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Increased Immunogenicity of Human Immunodeficiency Virus gp120 Engineered To Express Galα1-3Galβ1-4GlcNAc-R Epitopes

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The glycan shield comprised of multiple carbohydrate chains on the human immunodeficiency virus (HIV) envelope glycoprotein gp120 helps the virus to evade neutralizing antibodies. The present study describes a novel method for increasing immunogenicity of gp120 vaccine by enzymatic replacement of sialic acid on these carbohydrate chains with Galα1-3Galβ1-4GlcNAc-R (α-gal) epitopes. These epitopes are ligands for the natural anti-Gal antibody constituting ~1% of immunoglobulin G in humans. We hypothesize that vaccination with gp120 expressing α-gal epitopes ($gp120_{\alpha gal}$) results in in vivo formation of immune complexes with anti-Gal, which targets vaccines for effective uptake by antigen-presenting cells (APC), due to interaction between the Fc portion of the antibody and Fcγ receptors on APC. This in turn results in effective transport of the vaccine to lymph nodes and effective processing and presentation of gp120 immunogenic peptides by APC for eliciting a strong anti-gp120 immune response. This hypothesis was tested in α-1,3-galactosyltransferase knockout mice, which produce anti-Gal. Mice immunized with $gp120_{\alpha gal}$ produced anti-gp120 antibodies in titers that were >100-fold higher than those measured in mice immunized with comparable amounts of gp120 and effectively neutralized HIV. T-cell response, measured by ELISPOT, was much higher in mice immunized with $gp120_{\alpha gal}$ than in mice immunized with gp120. It is suggested that $gp120_{\alpha gal}$ can serve as a platform for anti-Gal-mediated targeting of additional vaccinating HIV proteins fused to $gp120_{\alpha gal}$, thereby creating effective prophylactic vaccines.

Many of the studies of recombinant protein and DNA human immunodeficiency virus (HIV) vaccines in primate models or in clinical trials report that these vaccines have not been found as yet to be satisfactory in eliciting a sterilizing protective immune response against infection with HIV or simian immunodeficiency virus (SIV) (3, 22, 31, 40). An effective prophylactic HIV vaccine has to induce a strong memory (anamnestic) immune response for the rapid production of neutralizing antibodies and a rapid cytotoxic T-lymphocyte (CTL) response. Such a combined immune response will enable prevention of cell infection primarily by neutralizing anti-gp120 (anti-Env) antibodies and destruction of infected host cells in early stages following transmission of the virus, when the number of infected cells is relatively low. In the absence of a rapid immune response, the infecting virus multiplies and mutates before anti-Env antibodies are produced in titers high enough to prevent spreading of the infectious virus into a large number of cells. These mutations in envelope glycoproteins enable HIV to escape the neutralizing antibodies without losing receptor binding activity (3–5, 22, 27, 31, 40, 41, 53, 58).

A major component on the envelope of HIV, which contributes to the masking of the virus from the immune system and which hinders the effective uptake of gp120 vaccines, is the multiple carbohydrate chains on this envelope glycoprotein (19, 30, 34). The HIV gp120 is quite unique among viral glycoproteins as it has a very high number of N (asparagine [Asn])-linked carbohydrate chains which form a "glycan shield" for this virus (58). There are ~24 N-linked carbohydrate chains on this glycoprotein with the size of 479 amino acids (30). As many as 13 to 16 of these carbohydrate chains are of the complex type which are capped with sialic acid (SA) (left chain in Fig. 1), and the rest are of the high-mannose type (19, 30, 34). The size of each of these carbohydrate chains is approximately 30% (~60 Å) of the diameter of the protein portion of the gp120 molecule in its globular form. Since they are hydrophilic, these carbohydrate chains protrude from the gp120 molecule and seem to contribute to the protection of HIV against neutralizing antibodies. This protective role of the multiple carbohydrate chains can be inferred from isolate clones of HIV type 1 (HIV-1) in AIDS patients, where at least half of the mutations in gp120 (i.e., the *env* gene) result in the appearance of new N-glycosylation sites (i.e., Asn-X-Ser/Thr) (58). These de novo-expressed carbohydrate chains provide a "glycan shield" that protects the virus from neutralizing antibodies (58).

We have developed a method to convert the carbohydrate chains on gp120 into a means for effectively targeting vaccinating gp120 to antigen-presenting cells (APC), thereby increasing their immunogenicity. This is achieved by enzymatic engineering of the complex-type carbohydrate chains on gp120 for the replacement of SA with α-gal (Galα1-3Galβ1-4GlcNAc-R) epitopes, as illustrated in Fig. 1. The in situ targeting of vaccinating gp120 molecules expressing α-gal epitopes (referred to as $gp120_{\alpha gal}$) to APC is mediated by the natural anti-Gal antibody. This natural antibody constitutes ~1% of serum immunoglobulin G (IgG) (20 to 100 μg/ml serum) (16), and it interacts specifically with α-gal epitopes on glycolipids and glycoproteins (10, 15). The α-gal epitope is absent in humans but is abundantly synthesized by the glycosylation enzyme α-1,3-galactosyltransferase (α1,3GT) within the Golgi appara-

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tus of cells in nonprimate mammals and in New World monkeys (10, 12, 18). Humans, apes, and Old World monkeys lack an active α1,3GT gene but produce the anti-Gal antibody in large amounts (10, 12, 18).

