Development of Resistance of Human Immunodeficiency Virus Type 1 to Dextran Sulfate Associated with the Emergence of Specific Mutations in the Envelope gp120 Glycoprotein

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SUMMARY

Polyanionic compounds are known to inhibit the binding of human immunodeficiency virus (HIV) to CD4+ cells and the subsequent fusion step between the virus and cells. We selected an HIV-1 strain resistant to dextran sulfate (DS) by cultivation of HIV-1 (NL4–3)-infected MT-4 cells in the presence of DS, 5000. DS did not inhibit the binding of DS-resistant virus to MT-4 cells or syncytium formation between MOLT cells and HUT-78 cells persistently infected with the DS-resistant virus. In addition, a monoclonal antibody with specificity for the V3 loop of envelope gp120 glycoprotein did not recognize the DS-resistant HIV-1 gp120 V3 loop. The following mutations were found in the gp120 molecule of the DS-resistant HIV-1 strain but not in the wild-type strain: S114N in the V1 loop region; S134N in the V2 loop region; K269E, Q278H, and N293D in the V3 loop region; N323S in the C3 region; a deletion of five amino acids (Phe-Asn-Ser-Thr-Trp) at positions 364–368 in the V4 loop; and R387I in the CD4 binding domain. Our results suggest that (i) DS interacts with specific amino acid residues in the gp120 molecule, (ii) the virus is able to overcome the inhibitory effect of DS on viral infectivity, (iii) cross-resistance developed against those polyanionic compounds that are structurally related to DS, and (iv) the molecular determinants of HIV cell tropism, syncytium-inducing ability, coreceptor (fusin/CC-CKR5) utilization, and polyanion resistance seem to be located in the env genome of HIV and specifically in the V3 loop domain.

Polyanions are potent inhibitors of HIV types 1 and 2 in vitro (1). DS and other polyanions (i.e., heparin) have been shown to inhibit virus binding (2) and to block mAb binding to the V3 principal neutralizing domain of gp120 (3) without interfering with the gp120/CD4 interaction (4). Anti-V3 mAbs such as NEA 9284 neutralize HIV-1 infection and inhibit syncytium formation but do not inhibit the binding of soluble gp120 to the CD4 receptor (5).

It has also been shown that, because of their overall negative charge, DS and heparin may interact in a nonspecific fashion with positively charged molecules such as the CD4 receptor (6). Although DS and heparin are potent inhibitors of HIV strains with tropism for lymphocytic cell lines, they do not inhibit the replication of MT viruses in primary macrophages (7).

Because lymphotropic HIV strains show a high overall V3 loop positive electric charge compared with noncytopathic, MT strains (8), the antiviral action of polyanionic compounds may be ascribed to their interaction with these positively charged residues, thus leading to alterations in the overall structure of the V3 loop and/or blocking the biological function of this gp120 domain.

We previously reported the development of HIV-1 resistance to the bicyclam JM3100 (SID791), which is a potent inhibitor of HIV-1 replication in different cell lines, including primary macrophages (9, 10). This bicyclam-resistant HIV-1 strain, which contained a total of 12 different mutations not present in the wild-type strain, showed cross-resistance to DS, heparin, and other polyanions (11, 12). This observation indicates that if the interaction of DS with its molecular target is specific, the virus may be able to overcome the inhibitory effect of DS on infectivity through mutations of the specific amino acids involved in the interaction with DS. Okada and Gurney (13) have shown that single amino acid substitutions at position 302 or 320 (positions 272 and 290 according to the published secondary structure shown in Ref.

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ABBREVIATIONS: HIV, human immunodeficiency virus; mAb, monoclonal antibody; SI, syncytium-inducing; HSA, human serum albumin; DS, dextran sulfate; DSr, dextran sulfate-resistant; CDS, cyclodextrin sulfate; PCR, polymerase chain reaction; CPE, cytopathic effect; MT, macrophage-tropic; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ATA, aurintricarboxylic acid; PBS, phosphate-buffered saline.
11) in the V3 domain are not sufficient to alter the antiviral activity of DS and heparin.

Although the positively charged amino acids and the entire conformation of the V3 domain may seem essential for virus tropism and SI phenotype, the role of the individual amino acids in the modus operandi of the V3 loop has not been delineated. Regardless of their therapeutic potential, polyanionic compounds may be considered important tools in attempts to resolve the molecular determinants of the V3 loop for HIV infectivity.

