Rapid selection for an N-linked oligosaccharide by monoclonal antibodies directed against the V3 loop of human immunodeficiency virus type 1

Kristian Schonning, Britt Jansson, Sigvard Olofsson and John-Erik Stig Hansen

Laboratory for Infectious Diseases, Department 144, Hvidovre Hospital, Kettegård Allé 30, 2650 Hvidovre, Denmark and Department of Clinical Virology, University of Göteborg, 413 46 Sweden

The V3 loop of the human immunodeficiency virus (HIV) surface protein, gp120, constitutes a principal neutralizing determinant. HIV strains lacking a naturally conserved N-linked oligosaccharide (at position 306) within the V3 loop are highly sensitive to neutralization. We subjected molecular clones of HIV-LAI lacking this 306-N-glycan to in vitro immune selection with MAbs directed against the V3 loop. In all, ten clones were characterized, and all proved resistant to V3-directed neutralization. Sequencing of the V3 loop revealed that six of the clones had become resistant at least partly by reacquisition of the 306-N-glycan. Only two of the clones possessed mutations within the binding site of the antibody itself, while the two remaining clones did not display changes within the V3 loop itself. Thus, HIV strains lacking the 306-N-glycan primarily develop resistance to V3-directed neutralization through acquisition of the specific oligosaccharide. This demonstrates that protein glycosylation can be a primary modifier of virus antigenicity of possible importance for the interaction of HIV with the host immune response.

Introduction

Carbohydrate moieties may dramatically influence the antigenicity of viral proteins (Alexander & Elder, 1984). The surface proteins of lentiviruses are characterized by a high carbohydrate content (Coffin, 1990). The surface protein of human immunodeficiency virus type 1 (HIV-1) contains more than 20 sites for N-linked glycosylation and carbohydrate constitutes more than half of its molecular mass (Myers & Lenroot, 1992). This abundant glycosylation may modulate the antigenicity of the underlying protein and may thus be important for establishment of persistent viral infection.

In addition to abundant glycosylation lentiviral surface proteins are characterized by hypervariability. The hypervariable V3 loop of gp120 constitutes an important neutralizing determinant for strains of HIV-1 adapted to T cell lines (Goudsmit et al., 1988; Javaherian et al., 1989; Rusche et al., 1988). Variability within this loop is observed both inter- and intra-individually (Balfe et al., 1990; Hahn et al., 1986; LaRosa et al., 1990; Wolfs et al., 1990). The emergence in vivo of variants resistant to neutralization is correlated with mutations within this loop (Arendrup et al., 1993). This indicates that although neutralizing antibodies are unable to clear the infection they may alter the population of virus present in the infected individual.

The hypervariable V3 loop contains a site for N-linked glycosylation at position 306 in the LAI strain of HIV-1; 95-6% of 385 complete V3 sequences available from NCBI-GenBank (release 8.40, August 1994) conserved the 306-N-glycan site. We and others have observed the spontaneous loss of this N-glycan when virus was propagated in vitro in the absence of selection pressure (Back et al., 1994; J.-E. S. Hansen, B. Jansson & G. J. Gram, unpublished data). The presence within a hypervariable region of a conserved N-glycan that is dispensable for virus infectivity may indicate a function for viral adaptation to the immune environment in vivo. Accordingly, Back et al. (1994) found that variants of the HXB2 strain of HIV-1 lacking this N-glycan displayed a more than 8-fold increase in sensitivity to V3-directed neutralization.

These considerations prompted us to examine if the presence of 306-N-glycan was a readily selectable feature when HIV variants lacking this oligosaccharide were subjected to an appropriate selection pressure.

