et al., 2001). In the control situation and in cultures treated with DNM, cell aggregates appeared at 15 h after VV9-1 infection. Aggregates specifically result from the binding of cell surface-associated gp120 to membrane CD4 present on adjacent lympocytes, as demonstrated in much detail elsewhere (Barbouche et al., 2000a; Fenouillet et al., 2001). Controls were also performed here and, for instance, aggregates did not appear when Env was expressed using VV1163, which codes for a soluble form of gp160 possessing CD4 binding capacity.

When VV9-1-encoded Env was expressed at the cell surface without DNM, cell aggregates involving Env-expressing cells and fusion partners developed within a few hours into multinucleated giant cells (syncytia). Syncytia reflect the membrane fusion process resulting from interaction of fusogenic gp41 with the target cell surface in a context of Env interaction with various membrane HIV receptors and catalysts, as demonstrated in detail elsewhere (Barbouche et al., 2000a; Fenouillet et al., 2001). For instance, large aggregates, but no syncytium, could be observed when Env was expressed at the surface of cells infected in the absence of DNM by using VV1134, which codes for a nonfusogenic transmembrane envelope with CD4 binding capacity (Kieny et al., 1988). DNM treatment inhibited cell-cell fusion by more than 80% and the size of the syncytia was drastically decreased. Similar results were obtained using an rVV expressing the 89.6 envelope that derives from a primary HIV-1 isolate,

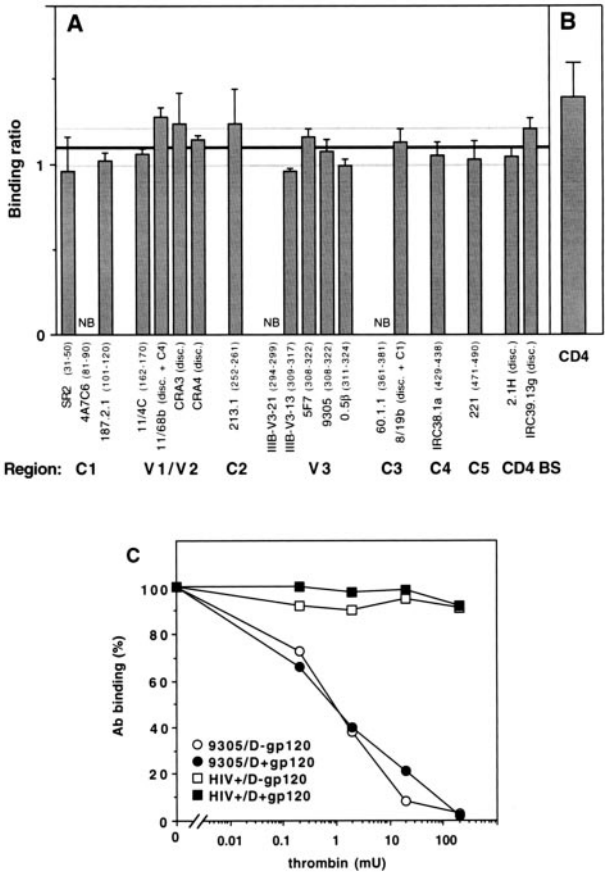

Fig. 4. Properties of secreted gp120. A, binding of mAbs. Equivalent amounts of secreted gp120 were incubated in D7324-coated wells and then with dilutions of mAbs. Bound mAbs were detected with anti-species ^{125}I-Abs. Bound radioactivity was assessed. For appropriate (see *Results*) Ab dilutions (n = 3–5; for each Ab dilution, duplicates were performed in at least two independent experiments), we determined the binding ratio of D− gp120 versus D+ gp120. NB, no binding. (The black line corresponds to the mean of the ratios calculated as indicated above; gray line, S.D.) B, CD4 binding. Cell supernatant was adsorbed on D7324-coated wells and then incubated with ^{125}I-CD4. Bound radioactivity was assessed. The binding ratio of D− gp120 versus D+ gp120 was determined (n = 3; the mean is presented). C, thrombin cleavage within V3. Secreted gp120 was captured on D7324-coated wells and further treated by various doses of thrombin; mAb 9305 or a pool of HIV$^+$ sera was used for detection (n = 2).

which can use CXCR4 as entry cofactor. These data indicate that cell surface D+ Env is able to bind surface CD4, but cannot mediate fusion.