Recently, a seven-transmembrane G protein-coupled receptor was identified as an HIV-1 entry cofactor termed fusinLESTR or CXCR-4 (14). Several lines of evidence suggest that the site of interaction of HIV and fusin/LESTR could be narrowed to the V3 loop region of the gp120 molecule, which is a major determinant of HIV tropism: the CXC chemokine SDF-1, a potent inhibitor of T cell-adapted HIV-1 (15), is the ligand for fusin/LESTR, whereas the chemokine RANTES interacts with the CC-CKR5 receptor. The latter prevents infection by primary monocyte-tropic viruses but is inactive against T cell-tropic strains (16, 17). MTs of HIV seem to be naturally resistant to DS inhibitory action (7). An understanding of the molecular determinants of DS resistance by HIV may help to clarify the mode by which HIV interacts with the second receptor of HIV infection.

Materials and Methods

Compounds. DS (Mr 5000), heparin, and ATA were purchased from Sigma Chemie (Deisenhofen, Germany). DS samples of various molecular weights were obtained from Pfeifer and Langen (Dormagen, Germany). The bicyclam derivates JM3100 and JM2763 and the polyoxometalates JM1493 and JM1497 were synthesized at Johnson Matthey (West Chester, PA), as described previously (18, 19). The 8-chloro-tetrahydroimidazo[4,5,1-JK][1,4]-benzodiazepine-2(1H)-one and -thione, R86183 (20), a-CDS, and b-CDS were provided by the Janssen Research Foundation (Beerse, Belgium). Negatively charged albumins (aconitic acid-HSA and succinic acid-HSA) (21) were provided by Prof. D. K. F. Meijer (Groningen State University, Groningen, The Netherlands). 39-Azido-39-deoxythymidine was obtained from Wellcome (Beckenham, UK). Polyvinylalcohol sulfate was provided by Dr. S. Görög (Chemical Works of Gedeon Richter, Budapest, Hungary). The oligonucleotide T30177 (AR177/Zintevir) was provided by Robert F. Rando (Aronex Pharmaceuticals, The Woodlands, TX) (22).

Viruses, cells, antiviral activity assays, and cytotoxicity assays. Anti-HIV activity and cytotoxicity measurements in MT-4 cells were based on viability of cells that had been infected or not infected with HIV and then exposed to various concentrations of the test compound. After the MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was quantified according to a tetrazolium-based colorimetric method (MTT method), as described by Pauwels et al. (23). The HIV-1 NL4–3 strain is a molecular clone obtained from the National Institutes of Health (Bethesda, MD).

Selection of HIV-1 (NL4–3) mutant strains. MT-4 cells were infected with HIV-1 (NL4–3) in medium containing DS at 2–4 times the EC50 value. Cultures were incubated at 37° until an extensive cytopathic effect was present (5–6 days). The culture supernatants were used for further passage in MT-4 cells in the presence of 2–5-fold-increasing concentrations of DS (Mr 5000).

Fig. 1. Development of HIV-1 (NL4–3) resistance to DS. MT-4 cells were infected with virus in the presence of five times the EC50 concentration of DS (passage 0). Every 5–6 days, supernatant of the cell culture was used to reinfect fresh MT-4 cells in the presence of the same or 2–5-fold-higher concentration of DS, depending on the CPE observed.

Fig. 2. Inhibition of virus binding to MT-4 cells. The cells were incubated with 1×105 pg of p24 antigen of wild-type HIV-1 NL4–3 (□) or DS' HIV-1 NL4–3 (▌). After 1 hr, cells were washed in PBS, and p24 antigen bound to the cells was determined by a commercial test.
Virus-binding assay. MT-4 cells (5 × 10^5) were infected with supernatant containing 1 × 10^5 pg of p24 antigen of either wild-type HIV-1 NL4–3 or DS r HIV-1 NL4–3 in the presence of different concentrations of DS. At 1 hr after infection, cells were washed three times with PBS, and the p24 antigen bound to the cells was determined by a commercially available test (DuPont, Wilmington, DE).

Syncytium formation assay. HUT-78 cells (2 × 10^6 cells/ml) persistently infected with wild-type or DS r HIV-1 NL4–3 virus were cocultured with MOLT-4 (clone 8) cells (1.8 × 10^6 cells/ml) in the presence of DS. After 24 hr, the number of giant cells (syncytia) was recorded microscopically as described previously (24).

