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Role of N-Linked Glycans of Envelope Glycoproteins in Infectivity of Human Immunodeficiency Virus Type 1

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We have shown that enzymatic removal of N-linked glycans from human immunodeficiency virus type 1 (HIV-1) recombinant envelope glycoproteins gp160 and gp120 produced in BHK-21 cells did not significantly reduce their ability to bind to CD4, the cellular receptor for the virus. Because recombinant proteins may behave differently from proteins present on virions, we investigated whether such viral envelope glycoproteins either in a purified form or present on viral particles could be deglycosylated by treatment with an endoglycosidase F-N-glycanase mixture which cleaves all accessible glycan moieties. Endoglycosidase analysis of the carbohydrate composition of purified viral gp120 (vgp120) indicated a glycosylation pattern similar to that for recombinant gp120 (rgp120), and treatment with endoglycosidase F-N-glycanase resulted in comparable molecular weight (M_W) reduction for both molecules. Similarly, after immunoblotting of the deglycosylated viral preparation, the characteristic 160- and 120-kilodalton (kDa) bands were replaced by 90- and 60-kDa bands, respectively. The apparent M_W of gp41 shifted to 35 kDa. These results are consistent with complete deglycosylation. The immunoreactive conformation of envelope glycoproteins remained unaltered after deglycosylation: they were recognized to the same extent by specific human polyclonal or mouse monoclonal antibodies, and no proteolysis of viral proteins occurred during enzymatic treatment. Deglycosylation of vgp120 resulted in a less than 10-fold reduction of the ability to bind to CD4, presented either in a soluble form or at the cell membrane. In addition, deglycosylation significantly reduced, but did not abolish, HIV-1 binding to and infectivity of CD4$^+$ cells as determined, respectively, by an indirect immunofluorescence assay and a quantitative dose-response infection assay. Taken together, these results indicate that removal of glycans present on mature envelope glycoproteins of HIV-1 diminishes but does not abolish either virus binding to CD4 or its capacity to infect CD4$^+$ cells.

The envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) are highly glycosylated molecules, N-linked glycans representing one-half of their molecular weight (M_W) (7, 23). Therefore, carbohydrates are likely to be prominent structures on the surface of HIV-1, but their precise function is currently poorly understood. This role is likely to be important because one-half of the asparagine residues of the *env* sequence are involved in its approximately 30 glycosylation sites and because these sites are rather conserved when one compares different HIV-1 isolates or HIV-1 with HIV-2 and simian immunodeficiency virus (2, 37).

The *env* gene product is a 160-kilodalton (kDa) precursor glycoprotein (gp160) that is proteolytically cleaved prior to viral assembly and budding (24) into outer-membrane gp120 (which mediates binding to CD4) and transmembrane gp41 (which is responsible for membrane fusion leading to virus entry into the cells and to cytopathogenicity) (4, 16, 25).

Glycosylation is certainly important for the bioactivity of glycoproteins (1, 8, 10, 28). Virus produced in the presence of glucosidase inhibitors, which results in aberrant glycosylation, displays markedly reduced infectivity and cytopathogenicity (12). It has also been suggested that carbohydrates at the surface of the mature molecule may play a direct role per se in the interaction of gp120 with CD4 (21, 32). However, by using purified recombinant gp120 and gp160 (rgp120/rgp160) produced in mammalian cells (BHK-21) infected with recombinant vaccinia virus, we have recently shown that enzymatic removal of glycans carried out in the absence of denaturating agents did not significantly affect the native conformation of rgp120/rgp160, since binding activity to CD4 was only slightly reduced (7).

Because recombinant proteins may display properties that are different from those of viral proteins in solution or included in the viral envelope, we have investigated here whether immunopurified soluble viral gp120 (vgp120) or gp120 and gp41 present at the surface of viral particles could be deglycosylated under the conditions previously reported (7) and whether this procedure altered binding to CD4 and virus infectivity. Our data show that enzymatic removal of glycans from envelope glycoproteins results in the reduction, but not in the abrogation, either of binding to CD4 or of viral infectivity for CD4$^+$ lymphoid cells.

MATERIALS AND METHODS

Soluble recombinant proteins. Soluble CD4 (sCD4) was a gift from R. Axel (Columbia University, New York, N.Y.). rgp120 and rgp160 were obtained from Transgène S.A. (Strasbourg, France).

Purification of viral gp120. HIV-1 LAV_{BRU} was purchased from Diagnostics Pasteur (Marnes-la-Coquette, France) as a viral pellet (1 mg/ml). It was solubilized in 5 ml of 10 mM Tris (pH 7.4)–100 mM NaCl–100 mM EDTA–1.5% Tween 20 (Bio-Rad Laboratories, Richmond, Calif.) in order to disrupt viral particles. After 10 h at 20°C, 50 ml of 20 mM phosphate-buffered saline (PBS) (pH 7.4)–150 mM NaCl containing 1 g of Sepharose CL4B (Pharmacia, Uppsala, Sweden) coupled to 1 mg of monoclonal antibody (MAb) 110-4 (Genetic Systems, Seattle, Wash.) according to the instructions of the manufacturer was added to immunopurify the solubilized

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gp120. After overnight incubation at 4°C in the presence of 6,000 cpm of radiolabeled rgp120 (50 μCi/μg) (as described in reference 7), which was added in order to monitor the purification yield, two centrifugations (200 × *g*) and washes with PBS (50 ml) were performed to remove unbound material. The dissociation of gp120 immune complex with MAb 110-4 was performed twice with 2 ml of 0.5 M formic acid–150 mM NaCl. The pH of the eluates was immediately raised to pH 7 by the addition of ammonium acetate, and the radioactivity of each eluate fraction was counted before lyophilization. rgp120 was similarly treated to verify the purification process and its possible consequences on the bioactivity of the molecule.