The CD4 binding capacity of membrane Env was further investigated in a molecular binding assay. The binding ratio of D+ Env versus D− Env was calculated as described above for the assessment of Ab binding to cell surface Env. Soluble ^{125}I-CD4 bound both cell-associated D+ and D− Env (Fig. 3B). The binding specificity was characterized as follows: 1) binding of ^{125}I-CD4 to 1×10^6 Env-expressing cells was about 50% of that obtained with 2×10^6 cells; 2) binding to uninfected cells, or to VV1163-infected cells, represented about 5% of the binding to VV9-1 infected cells; and 3) binding was inhibited by about 70% by preincubation of Env-expressing cells with unlabeled CD4 (1 μg/10^6 cells). Secreted D+ gp120 was also found to bind CD4 (Fig. 4B).

DNM and Coreceptor Binding. The HIV Env-induced cell-to-cell fusion process as reported above is a stepwise process requiring interaction between gp120 and the CXCR4 coreceptor after CD4 binding. Env binding to CXCR4 can be specifically monitored through a decreased binding to lymphocyte surface of SDF1-α, the natural ligand of CXCR4. Indeed, the binding site of SDF1-α on CXCR4 partially encompasses that of gp120 (Su et al., 1999; Zhou and Tai, 1999). The binding specificity of ^{125}I-SDF1-α to the CEM cell surface and the background signal were assessed using a high concentration of unlabeled SDF1-α (Fig. 5). SDF1-CXCR4 binding inhibition with D− Env was similar to that previously reported (Su et al., 1999). D+ Env displayed a weak binding inhibition capacity. Previous reports (Doranz et al., 1999a) have demonstrated that uncleaved soluble Envs only weakly interact with CCR5 or CXCR4. In agreement with these data, soluble oligomeric gp140 and monomeric gp160 were found to very weakly inhibit SDF1 binding compared with secreted D− gp120.

Effect of Combination Treatments Based on DNM and CXCR4 Ligands. We studied the effect on cell-to-cell fusion of DNM associated with SDF1 and SPC3, two peptide compounds that efficiently block syncytium formation through interference with Env binding to the lymphocyte surface chemokine receptor (Lacey et al., 1997; Barbouche et al., 2000b); SDF1 is the natural ligand of CXCR4 and SPC3 is a synthetic multiple antigen peptide ($[GPGRAF]_8$-K_4-K_2-K-βA) derived from the apex of the V3 loop of Env_{Lai}. The concentration of DNM, SDF1, and SPC3, which individually inhibited by about 50% cell-to-cell fusion in our conditions, was in the range of 0.4 mM, 30 nM, and 3 μM, respectively (Fig. 6). Lymphocytes preincubated for 3 days with 0.4 mM DNM were then treated with either 3 μM SPC3 or 30 nM SDF1. These conditions were far below the cytotoxic concentrations but they resulted in a potent inhibition of cell-to-cell fusion. Of particular interest was the "DNM + SPC3" association, which essentially abrogated fusion.

Discussion

The studies focusing on the influence of DNM treatment on Env properties have essentially used recombinant soluble monomeric envelope glycoproteins. The relevance of the results can be questioned because the oligomeric form of viral Env and its monomeric secreted counterparts display very different conformation (Moore et al., 1994; Burton, 1997). For instance, epitopes mapped to the C1 and C5 regions as well as epitopes recognized by neutralizing mAbs on V1, V2, and V3 or the CD4 binding site are less immunoreactive on oligomeric membrane Env than on monomeric soluble gp120 (Moore et al., 1994; Sattentau and Moore, 1995; Stamatatos and Cheng-Mayer, 1995; Burton, 1997).