Methods

Virus and cells. pBRU2, which contains the entire HIV-LAI genome and some additional pBR322 derived sequences, was subjected to site-directed mutagenesis to obtain molecular clones lacking the 306-N-glycan. The recognition signal for N-linked glycosylation is Asn-X-Ser/Thr. One mutant, which contained a T308A mutation disrupting
the site for glycosylation, was designated HIVA308 (Fig. 1). Another mutant, which contained an additional T324A mutation introduced as a marker mutation, was designated HIVA308/A324 (Fig. 1). Mutagenesis was done using the U.S. mutagenesis kit (Pharmacia) according to the manufacturer’s instructions, except that the plasmid was denatured with alkali (0.2 M-NaOH, 0.2 mM-EDTA, 30 min at 37 °C). Briefly, a 2.7 kb BamHI-SalI fragment of pBRU2 was inserted into pUC18. Mutagenesis was performed with two oligonucleotides, one constituting a primer introducing the desired mutation, the other constituting a selection primer eliminating a unique non-essential restriction site. The sequences of the mutagenic oligonucleotides were 5′ GACCCAACA-CTTCTCGAATCTCAAGATCTCCGGGTACCGAG Y and an antisense biotinylated primer, 5′ GAAGAAGAGTTGTTGCAATAGGAAAAATAG Y for the site for glycosylation, was designated HIVA308 (Fig. 1). Another mutation, which contained an additional T324A mutation introduced as a marker mutation, was designated HIVA308/A324 (Fig. 1). Mutagenesis was done using the U.S. mutagenesis kit (Pharmacia) according to the manufacturer’s instructions, except that the plasmid was denatured with alkali (0.2 M-NaOH, 0.2 mM-EDTA, 30 min at 37 °C). Briefly, a 2.7 kb BamHI-SalI fragment of pBRU2 was inserted into pUC18. Mutagenesis was performed with two oligonucleotides, one constituting a primer introducing the desired mutation, the other constituting a selection primer eliminating a unique non-essential restriction site. The sequences of the mutagenic oligonucleotides were 5′ GACCCAAC-CAATGCAAAGAAAAAGTAG 3′ for the T308A mutation and 5′ GAGAGCATTTGTTGCAATAGGAAAAATAG 3′ for the T324A mutation. The sequence of the selection primer was 5′ TTCGATTAGTAGAAGACATCTGCCGGTACCAG G 3′. Mutants were verified by DNA sequencing using the Sequenase version 2.0 sequencing kit (US Biochemical) and the mutated BamHI–SalI fragment was inserted into pBRU2.

Plasmid DNA was isolated (Qiagen) and transfected into the CD4* cell line H9 using a modified DEAE-dextran method as described elsewhere (Gram et al., 1994). Supernatants were harvested at the peak of antigen production, filtered (0.45 μm), divided into aliquots and stored at −80 °C until use.

The CD4* cell lines C8166 (Salahuddin et al., 1983), H9 (Popovic et al., 1984), MT2 and MT4 (Harada et al., 1985) were cultured in growth medium at 37 °C in 5% CO₂. Growth medium was RPMI 1640 supplemented with 10% fetal calf serum and antibiotics.

Monoclonal antibodies. NEA-9205, a mouse monoclonal antibody raised against a synthetic peptide representing amino acids 313 to 327 of HIV LAI gp120 was obtained from Dupont NEN. P4/D10, a mouse monoclonal antibody with a minimal epitope mapped to amino acids 314 to 323 of HIV LAI gp120 (Åkerblom et al., 1990), was kindly provided by L. Åkerblom.