Western blot (immunoblot) analysis of purified viral gp120 and of the viral preparation. (i) Viral gp120. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to estimate the purity of immunopurified vgp120. The products were from Bio-Rad. One microgram of vgp120 was heated at 80°C for 5 min in the presence of 20% SDS–1% β-mercaptoethanol and then submitted to SDS-PAGE (12.5%) (20). After transfer (36), the nitrocellulose membrane was saturated with PBS–5% casein, for 30 min at 25°C, and incubated overnight at room temperature with an anti-HIV-1-positive human serum (1:100 dilution). Staining was done according to the LAV BLOT kit instructions (Diagnostics Pasteur). rgp160 was analyzed in the same manner in order to compare the purity of the two molecules. Deglycosylated recombinant and viral gp120 treated by endoglycosidases (as described below) were analyzed in the same manner, and staining was performed by using MAb 110-4 diluted 1:100.

(ii) Virus preparation. A 50-μg portion of control or deglycosylated virus (as described below) suspended in buffer without bovine serum albumin (BSA), to avoid any interference with the band corresponding to BSA during migration, was heated at 80°C for 5 min in the presence of 20% SDS–1% β-mercaptoethanol and subjected to SDS-PAGE (12.5%). Transfer to nitrocellulose was done as previously described, and staining was performed either with serum from an HIV-1-seropositive patient or with a mixture of murine MAb to gp120 (110-4) and to gp41 (41-6) at a 1:100 dilution.

Deglycosylation procedures. (i) Analytical deglycosylation of recombinant and viral gp120. A 500-ng portion of purified viral or recombinant gp120 was incubated at 37°C in 30 μl of PBS in the presence of endoglycosydase H (endo H) (3 mU), endoglycosydase F-N glycanase free (endo F) (300 mU) or endoglycosydase F-N glycanase mixture (NG) (300 mU). Enzymes (17, 31, 35) (Boehringer Mannheim, Federal Republic of Germany) were added three times during a 20-h incubation. Samples were then submitted to Western blot analysis as previously described. Mock treatment of control samples was performed in each experiment.

(ii) Deglycosylation of vgp120 used in binding assays. A 5-μg portion of vgp120 was treated with NG (3 U) sequentially added three times during a 20-h incubation in a PBS final volume of 200 μl. A 500-ng portion was analyzed by Western blot, as described above, to verify deglycosylation.

(iii) Virus deglycosylation. Virus (100 μg) was treated at 37°C with NG in a PBS final volume of 200 μl; 3 U of enzyme were sequentially added three times during a 20-h incubation. Simultaneously, 6 μl of infectious virus supernatant (10^4 tissue culture infective dose per ml), corresponding to 6 ng of virus, was treated under the same conditions three times with 0.5 U of enzyme in a final volume of 40 μl. Control heat-inactivated or infectious virus was mock deglycosylated with the same treatment except for the enzyme.

Molecular-binding assay. The ability of deglycosylated vgp120 to interact with radiolabeled sCD4 was compared with that of the untreated molecule (as described in reference 7). A 50-μl portion of MAb 110-4 coupled to Sepharose was incubated for 2 h at 4°C with increasing amounts of rgp120 or of glycosylated and deglycosylated vgp120. Two washes with 4 ml of PBS–0.5% BSA–0.05% Tween 20 allowed removal of unbound vgp120. The complexes formed between MAb 110-4 and vgp120 were then incubated with a constant amount of ^{125}I-sCD4 (80,000 cpm) (5.5 μCi/μg) for 2 h at 4°C. After two more washes, the solid-phase-bound radioactivity was counted.

$CD4^+$ cells. Cells of the MT2 line (22) and clone 13 (CEM13) that were derived from the CEM line (American Type Culture Collection, Rockville, Md.) were cultured at 37°C in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum, 1% glutamine, and 1% antibiotics (streptomycin and penicillin) in a humidified atmosphere with 5% CO_2.

Analysis by indirect immunofluorescence assay of viral gp120 or of viral particles to $CD4^+$ cells. Increasing amounts of untreated or deglycosylated vgp120 or virus were incubated for 1 h at 20°C with 5×10^5 $CD4^+$ cells in a 100-μl volume of PBS–0.5% BSA–0.05% NaN_3. This temperature allows gp120 and virus binding but prevents enzyme activity (data not shown). The cells were washed and incubated in 50 μl of MAb 110-4 diluted 1:100 in buffer. After 1 h at 4°C, they were washed and incubated for 1 h at 4°C with a 1:25 dilution of anti-mouse immunoglobulin G (IgG) coupled to biotin (50 μl) (Amersham International, Amersham, United Kingdom). After being washed, the cells were stored in 50 μl of a 1:1,000 dilution of streptavidin-phycoerythrin (Becton Dickinson and Co., Mountain View, Calif.). After being washed, the cells were stored in 500 μl of buffer containing 1% paraformaldehyde. Cell membrane fluorescence intensity was measured with the FACS analyzer (Becton Dickinson).