Immunofluorescence binding assays. MT-4 cells were infected with wild-type or DS r HIV-1 (NL4–3) at a multiplicity of infection of 0.01. At 48 hr after infection, cells were washed with PBS and processed by flow cytometric analysis according to Schols et al. (3). The anti-gp120 mAb NEA 9284 and NEA 9305 (DuPont) specifically recognize the V3 loop epitopes NTRKSIRIQRG and RIQRG-PGRAFVTGK, respectively.

DNA sequence analysis of gp120. MT-4 cells were infected with wild-type or DS r HIV-1 and incubated at 37°C for 4 days. The cells were washed in PBS, and total DNA was extracted with a QUIAquick blood kit (Westburg, The Netherlands). PCR amplification was performed with ULTMA DNA polymerase with proof-reading capacity (Perkin-Elmer Cetus, Norwalk, CT) according to De Vreese et al. (11). The PCR product was electrophoresed in an agarose gel, and the relevant band was excised and purified with a QUIAquick cleaning kit. DNA sequencing was performed directly on the cleaned PCR product following the protocol provided with the ABI PRISM dye terminator-cycle sequencing kit and analyzed on an ABI PRISM 310 genetic sequencer (Perkin-Elmer Cetus). The sequences were exported to the software Geneworks and PCGene (IntelliGenetics, Oxford, UK) and analyzed in comparison with the published proviral DNA sequence of the virus strain NL4–3. The primer sets used for PCR amplification and sequence analysis are summarized in Table 1.

Cloning and marker rescue experiments. PCR fragments of the env gene of the wild-type and DS-selected strains were cloned in the pCR-Script SK+ cloning vector (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Marker rescue experiments were done as described by De Vreese et al. (11). Briefly, NL4–3 wild-type proviral DNA (GenBank database, accession no. M19921) was cleaved with single cutter restriction enzymes to excise the region corresponding to the V3–V5 DNA sequence for replacement by mutant DNA through homologous recombination. Restriction enzyme-digested NL4–3 wild-type DNA, precipitated by ethanol, together with PCR DNA was used to transfect MT-4 cells by electroporation: 10–15 μg of NL4–3 wild-type DNA and 5 μg of purified PCR DNA were mixed with 10^7 cells suspended in 200 μl of RPMI medium in

Fig. 3. Flow cytometric histograms of the binding of anti-gp120 mAb to MT-4 cells infected with NL4–3 HIV-1 wild-type or NL4–3 DS r HIV-1. At 48 hr after infection, cells were stained for flow cytometry with mAb NEA9284 or NEA9305, which recognize different epitopes of the gp120 V3 domain. The percentages of fluorescent positive cells are indicated in each histogram.
or at a 3–5-fold higher concentration, depending on the CPE either at the same concentration as in the previous passage was passaged in fresh, uninfected cells in the presence of DS standard MT-4/CPE test. Virus replication was monitored micro-

pathicity in MT-4 cells, as determined by the MTT method.

Mutations in the gp120 of DS r NL4-3 cells was isolated for sequencing. Culture was recovered for further testing, and DNA from infected until significant CPE was observed. The supernatant of the cell fresh medium containing 5

F). Immediately thereafter, the cells were resuspended in 15 ml of electroporation cuvettes and electropulsed (settings: 0.250 kV, 1050

μl).

electroporation cuvettes and electropulsed (settings: 0.250 kV, 1050 F). Immediately thereafter, the cells were resuspended in 15 ml of fresh medium containing 5 × 10^6 cells. Cells were examined daily until significant CPE was observed. The supernatant of the cell culture was recovered for further testing, and DNA from infected cells was isolated for sequencing.