Plaque selection procedure. Variants of HIVA308 and HIVA308/A324 resistant to neutralization were generated using a plaque selection procedure essentially as described by McKeating et al. (1989). In brief, six-well Nunc cell culture plates were treated with poly-L-lysine (50 μg/ml) for 1 h and washed three times with water prior to the addition of 2 × 10⁶ MT2 cells resuspended in serum free medium. Cells were left to adsorb for 30 min before inoculation. HIV LAI, HIV A308 and HIVA308/A324 were taken either directly from stock or after passage for 1 week in selecting anti-V3 MAb P4/D10 or NEA-9205 (6 or 0.8 μg/ml respectively). MAb selected virus was preincubated for 1 h with P4/D10 or NEA-9205 (30 or 4 μg/ml respectively) whereas mock treated virus was not. Cells were then inoculated for 2 h with 1 ml of virus suspension containing approximately 200 TCID₅₀ in the case of virus taken from stock and either P4/D10, NEA-9205 or nothing as appropriate. Non-adsorbed virus was removed by aspiration and to the cells was added an agarose overlay medium consisting of growth medium (RPMI 1640, 20% heat-inactivated fetal calf serum and antibiotics), 0.9% agarose (Sigma) and MAb P4/D10 or NEA-9205 (15 or 2 μg/ml). The plates were then incubated at 37 °C for 6 days when plaques were macroscopically visible. Uninfected cultures treated similarly did not produce plaques. Plaques were picked and transferred to 1 ml cultures of 200000 H9 cells with selecting antibody included in the medium and cultured for 4 days before cell free passage to 3 × 10⁶ H9 cells in 8 ml of growth medium to produce viral stocks.

Sensitivity to neutralization. The sensitivity to neutralization of viral stocks generated by the plaque selection procedure was determined by comparing the infectious titre of the individual stock in the absence and in the presence of antibody. Two identical 3-fold dilution series of virus were made and anti-V3 MAb P4/D10 was added to one of the dilution series to produce a final concentration of 7.5 μg/ml during inoculation. After 1 h, 200000 MT4 cells were added to each dilution and the cells were plated in quadruplicates of 40000 cells. After 4 days of culture supernatant was harvested and the amount of HIV-Ag present was determined using an in-house available biotin-avidin potentiated double antibody ELISA (Nielsen et al., 1987). Cultures giving an absorbance 2-fold above the mean of that of two uninfected cultures were considered positive for infection and the infectious titre in the presence and absence of antibody was determined using the Reed–Muench method (Reed & Muench, 1938). The sensitivity index was defined as the ratio between infectious titre determined in the absence of antibody and in the presence of antibody. A high index signifies resistance to neutralization; a high index signifies sensitivity to neutralization.

DNA sequencing. DNA sequencing of the V3 loop was done with DNA prepared from MT4 cells or H9 cells infected with parental stocks or stocks generated by the immune selection procedure respectively. Approximately 3 × 10⁶ cells were lysed in lysys buffer (100 mM-NaCl, 10 mM-Tris, 25 mM-EDTA, 0.5% SDS and 0.1 mg/ml Proteinase K) for 4 h at 55 °C. The sample was then extracted with phenol-chloroform, precipitated with ethanol and treated with RNase A. The concentration of nucleic acid was determined and 150 ng was subjected to PCR. PCR was done with a sense primer, 5′ GAAGAAGAGTACCAG G 3′, and an antisense biotinylated primer,
5' CCTCATATCCTCTCCAGGTCT 3', amplifying over 40 cycles of thermal cycling a 630 bp fragment containing the entire V3 loop. The biotinylated strand was isolated using streptavidin conjugated Dynabeads (Dynabeads M-280-Streptavidin; Dynal). The sequencing reaction was done using [35S]ATP as label and Sequenase version 2.0 following the manufacturer's instructions. The sequence reaction was denatured in stop solution and the unconjugated strand was separated from the Dynabeads and loaded onto a sequencing gel. Radioimmunoprecipitation and glucosidase treatment of immunoprecipitates. For radioimmunoprecipitation, 2.5 x 10⁶ C8166 cells were either infected with HIV or mock infected. Two days post-infection cells were resuspended in 1 ml of medium lacking methionine and cysteine and metabolically labelled for 7 h with 250 μCi Pro-mix (Amersham) containing a mixture of L-[35S] methionine and L-[35S] cysteine. Metabolically labelled culture supernatant (400 μl) was then absorbed with normal rabbit serum (Dako) and incubated with 4 μg of the anti-V3 MAb NEA-9205 at 4 °C overnight. Then rabbit anti-mouse Ig (Z412, Dako) coupled to Protein A-Sepharose (Pharmacia) was added and the mixtures incubated for an additional 3 h, after which the pellets were washed and eluted by boiling for 5 min in 10 μl 50 mM-sodium acetate (pH 5.3), 20 mM-EDTA, 0.1% SDS, 20 mM-DTT. To 20 μl aliquots of the eluate were added 2.5 μl 5% NP40 and either 0.125 U of endoglycosidase F/N-glycanase F (Boehringer Mannheim) or 2.5 μl of incubation buffer (50 mM-sodium acetate, 20 mM-EDTA, pH 5.3). Then samples were incubated overnight at 37 °C. After incubation samples were mixed with 35 μl of sample buffer (2% SDS, 250 mM-DTT, 0.2% tetraethyldiamine, 0.01% Pyronine Y and 10% glycerol in 0.4 M-Tris-HCl buffer), boiled for 3 min and subjected to SDS-PAGE in a 7% homogenous gel. After fixation, gels were incubated in amplification liquid (Amplify; Amersham), dried and placed on Kodak Biomax film for 4 days.

