Carbohydrate determinant NeuAc-Galβ(1-4) of N-linked glycans modulates the antigenic activity of human immunodeficiency virus type 1 glycoprotein gp120

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In the present study we investigated to what extent the peripheral carbohydrate structure of N-linked glycans influences the antigenic properties of human immunodeficiency virus type 1 glycoprotein 120 (gp120). Recombinant gp120 was purified from GMK cells infected with a recombinant vaccinia virus expressing gp120. Purified gp120 was then coated onto 96-well ELISA microplates and subjected to sequential removal of peripheral monosaccharide units. Modified or unmodified gp120 was then incubated with monoclonal antibodies recognizing specific epitopes of gp120 and with a reporter lectin to determine the extent of carbohydrate elimination. Antibody and lectin binding was quantified in an enzyme-linked system. We found that the carbohydrate structure NeuAc-Galβ(1-4) of N-linked glycans, defined both by lectin reactivity and by specific glycosidases, is involved in modulating the binding of antibody to a number of epitopes of peptide nature. The binding of antibody to one class of epitopes, situated in a region between amino acids 200 and 230, was strongly increased by removal of NeuAc-Galβ(1-4), whereas the binding to epitopes in the V3 region was decreased and the binding to epitopes in the far N-terminal region was not altered by the treatment. These results suggested that peripheral structures of N-glycans are involved in modulating the overall conformation of gp120.

Introduction

Human immunodeficiency virus type 1 (HIV-1) glycoproteins gp120 and gp41 constitute principal targets for the humoral immune response (for a review see Nara et al., 1991). The carbohydrate complement of the highly glycosylated gp120, which contains 24 utilized sites for N-linked glycosylation (Leonard et al., 1990), forms about 50% of its Mr (Geyer et al., 1988) and a broad variety of both high mannose- and complex-type oligosaccharides have been identified (Geyer et al., 1988; Mizuochi et al., 1988, 1990). In addition, studies with mouse anti-carbohydrate monoclonal antibodies (MAbs) indicate the presence of short-chain O-glycans on gp120; these are not expressed in normal uninfected cells although they are probably assembled by the host cell glucosyl transferases (Hansen et al., 1990, 1991). Some of these neo-antigens may constitute efficient targets for broadly reactive neutralizing antibodies (Hansen et al., 1990, 1991).

However, peripheral carbohydrate structures may also modulate the immunological activity of peptide epitopes. In previous reports we have found that enzymatic removal of the terminal sialic acid and penultimate β(1-4)-linked galactose residues from N-linked glycans of herpes simplex virus type 1 (HSV-1) glycoprotein gC-1 results in a dramatic decrease in the antibody-binding capacity of several clustered conformational epitopes of peptide nature (Olofsson et al., 1990; Sjöblom et al., 1987). This was accomplished by the use of special ELISA techniques designed to screen epitopes of viral glycoproteins for changes in their antibody binding capacity caused by small changes in the structure of peripheral carbohydrates (Olofsson et al., 1990; Sjöblom et al., 1987). The aim of the present study was to determine whether the numerous complex-type oligosaccharides could modulate the antigenic properties of gp120 in a similar manner. To this end, the same techniques were applied to purified recombinant gp120. We show that removal of the terminal sialic acid and
penultimate β(1-4)-linked galactose residues results in an increase in the antibody-binding capacity of epitopes located at the junction between the V2 and C2 regions, and decreased activity of epitopes in the V3 region.

**Methods**

**Virus and cells.** Recombinant vaccinia virus expressing the envelope glycoprotein gp120 was constructed according to the general scheme of Hu et al. (1986) with the following differences. (i) Expression of gp120 was under the control of the vaccinia virus 11K and not the 7.5K promoter, and (ii) a stop codon was introduced by site-specific mutagenesis immediately downstream from the sequence encoding the proteolytic cleavage site between gp120 and gp41, resulting in expression of gp120 as a secreted glycoprotein. Green monkey kidney (GMK) (Günlup, 1965) cells were used throughout the study. The cells were grown in 1 l roller bottles in Eagle’s MEM supplemented with 1% foetal calf serum and 10000 international units (IU)/ml penicillin and streptomycin.