Results

Selection of HIV-1 (NL4-3) mutant strains. An approach to identifying the site or sites of interaction of a compound with its molecular target is to develop resistance against the compound. HIV-1 (NL4-3) was passaged in MT-4 cells in the presence of DS at a starting concentration corresponding to ~5-fold the EC_{50} value (0.5 μg/ml) in the standard MT-4/CPE test. Virus replication was monitored micro-

scopically by the appearance of CPE and giant cell (syncytium) formation. Every 5–6 days, the replicating virus was passaged in fresh, uninfected cells in the presence of DS either at the same concentration as in the previous passage or at a 3–5-fold higher concentration, depending on the CPE observed. At concentrations of >500 μg/ml, uninfected cells were also incubated with DS to corroborate that CPE was not due to drug toxicity. After 17 passages (100 days), virus was recovered that was able to replicate at a concentration of 5 mg/ml DS (Fig. 1). Wild-type virus that had been grown in parallel in the absence of DS was not able to replicate under these conditions. The EC_{50} value (>125 μg/ml) of DS for the resistant strain was >1000-fold higher than that of the parental wild-type strain (EC_{50} = 0.1 μg/ml). We previously reported the development of HIV-1 resistance to the bicyclam JM3100 (SID791), a potent inhibitor of HIV-1 replication, which blocks viral entry after binding (12, 31). This bicyclam-resistant strain also showed cross-resistance to DS but took >60 passages (300 days) to emerge (10, 12).

Virus binding. DS inhibited the detection of p24 antigen bound to MT-4 cells after a 1-hr incubation of cells with wild-type virus supernatant. This effect was not observed when the DS r strain was used (Fig. 2).

Syncytium formation assay. Syncytium formation has been regarded as a good parameter for evaluation of virus fusion and the involvement of HIV glycoproteins in CD4+ cell depletion. DS did not inhibit syncytium formation between uninfected MOLT-4 and HUT-78 cells that were persistently infected with the DS r virus at a concentration of 125 μg/ml DS.

Immunofluorescence binding assays. The binding ability of the mAb NEA9305 to gp120 of wild-type virus was not altered compared with that of the DS r virus. The percentage of labeled cells detected (M1) did not vary significantly for the wild-type (M1 = 68%) and DS r (M1 = 54%) strains. However, when repeating this experiment with a mAb spe-

fic for another domain of the V3 loop of gp120 (mAb NEA 9284), the resistant virus was not recognized (M1 = 6%), which is in contrast to the wild-type strain (M1 = 71%) (Fig. 3).

Cross-resistance to other polyanions and polysul-

fates. Resistance of HIV-1 to DS was generated with DS of Mr 5000. In addition, the virus was fully resistant to DS of Mr 1500 and 3400 and less sensitive to higher-molecular-weight DS polymers DS40000 (4-fold) and DS70000 (4-fold) than the wild-type strain. The virus was partially cross-resistant to heparin (45-fold), α-CDS (25-fold), β-CDS (13-fold), ATA (17-fold), and, to a lesser extent, the polyoxometalates JM1493 (7-fold) and JM1497 (9-fold). The DS r virus was not cross-resistant to the bicyclams JM3100 and JM2763, the negatively charged analogs aconitic acid-HSA and succinic acid-HSA, polyvinylalcohol sulfate, and the oligonucleotide observed in the presence of DS at a starting concentration corresponding to ~5-fold the EC_{50} value (0.5 μg/ml) in the standard MT-4/CPE test. Virus replication was monitored micro-

TABLE 2

Anti-HIV activity of different compounds against wild-type and DS r HIV-1

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC_{50} a</th>
<th>CC_{50} b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type NL4-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS (M, 5000)</td>
<td>0.14</td>
<td>&gt;125</td>
</tr>
<tr>
<td>DS (M, 1500)</td>
<td>0.18</td>
<td>&gt;125</td>
</tr>
<tr>
<td>DS (M, 3400)</td>
<td>0.11</td>
<td>&gt;125</td>
</tr>
<tr>
<td>DS (M, 40000)</td>
<td>0.57</td>
<td>2.1</td>
</tr>
<tr>
<td>DS70000</td>
<td>0.48</td>
<td>2.1</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.16</td>
<td>49.9</td>
</tr>
<tr>
<td>β-CDS</td>
<td>0.13</td>
<td>3.2</td>
</tr>
<tr>
<td>γ-CDS</td>
<td>0.14</td>
<td>1.8</td>
</tr>
<tr>
<td>PVAS</td>
<td>1.2</td>
<td>3.3</td>
</tr>
<tr>
<td>JM1493</td>
<td>0.09</td>
<td>0.6</td>
</tr>
<tr>
<td>JM1497</td>
<td>13.1</td>
<td>117</td>
</tr>
<tr>
<td>ATA</td>
<td>0.092</td>
<td>1.5</td>
</tr>
<tr>
<td>T30177</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Aconitic acid-HSA</td>
<td>2.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Succinic acid-HSA</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Bicyclam JM2763</td>
<td>0.32</td>
<td>0.19</td>
</tr>
<tr>
<td>Bicyclam JM3100</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>3’-Azido-3’-deoxyxymidine</td>
<td>0.0002</td>
<td>0.0002</td>
</tr>
<tr>
<td>TIBO R86183</td>
<td>0.0024</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

a 50% Effective concentration based on the inhibition of HIV-induced cyto-

pathicity in MT-4 cells, as determined by the MTT method.