Results

Variants of HIV LAI lacking the 306 N-glycan

We subjected a molecular clone of HIV LAI, pBRU2, to site-directed mutagenesis to obtain clones lacking the 306 N-glycan. Two such clones were constructed: HIV A308, containing a threonine to alanine mutation at position 308, and HIV A308/A324, containing an additional threonine to alanine mutation at position 324 (Fig. 1). Virus stocks generated by transfection of H9 cells with the molecular clones were compared for kinetics of infection and sensitivity to neutralization. We could not demonstrate any differences in kinetics between mutants and wild-type virus (Hansen et al., 1996). In contrast, when tested for sensitivity to neutralization by the anti-V3 MAb NEA-9205, HIV A308 showed an approximately 200-fold increased sensitivity (IC₅₀ < 0.1 μg/ml) compared to wild-type HIV LAI (IC₅₀ > 20 μg/ml). HIV A308/A324 also displayed an increased sensitivity to neutralization (IC₅₀ = 0.5 μg/ml).

Generation of neutralization-resistant variants of HIV A308 and HIV A308/A324

To investigate the biological importance of the 306 N-glycan, we subjected HIV A308 and HIV A308/A324 to selection pressure using the anti-V3 MAbs P4/D10 and NEA-9205. HIV A308 and HIV A308/A324 were taken either directly from stock or passaged once in the presence of selecting antibody and subjected to a plaque selection procedure. Individual plaques were picked and propagated to produce virus stocks. In all, ten MAb-selected clones were characterized. The sensitivity to neutralization of the selected clones was determined by comparing the infectious titre of the individual virus stock in the absence and presence of anti-V3 MAb (Table 1). All antibody-selected variants were resistant to neutralization by the anti-V3 MAb P4/D10 (sensitivity index < 10) as was HIV LAI. Mock treated virus remained sensitive to neutralization (sensitivity index > 500 for HIV A308 and = 146 for HIV A308/A324), which excluded the possibility that the procedure itself rather than the selecting antibody was responsible for the selection of neutralization resistant mutants.