**Virus infection and preparation of antigen.** Confluent monolayers of GMK cells maintained in roller bottles were infected at a multiplicity of 10 according to the method of Bolmstedt et al. (1991). The culture supernatant was harvested when the c.p.e. was complete, and supplemented with 1 mM-PMSF (Sigma) prior to 15-fold concentration in an Amicon hollow fibre cartridge type H1PBO-20 according to the manufacturer’s instructions. Recombinant gp120 was purified from the concentrated culture medium according to the method of Fenouillet et al. (1990), and the purity of gp120 was determined to be about 95% by SDS-PAGE and Coomassie blue staining (Fig. 1).

**MAbs and polyclonal antisera.** The MAbs used are shown in Table 1. Two rabbit antisera against gp160 (cloned in vaccinia virus (Fuerst et al., 1987) and produced in Vero cells) were prepared by immunizing two New Zealand white rabbits (National Veterinary Institute, Sweden) three times at monthly intervals with 10 μg of gp160 iscoms (Åkerblom et al., 1991). Sera from HIV-1-seropositive humans were obtained from the Department of Clinical Virology, Göteborg, Sweden.

**In vitro neutralization test.** MT-4 cells (10⁶) in growth medium were inoculated with 25 TCID₅₀ HIVΔIII-V for 2 h. Prior to inoculation, the virus inoculum was preincubated for 1 h with a dilution series of MAbs (Hansen et al., 1991). After inoculation, cells were washed and quadruplicate samples of 200000 cells each were cultured in growth medium without MAb in 24-well culture plates. Infection was evaluated using an HIV antigen-capture ELISA and samples were taken from culture supernatants 4 days after inoculation.

**Neuraminidase/periodate treatment and enzyme-linked assays.** Sequential removal of carbohydrates from gp120 coated onto microtitre plates was carried out as described previously for HSV-1 gC-1 (Sjöblom et al., 1987). Polystyrene microplates (Dynatech PVC) were coated with purified preparations of gp120 (100 μl/well in 0.1 M-sodium carbonate buffer pH 9.6) by incubation for 24 h at 4 °C. The concentration of

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**Table 1. Characterization of MAbs**

<table>
<thead>
<tr>
<th>MAb designation (Ig class)</th>
<th>Immunogen</th>
<th>Epitope localization*</th>
<th>Mapping data</th>
</tr>
</thead>
<tbody>
<tr>
<td>110.4 (IgG1)</td>
<td>Purified gp from HIV (LAV-1) (Gosting et al., 1987)</td>
<td>V3 region (amino acids 302 to 323)</td>
<td>Linsley et al. (1988)</td>
</tr>
<tr>
<td>F58/H3 (IgG1)</td>
<td>Recombinant gp160 (HIV IIIb iscoms (Åkerblom et al., 1991))</td>
<td>V3 region (amino acids 304 to 323)</td>
<td>Åkerblom et al. (1990)</td>
</tr>
<tr>
<td>3D3, B8, 4C11, D8, 3F4, E3, 4C11, A9 (IgM)</td>
<td>MAB 3D3, B8: extract from purified virions (IIIb) Other MAbs: recombinant gp120 (GMK cells)</td>
<td>Junction of V2-C2 regions (amino acids 200 to 230)</td>
<td>Based on the reactivity of two consecutive peptides out of a panel of 33 partially overlapping peptides (S. Jeansson, unpublished results) Åkerblom et al. (1990); unpublished results</td>
</tr>
<tr>
<td>T9 (IgG1), T2 (IgG2A)</td>
<td>Recombinant gp160 (HIV IIIb iscoms (Åkerblom et al., 1991))</td>
<td>Amino acids 89 to 113 (T9); amino acids 90 to 126 (T2)</td>
<td>Åkerblom et al. (1990); unpublished results</td>
</tr>
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</table>

* Amino acid numbering according to the Los Alamos database.
glycoprotein was measured by the method of Lowry et al. (1951) and in most experiments the coating concentration was 0.1 μg/ml. The plates were subsequently treated with 50 μl/well sialidase (Behringwerke; 100 mU/ml) at 37 °C for 2 h and thereafter with serial dilutions of sodium periodate for 1 h at room temperature, according to Woodward et al. (1985). The plates were subsequently incubated with mouse MAb, rabbit hyperimmune sera to gp160/gp120 or selected antisera from seropositive individuals. The reactions were visualized as described below. All serum dilutions used in this assay were optimized according to Sjöblom et al. (1987).