Anti-Gal interacts very effectively with α-gal epitopes in vivo. This can be inferred from xenotransplantation studies. In vivo binding of anti-Gal to α-gal epitopes on transplanted pig heart or kidney is the main cause for the rapid rejection of such grafts in humans and in Old World monkeys (9, 11, 20, 45). This rejection is mediated by complement that is activated as a result of anti-Gal binding to α-gal epitopes on the graft endothelial cells. However, even if complement activation is inhibited, xenografts are rejected because of binding of various effector cells with Fcγ receptors (FcγR) (e.g., monocytes/macrophages, neutrophils, and NK cells) to the Fc portion of anti-Gal on xenograft cells and destruction of these cells by the antibody-dependent cell cytotoxicity mechanism (11, 57). In addition, because of its effective interaction with FcγR, anti-Gal can opsonize tumor cells expressing α-gal epitopes for very effective uptake by various APC including macrophages and dendritic cells (DC) which express these receptors, thereby increasing the immunogenicity of tumor vaccines (13, 28, 33).

We hypothesize that immunization with $gp120_{\alpha gal}$ (i.e., gp120 engineered to carry α-gal epitopes) will elicit a much stronger anti-gp120 immune response than immunization with gp120. The natural anti-Gal antibody, diffusing into the vaccination site from ruptured capillaries, forms immune complexes with $gp120_{\alpha gal}$ and effectively targets the vaccinating molecules to FcγR of APC, thereby greatly increasing uptake and transport of gp120 by APC to the lymph nodes. Moreover, the binding of $gp120_{\alpha gal}$/anti-Gal immune complexes to FcγR on dendritic cells further stimulates differentiation and maturation of these APC into professional APC that present gp120 peptides on both class I and class II major histocompatibility complex molecules for effective activation of gp120-specific $CD8^+$ and $CD4^+$ T cells, respectively (8, 32, 42, 44, 46).

Engineering gp120 into $gp120_{\alpha gal}$ is achieved by an enzymatic reaction combining the activities of neuraminidase and recombinant α1,3GT, as described in Fig. 1. The only available nonprimate mammalian experimental model in which immunogenicity of $gp120_{\alpha gal}$ could be analyzed is the knockout mouse for the α1,3GT gene (KO mouse), in which the α1,3GT gene was disrupted by targeted insertion of the neomycin resistance gene (51, 52). This mouse mimics the relevant human immune characteristics, as it lacks α-gal epitopes and it can produce anti-Gal in titers comparable to those in humans (29, 36, 38, 39, 50, 52). The studies described below indicate that the anti-gp120 immune response in KO mice immunized with $gp120_{\alpha gal}$ can be >100-fold higher than that in mice immunized with comparable amounts of gp120.

MATERIALS AND METHODS

Supplies. The $gp120_{BAL}$ protein produced in CHO cells was received as a generous gift from the NIH AIDS Research and Reference Reagent Program. Neuraminidase extracted from *Vibrio cholerae* was purchased from Sigma (St. Louis, MO). Recombinant α1,3GT was produced in the authors' laboratory in the *Pichia pastoris* expression system according to a procedure that was previously described (7). The monoclonal anti-Gal antibody designated M86 was obtained in tissue culture supernatants of hybridoma M86 cells, as previously described (14). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-mouse IgM antibodies were purchased from Accurate Laboratories (Westbury, NY). HRP-conjugated *Bandeiraea* (*Griffonia*) *simplicifolia* IB4 (BS lectin, specific for α-gal epitopes) was purchased from Vector Laboratories (Burlingame, CA). Synthetic α-gal epitopes linked to bovine serum albumin (α-gal BSA) were purchased from Dextra Laboratories (Reading, United Kingdom). Human anti-Gal was isolated from healthy human serum by using an affinity column of synthetic α-gal epitopes linked to silica beads (Synsorb, Alberta, Canada), as previously described (12). Mouse anti-Gal was also isolated on an affinity column by the same method as previously described (29), using serum from KO mice that were repeatedly immunized with pig kidney membrane (PKM) homogenate, in order to elicit anti-Gal production in the mice. The Ribi adjuvant, trehalose dicorynomycolate, was purchased from Corixa (Hamilton, MT).

Mice and immunization procedures. Mice used were α1,3GT KO mice on an *H-2*^{bxd} genetic background (52) which are bred and maintained at the animal facility of UMass Medical School. Studies were performed with both males and females and found to yield similar results. All experiments with mice were performed according to AAALAC guidelines. The mice were immunized intraperitoneally three times with 50 mg PKM homogenate for inducing anti-Gal production in titers similar to those of anti-Gal in humans (titers of 1:200 to 1:2,000 as measured by enzyme-linked immunosorbent assay [ELISA] with α-gal BSA as solid-phase antigen) (38, 39, 48–50). Following the demonstration of anti-Gal IgG production, the mice received a subcutaneous injection of gp120 or $gp120_{\alpha gal}$ in Ribi adjuvant. The injection was repeated after 2 weeks. The anti-gp120 immune response was evaluated 17 days after the second injection.

Enzymatic engineering of gp120 to express α-gal epitopes. The enzymatic reactions described in Fig. 1 are performed simultaneously in an enzyme buffer containing 0.1 M MES (methyl ethyl morpholinosulfonate), pH 6.0, and 25 mM $MnCl_2$ as previously described for the synthesis of α-gal epitopes on influenza virus hemagglutinin (24), on the bovine serum glycoprotein fetuin (7), and on the human serum glycoprotein α1, acid glycoprotein (49). The terminal SA is removed by neuraminidase (1 mU/ml), and α-gal epitopes are synthesized on gp120 by recombinant α1,3GT (30 μg/ml) and UDP-Gal (1 mM). The two enzymes are mixed in the same solution buffer and incubated with gp120 (1 mg/ml) for 2 h at 37°C. At the end of incubation, the $gp120_{\alpha gal}$ molecules are purified from the mixture of the enzymatic reaction by an affinity Sepharose column of *Bandeiraea simplicifolia* IB4 (BS lectin), which specifically binds α-gal epitopes (59). No other substances in the enzyme reaction bind to this lectin. The $gp120_{\alpha gal}$ bound to the BS-Sepharose beads is eluted with 100 mM of α-methyl galactoside which competes with α-gal epitopes for binding to the lectin. This enzymatic reaction and the subsequent affinity column process result in the isolation of >97% of the processed gp120 as $gp120_{\alpha gal}$ molecules.