HIV-1 infection of $CD4^+$ lymphoid cells. After enzymatic or mock treatment of the infectious virus preparation, 1×10^6 MT2 cells were mixed for 5 h with viral suspensions corresponding to 5, 0.5, or 0.05 μl of the original supernatant, in a 1-ml final volume of culture medium. The cells were than washed twice with 15 ml of RPMI 1640, and they were subsequently cultured in 24-well plates at 5×10^5 cells per ml. Each day a quarter of the supernatant was changed, and 150-μl aliquots were stored at −80°C for later measure of viral production.

Measure of viral production in the supernatants of infected cultures. Determination of viral-antigen production was performed on 10 μl of each sample by the immunocapture assay from Diagnostics Pasteur (ELAVIA Ag I kit) according to the specifications of the manufacturer.

For determination of reverse transcriptase activity, 50 μl of supernatant was incubated with 0.1% Triton X-100–150 mM KCl–10 mM dithiothreitol for 10 min at 4°C. Buffer (40 μl) (50 mM Tris hydrochloride [pH 7.8], 10 mM $MgCl_2$, 2 mM EGTA) containing 5 μg of poly(rA) oligo(dT) and 5×10^6 cpm of [^{3}H]dTTP (0.2 TBq/mM; Amersham) was added for 1 h at 37°C. The reaction was stopped with 20 μl of 120 mM $Na_4P_2O_7$–60% trichloroacetic acid at 4°C for 15 min. The content of the wells was precipitated on glass fiber filters with 12 mM $Na_4P_2O_7$–5% trichloroacetic acid, and radioactivity was counted in a liquid scintillation counter.

Immunocapture assay for the analysis of antibody recognition of deglycosylated virus. Microdilution plates (96-well)

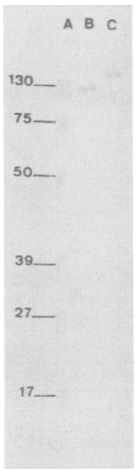

FIG. 1. Immunoblot analysis of purified viral gp120. Immunopurified vgp120 (lane B) and rgp160 (lane C) were submitted to SDS-PAGE and transferred to a nitrocellulose filter in order to estimate the purity of the viral gp120 preparation. Staining was performed by using the serum of an anti-HIV-1-seropositive patient. M_W standards are indicated in kDa (lane A).

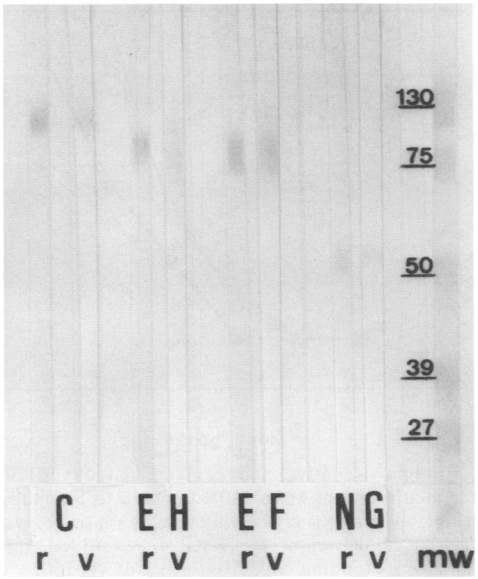

FIG. 2. Immunoblot analysis of recombinant and viral gp120 after endoglycosidase treatment. rgp120 and vgp120 were mock treated (C) or deglycosylated with either endo H (EH) or endo F (EF) or endo F-N glycanase mixture (NG). They were submitted to SDS-PAGE. After transfer to a nitrocellulose filter, staining was performed by using MAb 110-4.

(Nunc, Roskild, Denmark) were coated with MAb 110-4 diluted 1:50 in 50 μl of PBS for 2 h at 37°C. The wells were than saturated with 450 μl of PBS–5% casein for 1 h at 37°C, and 0.02 to 3 μg of stock virus in PBS–0.5% BSA was added for 2 h at 4°C; this procedure was followed by two washes, in order to determine a standard dose-response curve. A 50-μl portion of human anti-HIV purified IgG (a gift from T. Jouault) (14) (diluted 1:100) was added for 2 h at 4°C, and this addition was followed, after three washes, by 50 μl of antihuman immunoglobulin antibodies coupled to peroxidase (Diagnostic Pasteur) diluted 1:100 for 2 h at 4°C. After 5 washes, 100 μl of orthophenylenediamine (Diagnostics Pasteur) in 0.5% H_2O_2 was added, and the reaction was stopped with 50 μl of 4N H_2SO_4. $A_{492/620}$ was measured. Simultaneously, the dosage of 2 μg of mock-treated and of deglycosylated heat-inactivated virus was determined in order to compare their immunoreactivity by using the standard curve obtained with freshly thawed virus.