The effect of the combined sialidase–periodate treatment on the oligosaccharide side-chains was determined by the use of biotinylated *Ricinus communis* lectin (RCA) (Sjöblom et al., 1987). The reactions were visualized by addition of alkaline phosphatase-conjugated avidin (lectin assay) or alkaline phosphatase-conjugated goat antibodies against mouse IgG, mouse IgM, rabbit IgG or human IgG, using disodium *p*-nitrophenyl phosphate (Sigma) as a substrate. The absorbance was measured at 405 nm.

**Treatment with β-galactosidase and glycopeptidase F (PNGase)**

Purified gp120 was coated onto microtitre plates and subjected to neuraminidase treatment to remove sialic acid as described above. After washing the plates were treated with 10 U/well *Escherichia coli* β-galactosidase (Sigma, grade IX; in 0.1 M-phosphate buffer supplemented with 1 mM-MgCl2) (Sjöblom et al., 1987) or with 1 U/well PNGase (Boehringer Mannheim) in 0.2 M-phosphate buffer pH 7.5, 10 mM-EDTA for 24 h at 37 °C. The plates were washed and binding of antibodies and RCA to gp120 was assayed as above.

**Results**

To demonstrate the possible existence of carbohydrate-modulated epitopes, we coated purified recombinant gp120 onto microplates and removed the peripheral carbohydrates by sequential treatment with sialidase and increasing concentrations of periodate, as previously described for HSV-1 gC-1 (Sjöblom et al., 1987). The disappearance of sialic acid and galactose was monitored by the use of biotinylated RCA, with main specificity for terminal β(1-4)-linked galactose (Goldstein & Poretz, 1986) (Fig. 2). The sialidase treatment caused an increase in RCA-binding activity, whereas the subsequent periodate treatment caused a gradual decrease in RCA-binding activity, indicating disappearance of the penultimate galactose (Fig. 3a).

A panel of gp120-specific MAbs was analysed in the same system and we found that removal of peripheral sugars from gp120 had different effects depending on the epitope investigated (Fig. 3a, b) and that three classes of epitopes could be recognized (Table 2). Ep3D3.B8 represents a class of epitopes, designated I, for which sialidase–periodate treatment results in an increase in antibody binding (Fig. 3a). This takes place in parallel with the decrease in RCA-binding activity, suggesting that sialic acid and galactose units blocked antibody binding. The activity of class II epitopes, exemplified by EpF58 (Fig. 3b), was decreased by the sialidase–periodate treatment, indicating that sialic acid and galactose units blocked antibody binding.

**Table 2. Binding of MAbs to deglycosylated gp120**

<table>
<thead>
<tr>
<th>MAb designation</th>
<th>Neuraminidase*</th>
<th>PNGase†</th>
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<tbody>
<tr>
<td></td>
<td>M ± S.E.M.</td>
<td>M ± S.E.M.</td>
</tr>
<tr>
<td>Class I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D3.B8</td>
<td>0.67 ± 0.09</td>
<td>2.16 ± 0.18</td>
</tr>
<tr>
<td>4C11.D8</td>
<td>1.11 ± 0.04</td>
<td>1.56 ± 0.06</td>
</tr>
<tr>
<td>3F4.E3</td>
<td>0.49 ± 0.01</td>
<td>1.55 ± 0.12</td>
</tr>
<tr>
<td>4C11.A9</td>
<td>0.48 ± 0.02</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F58</td>
<td>2.05 ± 0.07</td>
<td>0.83 ± 0.10</td>
</tr>
<tr>
<td>110.4</td>
<td>2.38 ± 0.07</td>
<td>1.71 ± 0.11</td>
</tr>
<tr>
<td>Class III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>1.84 ± 0.06</td>
<td>1.74 ± 0.03</td>
</tr>
<tr>
<td>T9</td>
<td>2.12 ± 0.06</td>
<td>2.26 ± 0.11</td>
</tr>
</tbody>
</table>

*Absorbance determinations from gp120-coated ELISA plates treated with sialidase. Mean (M) and standard error of mean (S.E.M.; n = 3) values are given.
† Gp120 was treated with sialidase and PNGase as described in Methods, and absorbance values were measured.
galactose contribute to antibody binding. Class III epitopes, such as EpT9, were not affected by the sialidase-periodate treatment (Fig. 3a). The MAbs used in this study were tested for in vitro neutralizing activity using the HIVHIV-TLV strain and MT-4 cells. MAbs to Class II epitopes inhibited HIV infection in a dose-dependent manner (50% inhibition at 25 μg/ml antibody), whereas the other antibodies did not inhibit infection at any concentration (0 to 250 μg/ml).