To use a model that properly addresses the effect of DNM on Env presented at the surface of HIV virion, we have expressed at the surface of mammalian cells the native fusogenic Env by using an rVV vector. We have examined the glycoprotein from a lymphotropic HIV-1 isolate because most of the previous works on DNM have focused on this class of envelope. Moreover, many Abs directed against T-tropic Env have been characterized as tools to address the conformation of the HIV glycoprotein. DNM treatment was performed in

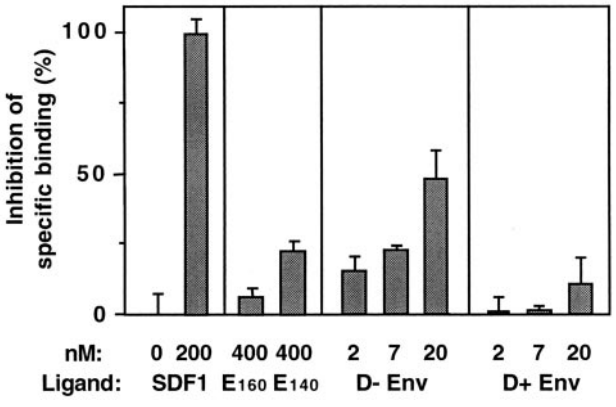

Fig. 5. Effect of Env on SDF1 binding to cell surface CXCR4. CEM cells were incubated for 1 h at 25°C in the absence (0) or presence of unlabeled SDF1 (200 nM) in buffer supplemented with 0.1% NaN_3. Labeled SDF1 was added for 1 h and the radioactivity associated with the cell pellet was assessed. The inhibition (%) of specific binding of SDF1 was determined. In parallel, living cells were incubated for 2 h at 37°C with high amounts of soluble uncleaved Envs (oligomeric gp140, E_{140}, or monomeric gp160, E_{160}) or with increasing quantities of either D− Env or D+ Env. Cells were then incubated with 0.1% NaN_3 and labeled SDF1 for 60 min. The radioactivity associated with the cell pellet was assessed. The inhibition (%) of specific binding of SDF1 was determined (n = 4).

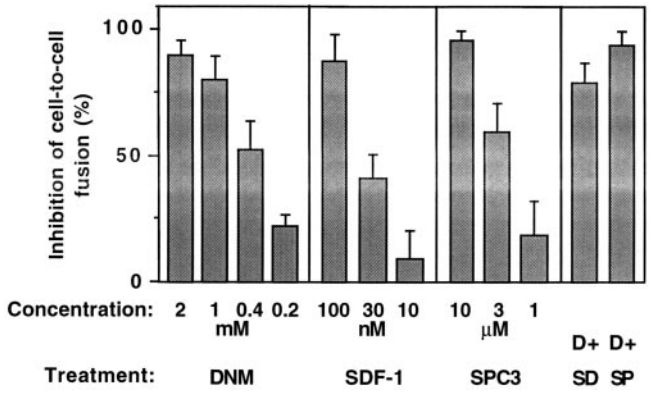

Fig. 6. Effect of DNM and CXCR4 ligands on membrane fusion. CEM cells were pretreated for 3 days by using various concentrations of DNM before infection using VV9-1. Alternatively, cells were treated using either SPC3 or SDF1 at 4 h postinfection. Fusions were assessed at 24 h postinfection. In some experiments, cells were treated using 0.4 mM DNM for 3 days before infection by using VV9-1; 4 h later, cells were incubated with either 30 nM SDF1 (D + SD) or 3 μM SPC3 (D + SP). Fusions were assessed at 24 h postinfection. Inhibition of fusion was determined as described under *Materials and Methods* (inhibition, 100%, no syncytium; 0%, syncytium formation obtained in control conditions; i.e., no inhibitor; n = 2).