RESULTS

Immunopurification of bioactive viral gp120. In order to obtain efficient disruption of viral particles and solubilization of gp120, we used different detergents, such as SDS or Triton X-100 (data not shown). Tween 20 at a final dilution of 1.5% was finally chosen for its ability to get a soluble and purified bioactive form of the molecule. The presence of 100 mM EDTA in the lysis buffer prevented possible protease activities, and no band except that of vgp120 was seen in a Western blot of the purified molecule (Fig. 1). We have chosen MAb 110-4 coupled to Sepharose CL4B to specifically isolate soluble gp120 after disruption of the virus because this MAb recognizes with high affinity an epitope located in the gp120 303 to 337 amino acid V3 region and its binding to vgp120 does not affect binding to CD4 (7). In order to estimate the purification yield of solubilized vgp120 at each step, a known amount of radiolabeled rgp120 (6,000 cpm) was added to the viral lysate before incubation with MAb 110-4 coupled to Sepharose. After overnight incubation, 4,600 cpm was still fixed on the beads, and 4,500 cpm was recovered after formic acid elution. Thus, the purification yield was approximately 75%. No other viral proteins were present in the eluate, as proven by Western blot analysis (Fig. 1). As a control, rgp120 was treated in a similar manner (incubation with 1.5% Tween in lysis buffer, immunopurification, elution, and lyophilization) and this treatment did not result in any significant difference of its binding capacity to ^{125}I-sCD4 (data not shown).

Quantitation of viral gp120 and characterization of its bioactivity. The amount of vgp120 obtained in the eluate was estimated by dot blot analysis followed by Coomassie blue staining or labeling with MAb 110-4 of the proteins, as compared with known amounts of rgp120 (data not shown). In this manner it could be determined that according to the virus batch, 10 to 50 μg of vgp120 was obtained from 1 mg of viral pellet in 0.8 ml of eluate. These results were in perfect agreement with those of the following experiments that were performed to estimate the bioactivity of vgp120 (i.e., the binding capacity to CD4). In these experiments, equivalent amounts of vgp120 and rgp120 were incubated with MAb 110-4 coupled to Sepharose and the preformed complexes were then analyzed for their capacity to bind ^{125}I-sCD4. Our results show that similar amounts of vgp120 and rgp120 bind sCD4 to the same extent (see Fig. 3). Moreover, comparable findings were noted for vgp120 and rgp120 binding to $CD4^+$ cells when analyzed by an immunofluorescence assay: the same mean of fluorescence intensity was obtained with an equivalent amount of rgp120 or vgp120 (see Fig. 4). The fact that in both types of assays the curves obtained with rgp120 and vgp120 were superimposable indicates, firstly, that vgp120 and rgp120 present similar avidity for CD4 and, secondly, that the purification method did not alter vgp120 bioactivity.

Comparative analysis of the glycosylation of recombinant gp120 and viral gp120. As shown in Fig. 2, rgp120 and vgp120 have the same M_W. Incubation of these molecules for 20 h at 37°C in the absence of enzyme did not modify the Western blot band pattern, as compared with Fig. 1, indi-

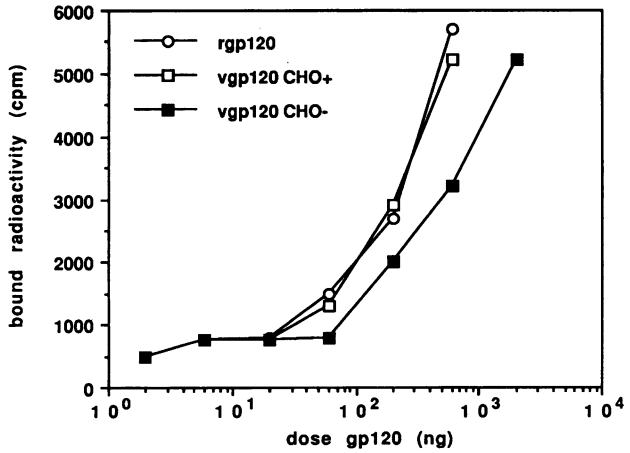

FIG. 3. Binding of native and deglycosylated vgp120 to ^{125}I-sCD4. After incubation of MAb 110-4 coupled to Sepharose CL4B with increasing amounts of glycosylated (CHO^+) or deglycosylated (CHO^-) vgp120, 80,000 cpm of ^{125}I-sCD4 was added. rgp120 was used to compare its binding capacity to that of vgp120.

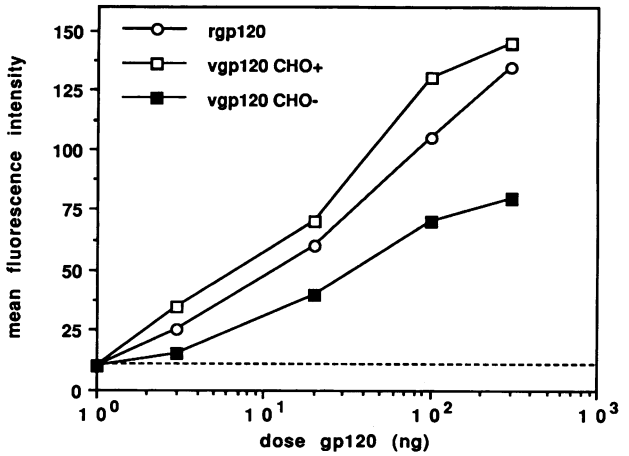

FIG. 4. Binding of native and deglycosylated vgp120 to $CD4^+$ cells. $CD4^+$ cells were incubated with increasing amounts of glycosylated (CHO^+) or deglycosylated (CHO^-) vgp120. rgp120 was used to compare its binding capacity to that of vgp120. Binding was determined by indirect immunofluorescence with 110-4 MAb and was measured with the FACS analyzer. The dashed line at the bottom indicates background fluorescence intensity.

cating, thus, that no proteolytic activity was present in either of these mock-treated samples.