ELISAs. Anti-Gal titers in mice immunized with PKM and the production of anti-gp120 antibodies were determined by ELISA as previously described (24, 29, 35, 36, 39). Briefly, ELISA wells were coated with α-gal BSA or gp120 molecules (10 μg/ml) overnight at 4°C. Plates were washed once with phosphate-buffered saline (PBS) and blocked with 1% BSA in PBS. Serum samples at various dilutions were plated at 50-μl aliquots in the wells for 2 h at 24°C. After washing, HRP-coupled goat anti-mouse IgG was added for 1 h. The color reactions were developed with orthophenylene diamine, and absorbance was measured at 492 nm. In assays using the monoclonal anti-Gal antibody M86 (14), HRP–anti-mouse IgM was used as secondary antibody.

Analysis of neutralizing anti-gp120 antibodies. The assay for neutralizing antibodies has been previously described (56) and is based on the study by Montefiori et al. (37). The assay was performed with the HIV-1 lab strain MN. The neutralization of HIV-1 lab strain MN was measured by the killing assay of the human T-cell lymphoma line MT-2. The virus stock of HIV-1 MN is produced in H9 cells. Virus in 50-μl aliquots containing 1,000 50% tissue culture infective doses was added to multiple dilutions of test sera and incubated at 37°C for 1 h in microtiter tissue culture wells. The T-cell lymphoma MT-2 cells (5×10^4 cells in 100 μl) were added to each well. Infection of the cells by HIV leads to extensive syncytium formation and virus-induced cell killing in 5 to 7 days in the absence of neutralizing antibodies. Neutralization was measured by staining viable cells with Finter's neutral red and measuring their adhesion to poly-L-lysine-coated wells in ELISA plates. Percent protection was determined by calculating the difference in absorbance at 540 nm between test wells (containing cells, serum sample, and virus) and virus control wells (cells and virus) and dividing this result by the difference in absorbance between cell control wells (cells only) and virus control wells. Neutralization was measured at a time when virus-induced cell killing in virus control wells was greater than 70% but less than 100%. Neutralizing antibody titers are expressed as the reciprocals of the serum dilutions required to protect 50% of cells from virus-induced killing. The background neutralization data measured with control sera from nonimmunized mice were subtracted from the data in the mice immunized with either gp120 or $gp120_{\alpha gal}$.

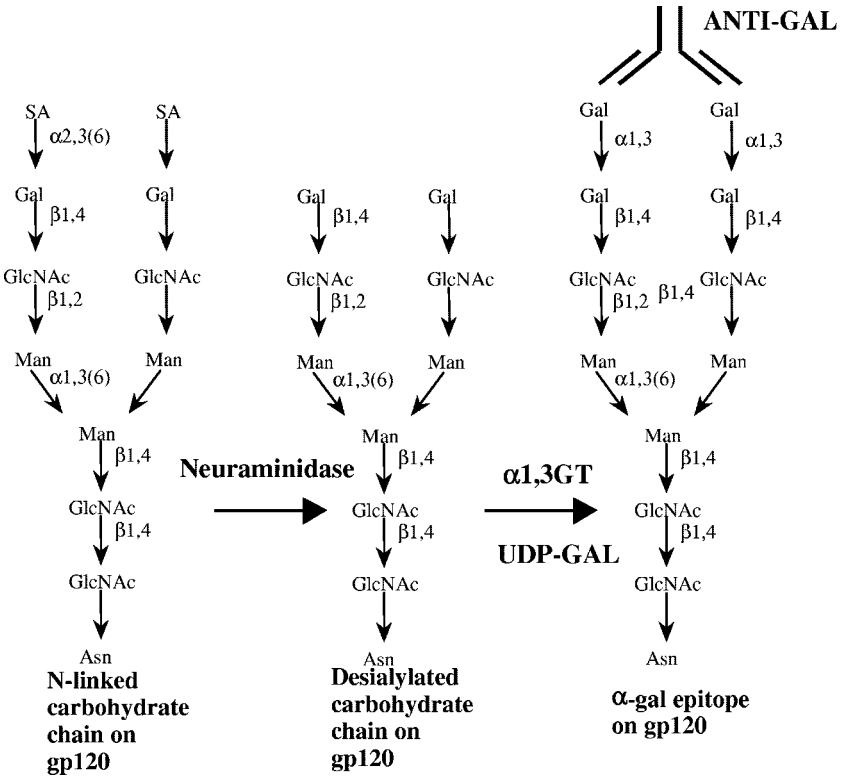

FIG. 1. Synthesis of α-gal epitopes on gp120. SA residues capping the N-linked carbohydrate chains of the complex type on gp120 (left chain) are removed by neuraminidase (middle chain). α-gal epitopes are synthesized by linking of galactosyls (Gal) from the sugar donor UDP-galactose (UDP-GAL), due to the catalytic activity of recombinant α1,3GT. These α-gal epitopes on immunizing $gp120_{\alpha gal}$ readily bind in situ the natural anti-Gal IgG molecules, thus forming immune complexes that target the vaccinating $gp120_{\alpha gal}$ to APC.

APC for ELISPOT assay. Bone marrow-derived DC were prepared as described previously (1). Briefly, bone marrow cells were cultured in RPMI medium containing granulocyte-macrophage colony-stimulating factor and interleukin-4. On day 5, immature dendritic cells were pulsed with 100 μM of gp120 protein for 18 h to allow protein processing. Cells were then washed and used for ELISPOT assay.