conditions that induce both expression of abnormally glycosylated Env (Fenouillet and Gluckman, 1991; Ratner et al., 1991) and inhibition of HIV spread in lymphocyte cultures (Gruters et al., 1987; Montefiori et al., 1988). In agreement with several reports (Fenouillet and Gluckman, 1991; Ratner et al., 1991), D+ Env still displayed complex sugar structures in these conditions. This was expected in view of the inability of DNM to block glucosidase activity by more than 30 to 40% (Elbein, 1987).

Env bioactivity was investigated using a semiquantitative assay that models HIV/cell fusion events and specifically discriminates between the CD4 binding step and the subsequent events leading to membrane fusion (Barbouche et al., 2000a; Fenouillet et al., 2001). D+ Env was found to bind efficiently CD4: the cell aggregation process resulting from the interaction between cells expressing D+ Env and CD4+ cells occurred normally and D+ Env bound soluble CD4 in molecular assays. This is consistent with previous data obtained using native Env and differs from others obtained with monomeric soluble mutated gp160 (Fenouillet and Gluckman, 1991; Jones and Jacob, 1991; Ratner and Vander Heyden, 1993; Fischer et al., 1995). In contrast, the membrane fusion capacity of D+ Env was altered, as indicated by its inability to induce syncytium formation. These results show that DNM blocks HIV Env-mediated membrane fusion at a post-CD4 binding step. Similar data were obtained with a primary isolate-derived envelope that can use various coreceptors for entry, including CXCR4 (Doranz et al., 1996).

Env conformation was probed using mAbs essentially mapped to C1, V1/V2, V3, or to the CD4 binding site. They strongly reacted with membrane Env and this is consistent with the accessibility of the corresponding domains on oligomeric Env (Moore et al., 1994). Few Abs mapped to C2, V4, and C5 were also tested and were found to react poorly with membrane Env. Because they are able to recognize denatured gp120, they are probably directed against regions that are hidden on mature folded Env (Moore et al., 1994). Probing Env conformation with anti-gp120 Abs therefore constitutes an approach that presents several limits. DNM treatment induced strong immunoreactivity changes for epitopes located on V1/V2 and C2 and on regions surrounding the apex of V3. The decreased binding of D+ Env to conformational Abs and its increased reactivity toward nonconformational Abs is consistent with misfolding of the glycoprotein. Indeed, the latter are directed against epitopes that are more reactive on denatured or abnormally folded Env compared with native Env (Moore et al., 1994). The increased reactivity of D+ Env indicates also that these immunoreactivity changes did not result from epitope masking by large DNM-induced carbohydrate structures but rather from DNM-induced misfolding of Env. Modification of the immunoreactivity of V1/V2 and C2 on D+ Env is in agreement with a previous report that investigated the reactivity of monomeric recombinant gp120 by surface plasmon resonance (Fischer et al., 1996). In contrast, we did not observe significant alteration of the immunoreactivity of the apex of the V3 domain presented on membrane D+ Env, in contrast to previous reports using soluble mutated Env and ELISA (Fenouillet and Gluckman, 1991; Jones and Jacob, 1991). Finally, D+ and D− gp120 displayed a similar immunoreactivity pattern in ELISA, which further shows that the use of plastic-bound Env is poorly relevant to address the properties of membrane Env.

We then tried to relate the functional characteristics of Env to its immunoreactivity pattern. The impaired ability of D+ Env to mediate membrane fusion is consistent with the altered immunoreactivity of V1/V2 and of the flanking regions of V3. Indeed, the role of these domains in post-CD4 binding events triggering fusion has been demonstrated: their mutations alter virus tropism and CD4 binding induces conformation changes within these regions to promote Env interaction with the chemokine receptors (Stamatatos and Cheng-Mayer, 1995; Palmer et al., 1996).