Endo H is known to cleave most of the high-mannose chains from the polypeptide backbone, while endo F, in addition, removes most of the biantennary species, unless they are fucosylated. Treatment of rgp120 and of vgp120 by either of these enzymes reduced the apparent M_W of both glycoproteins in the same proportion, i.e., to approximately 90 kDa (Fig. 2). Multiantennary and fucosylated structures are resistant to these enzymes and are removed only by NG, which cleaves all accessible glycans. The use of NG resulted in the reduction by 50% of the M_W of both molecules (i.e., 120 to 60 kDa (Fig. 2). Treatment by these enzymes did not result in any detectable proteolysis because no other band except the one corresponding to deglycosylated gp120 was detected.

Role of N-linked glycans of viral gp120 in the interaction with CD4. (i) Binding of glycosylated and deglycosylated vgp120 to sCD4. By using the molecular assay described above, only a moderate decrease of the binding of ^{125}I-sCD4 to deglycosylated vgp120 (about three- to fivefold) could be noted as compared with its untreated counterpart (Fig. 3).

(ii) Binding of glycosylated and deglycosylated vgp120 to the membrane of $CD4^+$ cells. Flow cytometry analysis also showed that both native and deglycosylated vgp120 bound to the membrane of $CD4^+$ cells (CEM13 cells) in a dose-dependent manner. However, 10 times more glycosylated vgp120 was required to obtain the same mean fluorescence intensity of labeling than with the native molecule (Fig. 4).

Altogether, these results indicate that the removal of glycans from vgp120 decreased about 3 to 10 times, but did not abolish, the capacity to bind to CD4, either in a soluble form or expressed at the cell membrane.

Treatment of viral particles with endoglycosidase F-N-glycanase mixture results in the complete deglycosylation of envelope glycoproteins. Viral preparations were treated with the enzyme (31, 35) under the same nondenaturating conditions used for vgp120 (7), i.e., in the absence of Triton X-100 and of SDS, and deglycosylation was assessed by immunoblotting. Indication of the complete removal of carbohydrate side chains was taken from the M_W modifications observed at the level of envelope glycoproteins (Fig. 5A). By using either human polyclonal anti-HIV-1 serum or mouse anti-gp120 and anti-gp41 MAb, the characteristic 160-, 120-, and 41-kDa bands were replaced, respectively, by 90-, 60-, and 35-kDa bands in the enzyme-treated preparations, although the 60-kDa envelope protein could not be clearly distinguished from $p64^{pol}$ when using human anti-HIV serum.

Deglycosylation of viral particles does not alter viral particle immunoreactivity. By using an immunocapture assay, we further investigated whether virus deglycosylation could lead to modifications of envelope glycoprotein immunoreactivity. Both MAb 110-4 and human polyclonal anti-HIV-1 IgG still recognized deglycosylated virus, since they bound to the same extent untreated and deglycosylated virus, indicating that at least the epitopes involved were unmodified (Fig. 5B).

However, because one of the functions that has been ascribed to carbohydrate chains is to protect the polypeptide backbone against proteolysis (18), it was important to verify that hypothetical protease activities did not alter envelope glycoproteins. That this did not happen stems from a series of observations. (i) At least the epitopes recognized by MAbs 110-4 and 41-6 and by polyclonal human anti-HIV-1 IgG appeared to be conserved in deglycosylated virus analyzed in Western blot (see Fig. 5A). (ii) Only the apparent M_W of envelope glycoproteins (and not that of the other viral proteins) was modified when examining an immunoblot obtained with deglycosylated virus, and no additional band that could correspond to products of proteolysis could be distinguished (Fig. 5A). (iii) In independent experiments, 2 ng of already deglycosylated ^{125}I-rgp160 (prepared and treated as described in reference 7) was incubated for 20 h with 20 μg of virus pellet in a 100-μl final volume of PBS–0.5% BSA before being analyzed by SDS-PAGE and autoradiography; deglycosylation of 2 ng of ^{125}I-rgp160 was also conducted in the presence or absence of infectious supernatant. In both cases, only the 90-kDa band that corresponded to the deglycosylated rgp160 (7) was observed, without any additional band which indicated the absence of both proteolysis and inhibiting activity of deglycosylation in the viral pellet (data not shown).