IFN-γ ELISPOT assay. ELISPOT assays for gamma interferon (IFN-γ)-secreting cells were performed with a commercial kit (Mabtech, Ohio), according to the manufacturer's protocol. Briefly, 96-well ELISPOT plates were coated with 100 μl/well of anti-IFN-γ monoclonal antibody AN18 overnight at 4°C. The plates were washed with PBS and blocked with PBS containing 10% fetal calf serum for 30 min at room temperature. Freshly isolated splenocytes (2×10^5 cells per well) were plated in triplicate together with dendritic cells (2×10^4) prepulsed with gp120 protein as described above. After overnight incubation at 37°C in 5% CO_2, cells were removed by washing with PBS and aliquots of 100 μl of anti-IFN-γ–biotin (monoclonal antibody R4-6A2; Mabtech) were added to each well for 2 h at room temperature. The plates were then washed with PBS, and 100 μl of streptavidin-alkaline phosphatase was added per well and incubated for 1 h at room temperature. After washing with PBS, 100 μl of chromogenic substrate (NBT-plus; Mabtech) was added to each well for 15 min to allow color development and formation of spots. The color reaction was stopped by the addition of water. Wells were then air dried, and spots were counted with an ELISPOT automated reader system (performed by Zellnet, Fort Lee, NJ). Calculated frequencies were based on the average of the triplicate wells. The results are expressed as gp120-specific IFN-γ-secreting T cells per 10^6 splenocytes, i.e., the number of spots after subtraction of the spot number in corresponding control wells that lack pulsed dendritic cells.

RESULTS

Synthesis of α-gal epitopes on gp120 by recombinant α1,3GT. Replacement of SA on the gp120 carbohydrate chains with α-gal epitopes is achieved by a two-step enzymatic reaction within one solution (Fig. 1). The studied recombinant gp120 was of the HIV_{BAL} strain and was produced in CHO cells that were transformed with the corresponding codon-optimized *env* gene. The SA was removed from the carbohydrate chains by neuraminidase to expose the penultimate *N*-acetyllactosamine residues (Galβ1-4GlcNAc-R) on the multiple complex type carbohydrate chains of gp120. The *N*-acetyllactosamines exposed on the carbohydrate chains function as an acceptor for recombinant α1,3GT, which links to them terminal α1-3-galactosyl residues, to form α-gal epitopes. The sugar donor providing galactose to α1,3GT is UDP-galactose (UDP-Gal). The latter enzymatic reaction is identical to that occurring within the Golgi apparatus of nonprimate mammalian cells for the synthesis of α-gal epitopes. The recombinant α1,3GT used for the generation of $gp120_{\alpha gal}$ is produced in the expression system of the yeast *Pichia pastoris* transformed by a New World monkey α1,3GT gene (7), which was originally cloned from marmoset cells (25). Because of the de novo-expressed multiple α-gal epitopes, $gp120_{\alpha gal}$ can be purified from the mixture of the enzymatic reaction by an affinity Sepharose column of *Bandeiraea simplicifolia* IB4 (BS lectin), which interacts specifically with these epitopes (59). This enzymatic reaction and the subsequent column separation process result in the purification of >97% of the gp120 as $gp120_{\alpha gal}$ molecules. As expected, the enzymatic manipulation did not result in changes in the size of gp120 as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2, upper

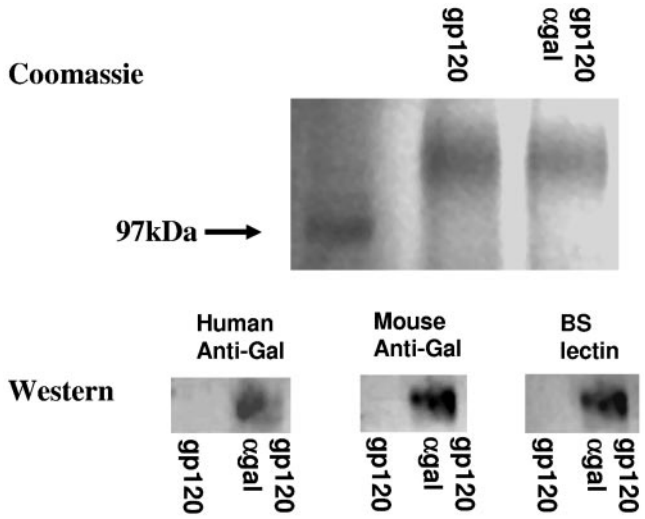

FIG. 2. Synthesis of α-gal epitopes on gp120 by recombinant α1,3GT as demonstrated by SDS-PAGE and Western blotting. Upper gel: Coomassie staining of SDS-PAGE gel. Lower gels: Western blot stained with human anti-Gal, mouse anti-Gal, and the α-gal epitope-specific BS lectin. Note that the antibodies and the lectin bind only to $gp120_{\alpha gal}$.

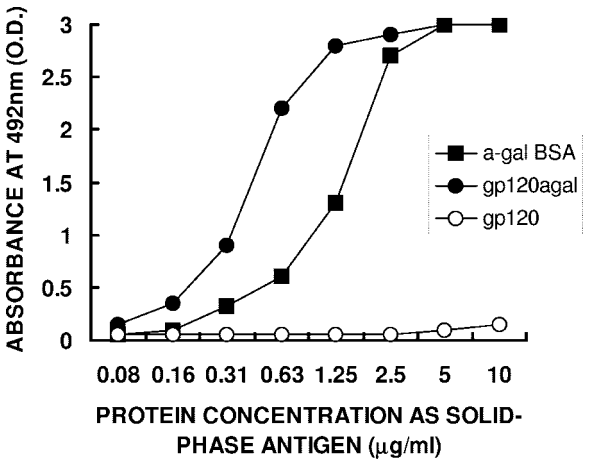

FIG. 3. Evaluation of α-gal epitope expression on $gp120_{\alpha gal}$. The figure shows binding of the monoclonal anti-Gal M86 to gp120, $gp120_{\alpha gal}$, and α-gal BSA that expresses 10 synthetic α-gal epitopes, as measured by ELISA with different amounts of glycoproteins coating the ELISA wells.

panel). The de novo expression of α-gal epitopes on $gp120_{\alpha gal}$ could be demonstrated in Western blots that are immunostained with human anti-Gal, mouse anti-Gal, and the α-gal epitope-specific BS lectin (Fig. 2, lower panel). The two antibodies and the lectin all interact specifically with α-gal epitopes, readily bound to $gp120_{\alpha gal}$ but not to the original gp120 molecules.