This suggests that D+ Env is unable to efficiently interact with the lymphocyte CXCR4 coreceptor. We therefore undertook to test this novel hypothesis. Various methods are available to address the interaction of Env with CXCR4 but all of them use soluble Env. First, direct binding of Env to cell surface CXCR4 can be investigated (Hesselgesser et al., 1997; Misse et al., 1998; Mondor et al., 1998; Doranz et al., 1999b). The detection of Env binding to CXCR4 uses either radiolabeled Env or fluorescence-activated cell sorting analysis. This assay is difficult to perform due to Env denaturation induced by labeling and to the low affinity of the Env/CXCR4 interaction. Second, the characteristics of Env binding to recombinant CXCR4 can be addressed via the assessment of the ability of soluble Env to interfere with binding to CXCR4 of its natural ligand SDF1 (Zhou and Tai, 1999). A major drawback of these experiments is that the recombinant CXCR4 molecule is expressed on the surface of a $CD4^-$ nonlymphocytic cell and this may explain why the binding capacity of Env does not reflect the ability of the Env/CXCR4/CD4 complex to trigger HIV entry (Doranz et al., 1999b). Here, we have used a third experimental approach that examines the inhibition by Env of SDF1 binding to lymphocyte membrane receptor CXCR4. This assay has several advantages: 1) SDF1 binding is assessed on functional lymphocyte CXCR4; 2) the use of nonmodified Env alleviates the possible denaturation of the protein during iodination; and 3) it is detected in the context of important catalytic activities (Fenouillet et al., 2001) associated with the living CD4+ lymphocyte surface. We have shown that incubation of CEM cells with D+ Env poorly interferes with SDF1 binding to CXCR4 compared with native D− Env. This is consistent with an altered interaction of D+ Env with CXCR4. The reduction in the Env-CXCR4 binding capacity induced by DNM is comparable with that observed in conditions that are considered as incompatible with Env/CXCR4 binding (Doranz et al., 1999b). This result further illustrates the influence of Env glycosylation on both its binding to CXCR4 and the mutual interactions between the V1/V2, V3, and C2 domains (Chen et al., 2001). The abnormal immunoreactivity of V1/V2 on D+ Env and its altered capacity to interact with CXCR4 and to mediate fusion are also in agreement with studies that highlight the influence of the glycans of the V1/V2 domain in coreceptor usage (Ogert et al., 2001; Pollakis et al., 2001).

The use of therapeutic agents as part of a combination therapy is often the most effective approach to reach very potent activity together with reduced toxicity. Because DNM treatment alters Env folding, and especially the CXCR4 binding competent conformation, we investigated the antiviral effect of DNM used in conjunction with natural or synthetic ligands of CXCR4. We have observed a severe alteration of membrane fusion when suboptimal doses of DNM and either SDF1-α, the natural ligand of CXCR4 and prototype of potent

anti-HIV agents, or SPC3, an anti-HIV V3-derived peptide construct interacting with CXCR4, were used.

In conclusion, our results have identified three major alterations of the properties of Env induced by DNM treatment: 1) immunoreactivity changes associated with the V1/V2, C2, and V3 regions; 2) altered capacity to bind CXCR4; and 3) inability to mediate fusion. These results allow us to propose that DNM exerts an anti-HIV activity in lymphocyte cultures through impairment of HIV entry after CD4 binding at the Env/CXCR4 interaction step. DNM analogs with reduced side effects, specifically active on α-glucosidase I-II activity and not on other metabolic pathways, are under development. Our preliminary results also indicate that such decoys are candidates to potently block HIV entry into lymphoid cells as part of combination treatment with CXCR4 ligands. Such an experimental combination therapeutic approach seems to be a unique opportunity to target both protagonists of the crucial CXCR4/Env binding step at the onset of the spread of syncytium-inducing viruses during the course of the disease.

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