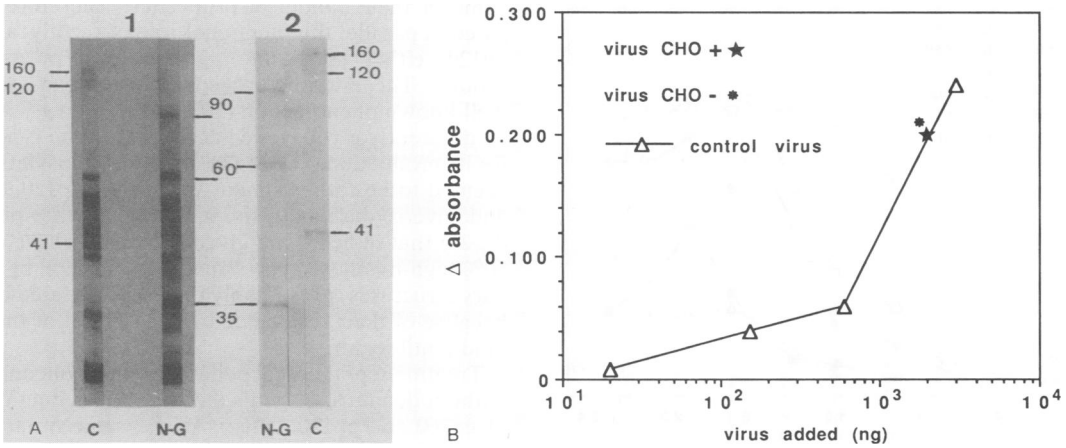

FIG. 5. (A) Immunoblot analysis of proteins from glycosylated and deglycosylated HIV-1 viral particles. Proteins from mock-treated (C) and N-glycanase-treated (NG) virus were submitted to SDS-PAGE and then transferred to nitrocellulose filters. Staining was performed by using the serum of an anti-HIV-seropositive patients (panel 1) or a mixture of two mouse MAb, one directed to gp120 (110-4), the other to gp41 (41-6) (panel 2). M_ws are indicated as kDa. (B) Immunoreactivity of glycosylated and deglycosylated HIV-1 for mouse MAb and human polyclonal antibodies. Increasing amounts of virus were incubated in 110-4 MAb-coated wells. Incubation with anti-HIV-1 human IgG was then performed and antihuman antibodies coupled to peroxidase were used to quantify virus binding. Binding of 2 μg of mock-treated glycosylated (virus CHO^+) and deglycosylated (virus CHO^-) HIV-1 particles to the mouse MAb was determined in the same run. Background absorbance was at an optical density of 0.100 nm, and absorbance is the difference between specific and nonspecific binding.

Deglycosylation reduces HIV-1 binding to $CD4^+$ cells. The influence of carbohydrate moieties of envelope glycoproteins in the interaction of HIV-1 particles with $CD4^+$ cells was investigated in an indirect immunofluorescence assay. Different amounts of mock-treated and deglycosylated virus were incubated with 5×10^5 MT2 cells, and virus binding to the cells was quantified by the fluorescence intensity obtained after labeling with MAb 110-4. Since MAb 110-4 recognized the glycosylated and deglycosylated viral gp120 to the same extent (7), any modification of labeling observed had to be related only with modification of virus binding to the cells. Preliminary experiments demonstrated that overnight incubation at 37°C did not modify virus binding to the cells as compared with viral preparations directly taken from −80°C storage (data not shown). Deglycosylation of viral particles did not abolish their binding capacity to $CD4^+$ cells, but 10 μg of deglycosylated virus had to be used to obtain the same mean fluorescence intensity of labeling that was obtained with 0.4 μg of control virus, indicating an approximately 25-fold reduction of binding capacity (Fig. 6).

Deglycosylation reduces but does not abolish the infectivity of HIV-1 for $CD4^+$ cells. MT2 cells are more susceptible to HIV-1 infection than CEM cells, and this is the reason these cells were chosen for infection experiments. The cells were infected with decreasing amounts of infectious virus, from 5 to 0.05 μl of a supernatant titrating 10^4 tissue culture infective dose per ml. In all instances, this resulted in the appearance of a cytopathic effect with syncytium formation in the culture, which correlated with detectable viral antigen production and reverse transcriptase activity in the supernatant. Differences in initial viral load resulted in variations of the culture time necessary to obtain measurable viral production and reverse transcriptase activity in the supernatant (Fig. 7). The deglycosylated viral preparation was able to infect $CD4^+$ cells, even though the antigen production and reverse transcriptase activity curves obtained with such virus compared with those obtained with 10-fold less control virus. Thus, removal of carbohydrate chains from envelope glycoproteins resulted only in loss of infectivity in the range of 1 order of magnitude.

DISCUSSION

We have previously demonstrated that deglycosylated recombinant envelope glycoproteins of HIV-1 produced in BHK-21 cells retain the main structural and biological properties of their glycosylated counterparts, bind with high affinity to CD4, and efficiently inhibit syncytium formation between HIV-infected and uninfected $CD4^+$ cells (7). These findings are at variance with those of Matthews et al., who reported that enzymatic deglycosylation of soluble viral gp120 from the supernatant of HIV-1-infected H9 cells markedly reduced its capacity to bind to membrane CD4 and to inhibit syncytium formation (23). This discrepancy may be due to differences in the purification method: here, Tween 20