The approximate number of the de novo-synthesized α-gal epitopes on the $gp120_{\alpha gal}$ molecules could be estimated by measuring the binding of the monoclonal anti-Gal antibody M86 (15) to different amounts of this molecule dried in ELISA wells and by comparison with this antibody binding to a standard glycoprotein expressing 10 synthetic α-gal epitopes, α-gal BSA. The two glycoproteins were dried at various concentrations as solid-phase antigens in ELISA wells, and binding of the monoclonal anti-Gal was measured in the different wells. As shown in Fig. 3, $gp120_{\alpha gal}$ bound comparable amounts of monoclonal anti-Gal in wells containing fourfold-less protein than in the wells containing α-gal BSA. Since α-gal BSA size is ~70% of that of $gp120_{\alpha gal}$, the data imply that there are ~3-fold-more α-gal epitopes on $gp120_{\alpha gal}$ than on α-gal BSA, i.e., ~30 α-gal epitopes/molecule. As indicated above, gp120 has 13 to 16 N-linked carbohydrate chains of the complex type, each with two branches (antennae) (Fig. 1) (19, 30, 34). Therefore, these data imply that most SA residues on gp120 were replaced by α-gal epitopes as a result of the enzymatic reactions with neuraminidase and α1,3GT. As expected, the monoclonal anti-Gal did not bind to the original gp120, since this glycoprotein lacks α-gal epitopes (Fig. 3).

Similarities between anti-Gal produced in humans and in α1,3GT-knockout mice (KO mice). Analysis of anti-Gal-mediated targeting of $gp120_{\alpha gal}$ versus gp120 vaccines requires an appropriate experimental animal model that produces anti-Gal. The α1,3GT-knockout mouse (KO mouse) is the only available nonprimate animal model because this mouse, in contrast to all other nonprimate mammals, lacks α-gal epitopes. KO mice naturally produce anti-Gal in titers much lower than those in humans. However, anti-Gal production in these mice can be increased to titers comparable to those in humans when they are immunized with xenogeneic cell membranes expressing α-gal epitopes, such as PKMs (36, 38, 39, 50). The similarity in anti-Gal activities in KO mice and in humans could be demonstrated in an ELISA measuring this antibody's binding to α-gal BSA. As shown in Fig. 4A there is a similarity in binding curves of anti-Gal at various dilutions of human or mouse serum samples. Furthermore, KO mice produce anti-Gal that has characteristics similar to those of human anti-Gal. Anti-Gal in humans is found as IgM, IgG, and IgA classes (16, 23), and anti-Gal IgG is found in all subclasses, IgG1 being the predominant one (43). Similarly, KO mice used in this study produced anti-Gal IgG that comprised all subclasses (IgG1, IgG2a, IgG2b, and IgG3), with IgG1 being the predominant subclass (Fig. 4B). IgG4 is not shown, since mice do not produce this subclass. Whereas anti-Gal IgM is also produced in mice, as in humans (23, 49), no significant production of anti-Gal IgA was detected in the PKM-immunized KO mice (Fig. 4B). This class of anti-Gal is readily found in the serum and in body secretions in humans (23). Because of the overall similarities in the characteristics of anti-Gal IgG and anti-Gal IgM in humans and in KO mice, these mice can serve as an appropriate model for determining whether formation of immune complexes between $gp120_{\alpha gal}$ and anti-Gal has any effect on the elicited anti-gp120 immune response.

Anti-gp120 antibody response in KO mice immunized with $gp120_{\alpha gal}$ or gp120. KO mice with confirmed production of anti-Gal IgG in titers comparable to those in humans were immunized subcutaneously twice at a 2-week interval with 5 μg of either gp120 or $gp120_{\alpha gal}$. Synthetic trehalose dicorynomycolate (TDM-Ribi) was used as adjuvant. This adjuvant was chosen because it has been approved by the FDA for experimental use in humans. Anti-gp120 antibody production was tested by ELISA in serum samples obtained 17 days after the second immunization. The titer is defined as the serum dilution