To exclude the possibility that a periodate-induced chemical modification of the polypeptide backbone was responsible for the altered antigenic properties observed, we subjected gp120 to enzymatic degalactosylation after sialidase treatment (Fig. 4). In these experiments we used *E. coli* β-galactosidase, which releases galactose in the β(1-4) but not in the β(1-3) conformation (Lundström et al., 1987) under the conditions used in our assay system. Removal of sialic acid and galactose induced an increase in binding of antibody by class I epitopes and a decrease in that by class II epitopes, whereas that by the RCA control (Fig. 4a) decreased. These results confirmed that the alterations in antibody binding by class I and class II epitopes were due to changes in carbohydrate composition, and that β(1-4)-linked galactose was the active carbohydrate determinant.

As the removal of galactose from N-linked glycans results in exposure of penultimate wheatgerm agglutinin (WGA)-binding GlcNAc units (Sjöblom et al., 1987), it was necessary to exclude the possibility that GlcNAc was a physical part of class I epitopes. In these experiments we tried to block antibody binding by adding N,N'-diacetylchitobiose, of which the inhibitory activity on WGA binding is at least 20-fold greater than that of monomeric GlcNAc (Goldstein & Poretz, 1986). We were not able to block the binding between MAb 3D3.B8 and degalactosylated gp120 with 0.5 mM-N,N'-diacetylchitobiose (Fig. 5), a concentration inhibiting the binding between WGA and gp120 (Fig. 5, inset). In addition, concentrations as high as 25 mM-GlcNAc or -galactose did not interfere with antibody binding to gp120 (data not shown). These results support the conclusion that galactose is an indirect modulator of antibody binding and that GlcNAc is not a physical part of the binding epitope.
To obtain direct evidence that N-linked glycans were engaged in the observed modulation of antibody binding, we analysed gp120 after removal of all N-linked oligosaccharides by PNGase treatment. This enzyme liberates both high mannose-type and complex-type N-linked glycans, disrupting the N-glycosidic linkage (Tarentino et al., 1985). An increase in antibody binding by class I epitopes and a decrease in that by class II epitopes was noted after removal of N-glycans by using PNGase (Table 2). These results confirmed that the antigenicity-modulating β(1-4)-linked galactose was associated with complex-type N-linked oligosaccharides of gp120.

To determine the influence of terminal galactose and sialic acid on the overall antibody binding of gp120, we analysed the reactivity of a gp120/gpl60-specific rabbit hyperimmune serum in the same assays as described above. The reactivity of this antiserum was neither increased nor decreased by removal of NeuAc-Galβ(1-4) (Fig. 3b, Fig. 4e), indicating class III behaviour of the epitopes reacting with this antiserum. Similar results, i.e. class III behaviour, were also found for two human sera (data not shown). However, removal of N-linked glycans from gp120 with PNGase resulted in a significant increase in the reactivity of two rabbit hyperimmune sera to gp160/gp120 and that of two sera from HIV-infected patients (Fig. 6).

**Discussion**

In the present paper we have shown that the terminal sialic acid and galactose units of N-linked glycans are involved in positive and negative modulation of a number of defined gp120 epitopes. The conclusion that galactose is involved is based on two independent lines of evidence. The periodate-induced change in antigenicity correlates with a decrease in gp120 reactivity with the galactose-specific lectin RCA, and enzymatic removal of galactose with *E. coli* β-galactosidase results in a change in antibody binding of particular epitopes. Previous studies in our laboratory have shown that cleavage of Galβ(1-4) but not Galβ(1-3) takes place under the experimental conditions used (Lundström et al., 1987). The conclusion that N-linked glycans are involved is supported by experiments with PNGase, which releases N-linked glycans. Therefore we conclude that the carbohydrate structural determinant NeuAc-Galβ(1-4) is involved in the modulation of antibody binding by gp120.