b 50% cytotoxic concentration, based on the viability of MT-4 cells by the MTT method.

Cross-resistance to other polyanions and polysul-

fates. Resistance of HIV-1 to DS was generated with DS of Mr 5000. In addition, the virus was fully resistant to DS of Mr 1500 and 3400 and less sensitive to higher-molecular-weight DS polymers DS40000 (4-fold) and DS70000 (4-fold) than the wild-type strain. The virus was partially cross-resistant to heparin (45-fold), α-CDS (25-fold), β-CDS (13-fold), ATA (17-fold), and, to a lesser extent, the polyoxometalates JM1493 (7-fold) and JM1497 (9-fold). The DS r virus was not cross-resistant to the bicyclams JM3100 and JM2763, the negatively charged analogs aconitic acid-HSA and succinic acid-HSA, polyvinylalcohol sulfate, and the oligonucleotide observed in the presence of DS at a starting concentration corresponding to ~5-fold the EC_{50} value (0.5 μg/ml) in the standard MT-4/CPE test. Virus replication was monitored micro-

fates.

TABLE 3

Mutations in the gp120 of DS r NL4-3

<table>
<thead>
<tr>
<th>Amino acid position (region) a</th>
<th>Codon</th>
<th>Amino acid</th>
<th>Codon</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>113 (V1)</td>
<td>AGT</td>
<td>S</td>
<td>AAT</td>
<td>N</td>
</tr>
<tr>
<td>134 (V2)</td>
<td>AGC</td>
<td>S</td>
<td>AAC</td>
<td>N</td>
</tr>
<tr>
<td>269 (V3)</td>
<td>AAA</td>
<td>K</td>
<td>GAA</td>
<td>E</td>
</tr>
<tr>
<td>278 (V3)</td>
<td>CAG</td>
<td>Q</td>
<td>CAT</td>
<td>H</td>
</tr>
<tr>
<td>293 (V3)</td>
<td>AAT</td>
<td>N</td>
<td>GAT</td>
<td>D</td>
</tr>
<tr>
<td>323 (C3)</td>
<td>AAT</td>
<td>N</td>
<td>AGT</td>
<td>S</td>
</tr>
<tr>
<td>Δ364-368 (V4)</td>
<td>TAT</td>
<td>FNSTW</td>
<td>Deletion</td>
<td></td>
</tr>
<tr>
<td>387 (CD4BD) b</td>
<td>AGA</td>
<td>R</td>
<td>ACA</td>
<td>I</td>
</tr>
</tbody>
</table>

a Numbering of amino acid and domain classification according to the published secondary structure of the mature HIV-1 gp120 protein (11).

b CD4BD: CD4 binding domain of the gp120 molecule as in Ref. 39.
Table 4: Inhibition of env recombinant viruses by DS

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Recombination site</th>
<th>EC50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL4-3/4C20</td>
<td>Wild-type gp120-V3/V5 region(NheI/BsaBI)</td>
<td>0.2</td>
</tr>
<tr>
<td>NL4-3/2C3</td>
<td>DS5’ gp120-V3/V5 (NheI/BsaBI)</td>
<td>40</td>
</tr>
</tbody>
</table>

Recombinant viruses containing genome segments of wild-type and DS’ strains obtained in the drug selection experiment were analyzed for drug sensitivity in MT-4 cells by the MTT method.

T30177. The EC50 values of these compounds for wild-type and DS’ virus are shown in Table 2.