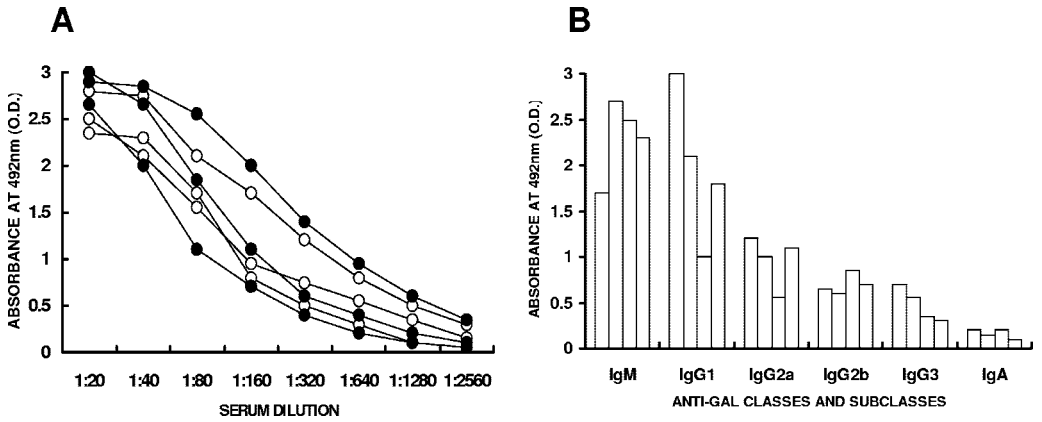

FIG. 4. Characteristics of mouse anti-Gal in comparison to human anti-Gal. A. Comparison of anti-Gal IgG activity in human sera (●) and sera of mice immunized with PKMs (○). Data are for 3 out of 30 humans and 30 KO mice with similar results. B. Classes and subclasses of anti-Gal in KO mice as measured by ELISA with α-gal BSA as solid-phase antigen. The antibody activity was measured at a serum dilution of 1:100. Data from 4 representative mice out of 15 with similar results are shown.

yielding 50% maximal binding (i.e., ~1.5 optical density [OD] units). All mice immunized with $gp120_{\alpha gal}$ displayed extensive production of anti-gp120 antibodies with titers ranging between 1:320 and 1:2,560. Anti-gp120 antibody production was much lower in mice immunized with the original (i.e., unprocessed) gp120. Three of these mice displayed marginal production of anti-gp120 antibodies at the lowest serum dilution of 1:20 (0.5 to 1.0 OD), whereas the remaining two immunized mice displayed no significant anti-gp120 antibody production (<0.5 OD) (Fig. 5A). These findings imply that $gp120_{\alpha gal}$ is at least 100-fold more immunogenic than gp120 in its ability to induce the production of anti-gp120 antibodies. The differential immunogenicity of $gp120_{\alpha gal}$ versus gp120 was less distinct with lower or higher vaccine doses (Fig. 5B and 5C). Two immunizations with 0.5 μg $gp120_{\alpha gal}$ elicited a strong anti-gp120 antibody response in two of the five mice tested and less response in the remaining three mice (Fig. 5B). Nevertheless, all mice in this group displayed higher anti-gp120 antibody responses than mice vaccinated with 0.5 μg gp120, in which no significant antibody response was detected (Fig. 5B). Immunization with 50 μg gp120 (10-fold higher than in Fig. 5A) resulted in distinct production of anti-gp120; however, immunizations with equal amounts of $gp120_{\alpha gal}$ resulted in a ~30-fold-higher antibody response on average (Fig. 5C). It is not clear at present whether the overall higher anti-gp120 antibody response in mice immunized with 5 μg versus 50 μg of $gp120_{\alpha gal}$ is the result of the use of different $gp120_{\alpha gal}$ batches or is because of an inherent higher immunogenicity of the 5-μg dose. Evaluation of the kinetics of anti-gp120 antibody production indicated that this activity could be detected in the serum of the immunized mice only 2 weeks after the second immunization, whereas no such antibodies were identified after the first immunization with $120_{\alpha gal}$ or gp120, even at the high dose of 50 μg (not shown).

The increased immune response to $gp120_{\alpha gal}$ is associated with the presence of anti-Gal antibody molecules which form immune complexes with the immunizing glycoprotein. This can be inferred from analysis of anti-gp120 antibody response in wild-type C57BL/6 mice, i.e., the parental strain of KO mice which cannot produce anti-Gal, despite repeated PKM immunizations, because they express α-gal epitope (49). Wild-type mice immunized twice with either 5 μg gp120 or 5 μg $gp120_{\alpha gal}$ displayed low titers of anti-gp120 antibodies, as there are no antibodies in these mice that can target the vaccine to APC (Fig. 5D).

Neutralizing activity of anti-gp120 antibodies in immunized mice. The major potential protective activity of anti-gp120 antibodies is in neutralizing HIV, thereby preventing infection of the host cells. Thus, it was of interest to determine whether anti-gp120 antibodies produced in KO mice immunized with $gp120_{\alpha gal}$ can display neutralizing activity. The neutralizing activity of these antibodies was evaluated with the HIV-1 lab strain MN which is convenient for manipulation in the laboratory and for the analysis of neutralizing activity by antibodies. The neutralization was measured by the killing assay of the human T-cell lymphoma line MT-2 (37, 56). The analysis of the neutralizing activity with HIV_{MN} is a conservative approach for evaluating the production of protective antibodies against HIV_{BAL}. Although the $gp120_{BAL}$ sequence may differ by a few amino acids from that of $gp120_{MN}$, demonstration of neutralizing antibodies with HIV_{MN} will imply an even higher neutralizing activity against HIV_{BAL}. In accord with the low titers of anti-gp120 antibodies in the serum from the mice immunized with gp120 (Fig. 5A), serum from these mice displayed no neutralizing activity above that of the nonimmunized mice (mice 1 to 6 in Fig. 6). In contrast, the serum from the mice immunized with $gp120_{\alpha gal}$ (mice 7 to 12 in Fig. 6) displayed a very effective neutralization activity, which was similar to that observed in the positive control of rabbit serum containing anti-HIV neutralizing antibodies (originating from a rabbit receiving multiple immunizations with gp120) (56). These data demonstrate a correlation between the high titer of anti-gp120 antibody production as a result of immunization with $gp120_{\alpha gal}$ and the neutralizing activity of the elicited antibodies in the immunized mice.