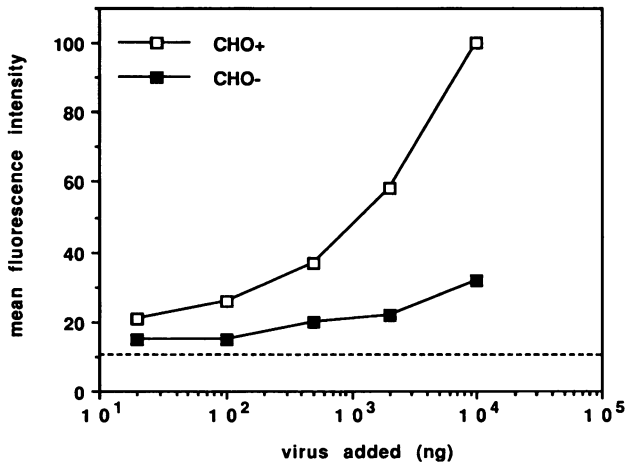

FIG. 6. Binding of native and deglycosylated HIV-1 particles to $CD4^+$ cells by using an indirect immunofluorescence assay. $CD4^+$ cells were incubated with increasing amounts of native glycosylated (CHO^+) or deglycosylated (CHO^-) virus particles. Binding was determined by indirect immunofluorescence with MAb 110-4. Fluorescence intensity was measured with the FACS analyzer. The dashed line indicates background fluorescence intensity.

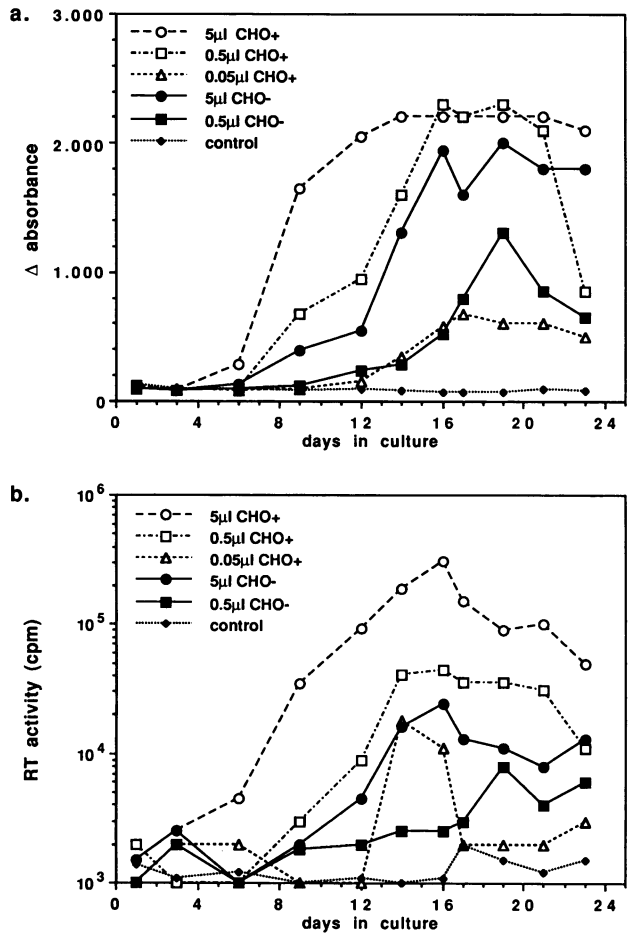

FIG. 7. Comparison of the infectivity of native and deglycosylated HIV-1 viral particles. $CD4^+$ cells were infected with 5, 0.5, or 0.05 µl of infectious glycosylated (CHO^+) or deglycosylated (CHO^-) viral supernatant. Aliquots of cell culture supernatants were then sequentially analyzed to comparatively measure virus production. (a) Viral antigen production was evaluated by using an immunocapture assay. Absorbance is indicative of the amount of virus produced. (b) Reverse transcriptase (RT) activity as measured by the incorporation of [^{3}H]thymidine.

was used instead of Triton X-100, and deglycosylation was conducted without denaturing agents that could change the structure of the deglycosylated molecule.

Moreover, the gp120 preparations were produced in different types of cell lines, which is likely to result in differences in glycosylation patterns (18) and, hence, to disturb the biological properties of the molecules (8).

In order to study the carbohydrate composition of vgp120 and the role it plays in bioactivity of the molecule, we developed an immunopurification method by using a MAb which is known to recognize with high affinity the V3 region of the molecule independent of the presence of glycans (7). This one-step method resulted in a high yield (75%) of purified bioactive vgp120, but the amount of vgp120 obtained (vgp120 weight/virus weight) varied approximately from 1 to 5%, which might be accounted for by differences in the amount of contaminating cell debris that were noted when examining viral pellets by electron microscopy.

Envelope glycoproteins in soluble form or expressed in situ at the viral particle surface may differ with respect to some of their biological properties. Therefore, we investigated in parallel the functional role of the glycan moieties of gp120, either in soluble purified form or as present on virions. The microheterogeneity of glycan structures is a well-known phenomenon (18), and a given glycoprotein may be differently glycosylated according to the type of cell used for its production (8). Unexpectedly, glycosylation of vgp120 seemed to be rather similar to that of rgp120, as judged by endoglycosydase analysis, in line with other published data (9, 26) that indicate the glycans of vgp120 are composed of 50% high-mannose-type structures and 50% bi-and triantennary structures, presumably of the fucosylated complex type because of their resistance to endo F and of the capacity to bind lentil lectin.