**DNA sequence analysis of the env gene of DS’ virus.** A minimum of four independent PCR products were sequenced to avoid possible introduction of false mutations during the PCR amplification. The gp120 sequence of the wild-type strain passaged in the absence of DS differed from the original viral sequence at two positions (N268Y and N293D). In addition, we identified several mutations in the gp120 gene sequence of the DS’ strain that were not present in the wild-type strain. Three mutations were clustered in the V3 loop region (Fig. 3), two of which corresponded to mutations found in the HIV-1 NL4-3 strain that is resistant to the bicyclam JM3100 (11). Other mutations, including a deletion of five amino acids, were found in the V1, V2, C3, and V4 and the CD4 binding domain of gp120 (Table 3).

**Marker rescue.** In marker rescue experiments, recombinant viruses were generated in which the DNA sequence encoding the V3–V5 region of the parental NL4–3 proviral genome was replaced by a corresponding sequence of the DS’ (2C3) or wild-type (4C20) strain recovered from the selection experiments. Determination of the drug sensitivity of the recombinant viruses revealed that the resistant phenotype was recovered in the DS-recombinant strain (2C3) but not, as expected, in the wild-type-recombinant strain (4C20). The virus strain 2C3 was ~200-fold resistant to DS in comparison with the 4C20 strain (Table 4); however, it was less resistant to DS than the original DS’ virus. In all recombination experiments, virus originated from trace amounts of uncleaved pNL4–3 proviral DNA present in the preparation (11). This wild-type virus in the pool of the DS’ stock may interfere with the EC50 determination, resulting in a higher sensitivity to DS than the original DS’ strain but still showing the resistant phenotype. Sequence analysis of the 2C3 strain confirmed the presence of the mutations obtained by selection in the V3–V5 region.

### Discussion

DS is a potent inhibitor of HIV replication in lymphocytic cell lines. Studies on the mode of antiviral action of DS and sulfated polymers and their ability to inhibit virus adsorption and virus/cell fusion have allowed us to recognize important features in the mechanism of HIV infection, such as mapping of the site of interaction of gp120 with the CD4 receptor (9, 26), identification of phenotypic characteristics of different HIV variants (9, 7), and interaction of the envelope gp120 and gp41 glycoproteins with cell surface proteoglycans (27). This in turn has opened new insights for the development of more potent and selective inhibitors of HIV replication. Several compounds are being studied that are targeted at early stages (virus binding/virus fusion) of HIV replication but possess diminished adverse effects (9, 22, 28, 44). Some of these compounds share similarities in their mode of action to DS, as in the case of the bicyclam JM3100 (11), which may show phenotypic and genotypic similarities for both DS and bicyclam resistance development.

It has been reported that DS also interacts, albeit in a relatively nonspecific fashion, with the CD4 receptor, shielding it from virus infection. In this study, we have shown that the development of the DS’ phenotype can be attributed to the specific amino acid changes that occurred due to the selective pressure of the compound on virus replication. This suggests that there is a specific interaction of DS with HIV and that the virus is able to overcome, through genotypic changes, the inhibitory effect of DS on virus binding, on HIV-induced cell-to-cell fusion, and on HIV infectivity. These mutations seem to be located in the gp120 molecule.

Although other mutations in other proteins of the virion may be present, marker rescue experiments in which the DNA sequence encoding the V3–V5 region of the DS’ strain was recombined into the wild-type NL4–3 proviral sequence showed that these mutations are sufficient to restore the DS’ phenotype. In addition, the observations that an epitope in the V3 region of the mutant virus was not recognized by an mAb directed to it and that DS did not inhibit syncytium formation between uninfected cells and cells persistently infected with the DS’ virus point to the gp120 as the target molecule for DS.

Of the six mutations detected in the gp120 of the DS’ phenotype, three (K269E, Q278H, and N293D) seemed to be located in the V3 domain (Fig. 3). Two of these mutations (Q278H and N293D) were also detected in the bicyclam-resistant strain that showed cross-resistance to DS, suggest-
ing that these mutations may indeed be of key relevance in determining the DS' phenotype. These same two mutations (Q278H and N293D) are clustered in a stretch of 11 amino acids in the V3 domain that seems to play a major role in the MT and SI phenotypes (30–33). In MT strains such as HIVBal, an aspartic acid residue (D) is present at position 293. MT strains also have a deletion at position 278. Furthermore, recent studies have shown that MT HIV strains are not susceptible to the inhibitory effect of DS (7) and that a naturally occurring mutation at position 293 (D to N) is required for full expression of the SI phenotype (8). Therefore, although the DS' virus is still able to replicate in lymphoid cell lines such as MT-4, the mutations N293D and Q278H, which seem to be of key relevance in the DS' phenotype, may also be of importance in determining the tropism phenotype of the HIV strain.