Analysis of gp120-specific T cells by ELISPOT. Detection of IFN-γ-secreting cells in the immunized mice was determined by ELISPOT assay as described in Materials and Methods and reference 2. The number of IFN-γ-secreting cells in $gp120_{\alpha gal}$-immunized mice was significantly higher than that in gp120-

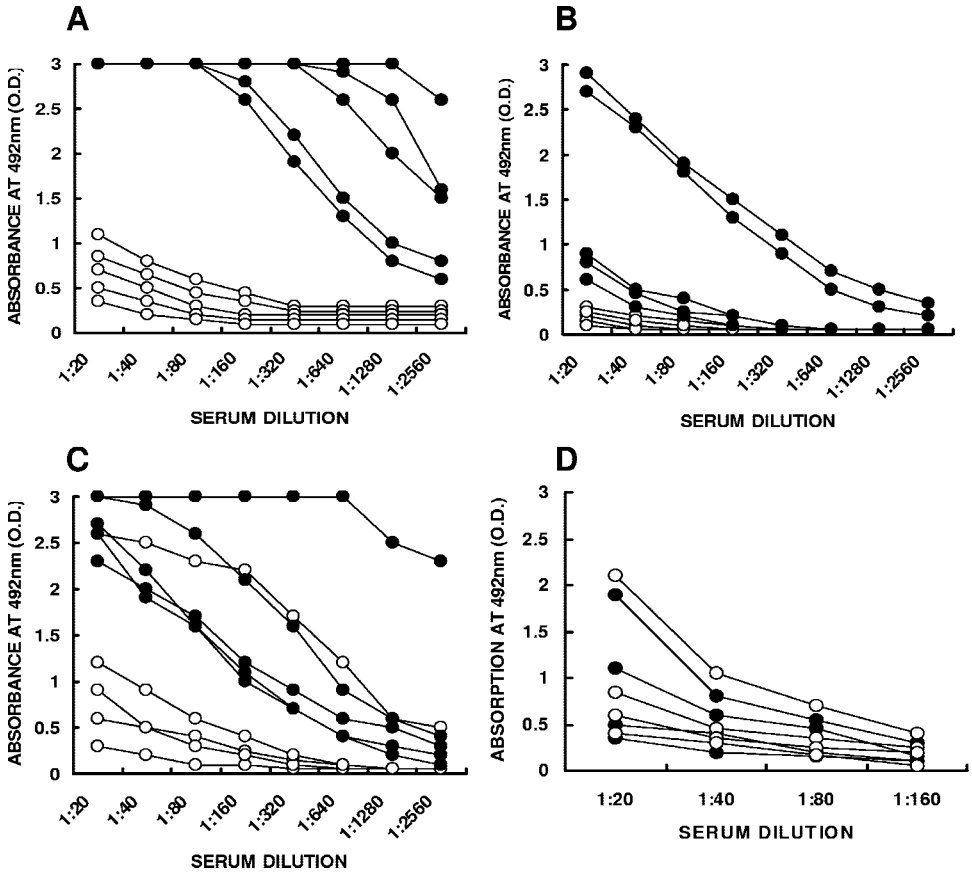

FIG. 5. Elicited anti-gp120 antibodies in response to immunization with gp120 or $gp120_{\alpha gal}$. The figure shows production of anti-gp120 antibodies in KO mice immunized twice at 2-week intervals (A to C) or in wild-type (WT) mice (D), immunized with either gp120 (○) or $gp120_{\alpha gal}$ (●). (A) 5.0 μg/vaccine; (B) 0.5 μg/vaccine; (C) 50 μg/vaccine; (D) 5.0 μg/vaccine. Note that KO mice in panels A to C immunized with gp120 produced anti-gp120 antibodies in low titers or completely lacked such antibodies, whereas a significant increase in anti-gp120 antibody production was observed in mice immunized with $gp120_{\alpha gal}$. In contrast, no differences in anti-gp120 antibody production are detected in panel D in wild-type mice immunized with the two glycoproteins, as the wild-type mice are incapable of producing anti-Gal despite repeated PKM immunizations.

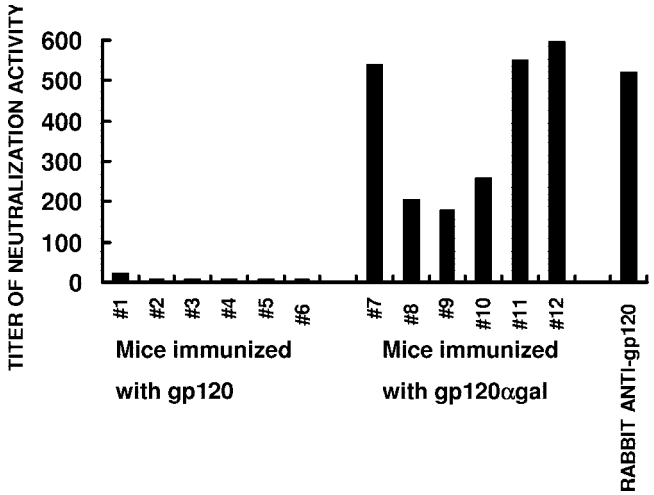

FIG. 6. HIV neutralization activity in mice immunized with $gp120_{\alpha gal}$ or with gp120. The figure shows the titer of neutralization activity in various mice immunized twice with 5 μg gp120 (mice 1 to 6) or $gp120_{\alpha gal}$ (mice 7 to 12). Titer is defined as the reciprocal of the serum dilution displaying 50% neutralization.

immunized mice that were tested simultaneously. The ELISPOT wells with lymphocytes from three out of six mice in each group are shown in Fig. 7A. The numerical values of IFN-γ-secreting T cells per 1×10^6 cells in six mice tested in each group, after subtraction of the number of spots in the corresponding control wells lacking the gp120 pulsed dendritic cells, are presented in Fig. 7B. Despite the variability between the individual mice, the number of spots representing gp120-specific T cells is much higher in the group immunized with $gp120_{\alpha gal}$ than in the group of mice immunized with gp120. The mean number of spots in $gp120_{\alpha gal}$-immunized mice was calculated to be 332 spots/10^6 cells, whereas the mean number of spots in mice immunized with gp120 was only 23 spots/10^6 cells. These findings imply that T-cell activation against the gp120 peptides was much higher in $gp120_{\alpha gal}$-immunized mice than in gp120-immunized mice.