We found that the epitopes investigated could be classified into three groups, depending on the consequences of desialylation and degalactosylation for each epitope (Fig. 7). Antibody binding to one class of epitopes, mapped to amino acids 200 to 230, was found to be enhanced by elimination of NeuAc-Gal from N-linked glycans. This region, situated downstream from the V2 region, is conserved (Modrow et al., 1987) and is characterized by a high degree of secondary structure and several complex-type N-linked glycans, as determined for recombinant gp120 (Leonard et al., 1990).
possibility that carbohydrate determinants were engaged in antibody binding was ruled out by the findings that the MAbs react with synthetic peptide sequences of gpl20 and that even high concentrations of galactose or GlcNAc do not inhibit binding between the antibody and degalactosylated gp120. In contrast, the antigenic activities of class II epitopes, EpiF58 and Epi110.4, were decreased by removal of sialic acid and galactose. There is no reason to believe that the oligosaccharides themselves participate in direct paratope binding, because MAbs F58 and 110.4 both react with synthetic peptides. Our results regarding class II epitopes are in agreement with those of Fenouillet & Gluckman (1991), who reported that binding of MAb 110.4 to gp160 produced in the presence of deoxynojirimycin was 10-fold less than that to fully glycosylated gp160.

Removal of NeuAc and galactose from gp120 does not change the net reactivity of polyclonal sera with gp120, irrespective of origin. This was not unexpected and suggests that the number of epitopes inducible and the number of epitopes blocked by NeuAc-Gal, respectively, are similar, and that the polyclonal sera react equally well with both types of epitope. As discussed in more detail below, it is important to determine whether these two classes of epitope differ in their properties as targets for neutralizing antibodies. The reactivity of both human and rabbit sera was slightly increased by PNGase treatment of gp120, suggesting that the inner part of N-glycans exerts some extra blocking activity on at least some epitopes which is not increased by removal of NeuAc-Gal.

Enhancement of binding by class I epitopes required exactly the same change in carbohydrates as did the decrease in binding by class II epitopes, therefore it is tempting to speculate that removal of the determinant NeuAc-Galβ(1-4) induces a larger shift in the overall conformation of gp120, including greater exposure of class I epitopes and decreased exposure of class II epitopes. Both NeuAc and Gal have been reported to influence the conformation of viral and cellular glycoproteins in solution (Rademacher et al., 1988). The major contribution of sialic acid to glycoprotein conformation is associated with the strong negative charge. Moreover, galactose, located in the flexible Galβ(1-4)GlcNAcβ(1-6) antenna of large complex-type oligosaccharides may interact directly with the protein surface of the Fc part, contributing to a biologically active conformation of this part of human IgG (Rademacher et al., 1988). We have data supporting a similar function for galactose in HSV-1 gC-1 (Olofsson et al., 1990). The degree of sialylation and galactosylation varies in different types of cell, and gp120, as described for other glycoproteins (Rademacher et al., 1988), probably exists in several glycoforms with slightly different biological properties determined by the host cell. Independent support for the conclusion that the accessibility of epitopes in the V3 region is susceptible to induced changes in the overall conformation of gp120 comes from Clements et al. (1991). These authors have shown that treatment of gp120 with non-ionic detergents results in complete resistance of the thrombin-sensitive site of the V3 loop to the action of this proteolytic enzyme.

Although the carbohydrate-induced modulation of antigenic activity reported in the present study is an in vitro phenomenon, a number of other studies emphasize the significance of N-linked glycans for the biological properties of gp120. Thus, Wolinsky et al. (1992) have shown that transmission of HIV from mothers to infants is strongly selective for virus variants lacking one N-linked glycan in the V3 region. Moreover, Fung et al. (1992) have found that a broadly neutralizing MAb, reacting with a conformational epitope in the V2 region, is dependent on gp120-associated N-linked glycans for epitope binding. Carbohydrate-induced modulation of antibody binding, as described above, has been suggested to be a viral strategy for escaping immune responses against conserved regions (Bolmstedt et al., 1991; Lee et al., 1992). Benjouad et al. (1992) have found that hyperimmune sera raised against desialylated gp160 show increased reactivity to a synthetic peptide representing a region of gp120 situated close to the class I epitopes defined in the present study. Furthermore, this antiserum against desialylated gp120 showed increased cross-reactivity with HIV-2 gp140. In this context it is interesting to note that the region harbouring the class I epitopes, the reactivity of which is enhanced by removal of NeuAc-Galβ(1-4), is highly conserved among HIV isolates (Modrow et al., 1987). Experiments to clarify the biological significance of this concept have been initiated.

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