DNA sequencing of MAb selected clones

To determine to what extent N-linked glycosylation within the V3 loop was responsible for the resistance to neutralization observed among the MAb selected clones described in Table 1, DNA sequencing of the V3 loop was undertaken (Table 2). Six neutralization resistant clones of HIV A308 were sequenced. Four of these had regained the 306 N-glycan through a reversion mutation (Table 2: HIV A308 MAb selected clones 1, 4, 5 and 6), while in two Ala²⁹¹ within the antibody binding site had mutated to Thr (Table 2; HIV A308 MAb selected clones 2 and 3), a mutation previously described to occur among anti-V3 MAb selected mutants (Masuda et al., 1990). Four clones of HIV A308/A324 resistant to neutralization were sequenced. Two of these had regained the 306 N-glycan through an Ala²⁹⁸ to Thr mutation (Table 2: HIV A308/A320 MAb selected clones 1 and 4), while they maintained Ala²⁹¹, excluding any possibility of contamination. We did not find any mutations in the V3 loop in two of the resistant clones (Table 2: HIV A308/A324 MAb selected clones 2 and 3), which could indicate that a change in a distant part of the envelope gene outside the V3 domain might be responsible for development of resistance. Mock treated clones remained unchanged. Thus, of ten clones resistant to neutralization six had become so at least partly by acquisition of a site for an N-glycan within the V3 loop.

Electrophoretic mobility of MAb selected variants of HIV A308/A324

To confirm that the glycosylation site at position 306 was indeed utilized when present in neutralization resistant variants, we immunoprecipitated gp120 obtained from HIV A308/A324 MAb selected clones 1 and 2 as well as mock
Table 1. Sensitivity to neutralization of anti-V3 MAb selected and mock treated clones

The sensitivity to neutralization of MAb selected and mock treated clones was determined by comparing the infectious titre of the individual clone in the absence of antibody (a) and in the presence of antibody (b). The sensitivity index was defined as the linear ratio between infectious titre determined in the absence of antibody and in the presence of antibody. A low index signifies resistance to neutralization; a high index signifies sensitivity to neutralization.

<table>
<thead>
<tr>
<th>Infectious titre (log TCID₅₀/ml)</th>
<th>(a) Without anti-V3 MAb</th>
<th>(b) With anti-V3 MAb</th>
<th>Sensitivity [antilog (a−b) (without MAb/with MAb)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIVₜₘₜ Mock treated Clone 1</td>
<td>4.88</td>
<td>4.48</td>
<td>2.5</td>
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<tr>
<td>HIVₜₘₜ Mock treated Clone 2</td>
<td>5.07</td>
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<td>4.58</td>
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<tr>
<td>HIVₜₘₜ MAb-selected Clone 2</td>
<td>4.64</td>
<td>3.86</td>
<td>6.0</td>
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<tr>
<td>HIVₜₘₜ MAb-selected Clone 3</td>
<td>4.02</td>
<td>3.23</td>
<td>6.3</td>
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<tr>
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<td>3.78</td>
<td>3.78</td>
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<tr>
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<td>4.34</td>
<td>1.7</td>
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<td>HIVₜₘₜ MAb-selected Clone 6</td>
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<td>5.06</td>
<td>1.0</td>
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<tr>
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<td>146</td>
</tr>
<tr>
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<td>2.11</td>
<td>2.11</td>
<td>1.0</td>
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</table>

Table 2. DNA sequencing of the V3 loop of gp120 of parental stocks and immune selected clones

The site for N-linked glycosylation present in HIVₜₘₜ is indicated by (*); the sites of the mutations introduced to make HIVₜₘₜ and HIVₜₘₜ are indicated by (△).

- HIVₜₘₜ parental: Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly Asn Met Arg Gln Ala His Cys
- HIVₜₘₜ mock treated: Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala Phe Val Thr Ala Ile Gly Lys Ile Gly Asn Met Arg Gln Ala His Cys
- HIVₜₘₜ MAb selected: Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala Phe Val Thr Ala Ile Gly Lys Ile Gly Asn Met Arg Gln Ala His Cys