The overall charge of the V3 loop decreased from +9 in the wild-type strain to +6 in the DS' strain (Fig. 3). De Jong et al. (8, 34) have shown that non-SI strains have a reduced overall net charge (≤+4). Through a computer-based model of V3-loop sequences, Battacharyya et al. (35) proposed that positive charge residues in the V3 loop correlate with the HIV type 1 SI phenotype and the V3 loop ability to bind a disulfated sugar moiety. From the reduced positive charge of the V3 loop of the DS' strain, we may thus infer that polyanionic compounds select for HIV mutant strains that are of non-SI phenotype.

Preliminary studies (36) have shown that the V3 domain of gp120 determines the coreceptor (fusin for SI strains, CCKR5 for NS1 strains) that is used. DS inhibited the binding of MIP-1α (a chemokine that uses CCKR5 as a cell receptor) to phytohemagglutinin-stimulated peripheral blood mononuclear cells.2 Tests are under way to verify whether the differences detected between the DS' and wild-type strains may be of relevance in determining coreceptor utilization and play a role in replication efficiency and cell tropism by HIV.

In addition to the mutations present in the V3 loop of the DS' strain, at least three other mutations (S114N, S134N, and R387I) were detected in the gp120 molecule, and a deletion of five amino acids in the V4 loop was found in the JM3100-resistant strain. The roles of these mutations in (i) the DS-resistance phenotype and (ii) the infectivity of the mutant viral strains remain to be assessed. Their presence argues in favor of the multifactorial interaction of sulfated polysaccharides with the gp120 molecule. DS may interact with multiple, specific sites of the gp120 molecule, accounting for the different effects that have been encountered with this type of compounds, viz. inhibition of virus adsorption and inhibition of virus/cell fusion (syncytium formation). These effects may be mediated by separate segments of the gp120 molecule.

We have also shown that the DS' (M6, 5000) strain was fully cross-resistant to DS samples of lower molecular weight but remained sensitive to DS polymers of higher molecular weight, albeit at slightly higher concentrations than required to inhibit the wild-type strain. The DS' strain also showed cross-resistance to heparin, α-CDS, sulfated β-CDS, ATA, and the poloxometalates JM1493 and JM1497. These data support the notion that polyanions, whether polysulfates, polycarboxylates, or poloxometalates, share similarities in their mechanism of action. The cross-resistance to ATA deserves special attention. This compound has been reported to block the binding of mAb directed to the gp120 binding domain of the CD4 receptor, as well as the binding of mAb to the V3 loop of gp120 (3, 37). Our present observation regarding the cross-resistance of the DS' HIV strain to ATA suggests that ATA and its analogues (38) may indeed have a bimodal mode of action targeted at both CD4 and gp120.

We did not observe any cross-resistance of the DS' HIV-1 strain to the bicyclams JM2763 and JM3100. However, the JM3100-resistant strain previously reported (14) proved to be cross-resistant to DS of low molecular weight (11, 12). In separate experiments, we found that a chimeric HIV-1 clone derived from the JM3100-resistant strain [constructed according to de Jong et al. (36)], which differs from the wild-type strain only in the gp120 V3 region, has a reduced (4-fold) sensitivity to DS (M6, 1500) (data not shown). Thus, the mutations (≤12) needed to generate resistance to bicyclams also lead to cross-resistance to DS, whereas the mutations [up to six (as shown here)] that engender resistance to DS do not suffice to promote resistance to bicyclams. Resistance to bicyclams was found to develop much more slowly than resistance to DS, and resistance to bicyclams was much less pronounced than resistance to DS. These observations, on the one hand, point to the greater therapeutic potential of the bicyclams compared with that of DS and, on the other hand, indicate that the bicyclams differ from DS in the gp120 sites with which they interact and/or the gp120 conformational changes they induce.

Finally, because gp120 seems to play an important role in virus tropism, infectivity, and pathogenicity, the results presented here open the possibility that new anti-HIV compounds directed to early stages of infection but with greater potency may not only inhibit virus replication but also exert selective pressure in favor of less infectious or less cytopathic strains, thus prolonging the asymptomatic phase in HIV-infected individuals.

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References