Treatment of the viral pellet by a high concentration of NG without denaturing agents led to a 50% reduction of the M_W of gp160 and gp120. This is in the same order as what has been previously reported by other authors (23) and by ourselves (7) in experiments in which evidence of complete deglycosylation was brought out by SDS-PAGE and Coomassie blue staining or autoradiography, high-pressure liquid chromatography analysis, and gas-liquid chromatography. Moreover, in an independent experiment, 2 ng of ^{125}I-rgp160 mixed with viral pellet was totally deglycosylated by NG and no proteolysis was observed, indicating that the viral pellet per se did not affect enzyme activity. Likewise, a shift in the M_W of transmembrane gp41 that may be accounted for by the removal of four glycan chains corresponding to four potential glycosylation sites was observed (37). Altogether, these results indicate that complete deglycosylation of gp41 as well as of gp120 may be achieved under native conditions.

Similar to our previous report regarding rgp120 (7), deglycosylation of vgp120 reduced binding to sCD4 as well as to $CD4^+$ cells. The latter decrease was more important, maybe due to the technical differences related to the assays used (number of washes and amplification procedure). In the same manner, deglycosylation of viral particles significantly reduced, but did not abolish, HIV-1 binding to and infection of $CD4^+$ cells. Approximately 10 to 25 times more deglycosylated than control virions were necessary to obtain comparable levels of cell membrane binding or virus production as expressed as HIV-1 antigen production or reverse transcriptase activity in culture supernatants. Nevertheless, and as far as it can technically be done, it cannot be excluded that this effect may be due to incomplete deglycosylation. On the other hand, it is possible that reduced cell-binding capacity and infectivity of HIV-1 are not due to removal of carbohydrates from envelope glycoproteins but rather to other reasons; however, no proteolysis of either glycoproteins or other structural proteins was noted by Western blot analysis, and the immunoreactive conformation of envelope glycoproteins was conserved. In addition, incubating the cells with virus at 20°C in the presence of a NG amount equivalent to that used for deglycosylation did not modify the amount of virus that attached to the cells (data not shown). Another reason may be increased shedding of deglycosylated gp120 from the particles under the conditions used. Indeed, the viral preparations used were rather crude and certainly represented a mixture of cell lysate containing soluble viral proteins and of more or less mature viral particles, as testified by the detection of precursor gp160 in the immunoblot of the viral preparation (shown in Fig. 1). We can exclude the possibility that this 160-kDa species observed was a tetramer of gp41 (30) because after deglycosylation, this form was reduced to 90 kDa, as previously

reported for the deglycosylated gp160 (7), and not to 140 kDa, as could be expected from the number of glycosylation sites of gp41. Control and deglycosylated preparations apparently contained the same amount of gp120 that was similarly recognized by specific human and mouse antibodies, and gp120 spikes were noted to the same extent on electron microscopy photographs of enzyme-treated and control viral particles (data not shown).

HIV-1, the envelope glycoproteins of which are deglycosylated, can thus still bind to $CD4^+$ cells with an affinity sufficient to result in membrane fusion, virus entry, and infection. Therefore, unlike what has been shown for glycoprotein hormones (3), carbohydrates of gp120 or of gp41 do not seem to play a role in the events that occur after virus binding to CD4.

However, in some experimental models, carbohydrates of gp120 have been shown to play a role in HIV-1 infectivity. For example, mannose-binding lectins are able to bind to gp120, and this may lead to in vitro blockage of infection (21, 32), but this fact can easily be explained by steric hindrance rather than by the direct involvement of carbohydrates in gp120-CD4 interaction, as discussed by the authors themselves. More importantly, it has been shown that the use of compounds that inhibit the first steps of glycosylation (5, 33)—such as tunicamycin (an inhibitor of the dolichol pathway), castanospermin and deoxynojirimycin (inhibitors of glucosidases I and II), or deoxymannojirimycin (an inhibitor of mannosidase I) but not swainsonin (an inhibitor of mannosidase II)—markedly reduced viral infectivity and cytopathogenicity (12, 15, 27). However, neither inhibitors of glucosidases nor of mannosidase I appeared to impair viral production, as assessed by the measure of antigen load or reverse transcriptase activity in infected culture supernatants (12, 27) but not by electron microscopy examination. It is therefore possible that aberrant glycosylation can affect the cellular routing, processing, and folding of envelope glycoproteins, which may then result in alteration of their biological activity. That glycan moieties play a role in the conformation of glycoproteins has already been described for the G protein of vesicular stomatitis virus (11) and for the C protein of influenza virus (13, 34). Indeed, recent reports indicate that such could be the case for HIV-1 envelope glycoproteins. Glucosidase trimming inhibitor deoxynojirimycin, but not mannosidase inhibitors, blocked the proteolytic processing of gp160, which was then totally susceptible to endo H digestion, suggesting that proteolytic cleavage of gp160 into gp120 and gp41 occurs at a stage when it mostly presents high-mannose oligosaccharide structures (19, 29). On the other hand, neither inhibitors of glucosidases I and II nor of mannosidase I did impair the CD4-binding activity of recombinant gp120 expressed in CHO cell lines (6), indicating that correct folding of gp120 is independent of further glycosylation steps.

Altogether, one can conclude from these results that after biosynthesis, carbohydrates present on the mature envelope glycoproteins of HIV-1 are not of paramount importance for its infectivity. However, the release of infectious virus undoubtedly appears to depend on proper trimming of glucose residues and on primary trimming of mannose residues (29). The precise mechanisms by which these processes interplay in the production of infectious particles remain to be elucidated. Studies with normal and glycosylation inhibitor-treated virus should lead to final conclusion on this point.

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