Treated HIVₜₘₜ with NEA-9205. NEA-9205 efficiently precipitated gp120 from both sensitive and resistant clones indicating that the epitope of NEA-9205 was present (Fig. 2). Aliquots of the immunoprecipitate were incubated with and without endoglycosidase F/N-glycanase F. If not treated with endoglycosidase, HIVₜₘₜ MAb selected clone 1 displayed a higher apparent molecular mass (118 kDa) than both HIVₜₘₜ MAb selected clone 2 (115 kDa) and HIVₜₘₜ mock treated clone 1 (115 kDa) (Fig. 2). This shift in electrophoretic mobility was not observed after treatment with endoglycosidase to remove N-linked glycans. As shown in Fig. 2 endoglycosidase treated gp120 from all clones displayed similar electrophoretic mobility. The apparent molecular mass of deglycosylated gp120 was calculated to be 57 to 58 kDa, a size consistent with complete removal of N-linked oligosaccharides. Thus, in agreement with the sequencing results the site
for the \( ^{306}N \)-glycan was present and utilized only in HIV\(_{\text{A308/A324}} \) selected clone 1.

**Discussion**

The modulation of sensitivity to neutralization by an \( N \)-linked oligosaccharide within the V3 loop has been reported previously (Back et al., 1994). We confirm this finding by demonstrating an even more dramatic shift in resistance to neutralization mediated by an oligosaccharide in HIV\(_{\text{LAI}} \). We observed a several hundred-fold increase in sensitivity to V3-mediated neutralization of a mutant of HIV\(_{\text{LAI}} \) lacking the \( ^{306}N \)-glycan (cf. Table 1), while Back et al. (1994) reported a more than 8-fold increase in sensitivity of a mutant of HXB2. Furthermore, we extend the previous report by showing that HIV variants lacking the \( ^{306}N \)-glycan readily and primarily develop resistance to neutralization by antibodies directed to a principal neutralization determinant through acquisition of an oligosaccharide. In the HIV-infected individual such antibodies are abundant (Chamat et al., 1992; Profy et al., 1990), suggesting that our demonstration of viral adaptation to immune pressure by the acquisition of an oligosaccharide may be of pathogenic importance.

We believe that the acquisition of carbohydrate is a general mechanism by which a virus reduces its sensitivity to neutralization, and we suggest that this mechanism is not limited to HIV strains adapted to cell lines, such as the LAI isolate. First of all, the presence of the \( ^{306}N \)-glycan in a hypervariable region is conserved among HIV isolates. It might also be noted that Bou-Habib et al. (1994) passaged a monocytotropic HIV isolate in the absence of selection pressure to obtain T cell tropic variants. A T cell tropic variant proved sensitive to V3 mediated neutralization in contrast to the original monocytotropic isolate. Among the changes noted in the V3 loop of this isolate was disruption of a site for \( N \)-linked glycosylation homologous to the \( ^{306}N \)-glycan. On the basis of our results we suggest that the increased sensitivity to neutralization observed in the Bou-Habib study was caused by the loss of the \( N \)-linked oligosaccharide. Thus, the selective modulation of sensitivity to neutralization may also extend to monocytotropic HIV strains.

The acquisition of \( N \)-linked oligosaccharides has previously been shown to cause decreased sensitivity to neutralization, e.g. for rotavirus (Caucs et al., 1987) and influenza virus (Skehel et al., 1984). Late variants isolated from macaque monkeys inoculated with molecular clones of simian immunodeficiency virus exhibit the acquisition of new sites for possible \( N \)- as well as \( O \)-linked glycosylation (Overbaugh & Rudensey, 1992). These changes may be a consequence of adaptation of the virus to the immune environment in the host. Similarly, based on the study of sequence polymorphisms, a mechanism for the insertion of asparagine residues has been suggested for HIV (Bosch et al., 1994). If followed by point mutations this mechanism would create new sites for \( N \)-linked glycosylation which might be of selective advantage. Our study demonstrates that HIV variants containing an \( N \)-linked oligosaccharide are readily selected in cell culture if subjected to an immune selection pressure against a principal neutralizing determinant. This provides experimental support for the hypothesis that glycosylation is involved in virus evasion of immune
neutralization and consequently in the persistence of lentiviral infections.

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