DISCUSSION

This study demonstrates a method for increasing the immunogenicity of gp120 by replacing its multiple SA residues with α-gal epitopes. These epitopes can bind the natural anti-Gal antibody (present in all humans as 1% of IgG), when injected

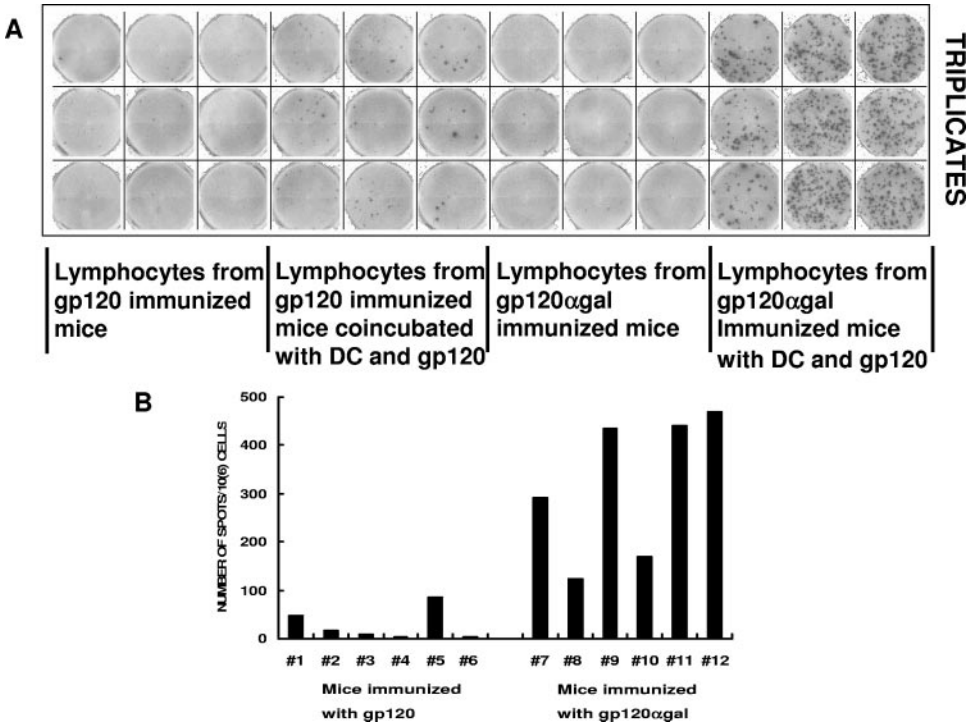

FIG. 7. ELISPOT analysis for IFN-γ secretion in mice immunized with gp120 or $gp120_{\alpha gal}$. A. Actual wells with splenocytes from three mice, each tested in triplicate (vertical lanes) in the absence or presence of gp120-pulsed DC. B. Presentation of ELISPOT data for six mice immunized twice with 5 μg gp120 (mice 1 to 6) and six mice immunized twice with 5 μg $gp120_{\alpha gal}$ (mice 7 to 12), as the number of spots per 10^6 splenocytes.

as a vaccine in humans. The formation of immune complexes with anti-Gal results in targeting of the vaccinating $gp120_{\alpha gal}$ molecules to APC, thereby inducing an effective anti-gp120 immune response. The incubation of gp120 with an enzyme mixture of neuraminidase and recombinant α1,3GT and with the sugar donor UDP-Gal results in synthesis of multiple α-gal epitopes on most N-linked carbohydrate chains of the complex type on gp120, as indicated by the subsequent extensive binding of the monoclonal anti-Gal antibody (Fig. 3). The increased immunogenicity of $gp120_{\alpha gal}$ was demonstrated in the only nonprimate mammalian experimental model available for studies of anti-Gal-mediated immune response, the α1,3GT-knockout mouse (KO mouse). Our previous studies demonstrated in this model increased immunogenicity of tumor vaccines consisting of tumor cells engineered to express α-gal epitopes (28). In tumor vaccine studies, the efficacy of vaccines expressing α-gal epitopes can be demonstrated by the immune protection following challenge of the mouse with live tumor cells. The present study demonstrates increased immunogenicity of a soluble protein expressing α-gal epitopes, the HIV envelope glycoprotein gp120. The increased production of anti-gp120 antibodies in response to vaccination with $gp120_{\alpha gal}$ versus vaccination with gp120 was observed in all three doses of 0.5, 5, and 50 μg per vaccine. The greatest difference, of >100-fold, in the antibody response was observed in mice immunized with 5 μg of the glycoprotein. Similarly, we observed a parallel increase in T-cell response, as assessed by ELISPOT. However, we could not analyze immune protection from challenge postvaccination as there are no HIV strains that are infective in mice.

The principle of increasing immunogenicity of a given antigen by 10- to 1,000-fold, by complexing the antigen with its corresponding antibody, was demonstrated with a variety of antigens, including tetanus toxoid (21, 32), hepatitis B virus antigen (6), and Eastern equine encephalomyelitis virus antigen (26). Accordingly, recent studies demonstrated that immune complexes between SIV and anti-SIV antibodies were targeted to APC, resulting in enhanced cross-presentation of SIV peptides, as indicated by effective activation of cytotoxic T cells by MHC class I-presented peptides, in SIV-infected monkeys (55). As expected, enzymatic destruction of the Fc portion of the anti-SIV antibodies, or blocking of the FcγR on APC, abrogated this enhancing effect of immune complexes (55). Similarly, natural antibodies in mice were found to function as an endogenous adjuvant forming immune complexes with *Leishmania* vaccine and inducing a strong $CD8^+$-T-cell response against the intracellular form of the *Leishmania* parasite (47). Thus, the interaction between the Fc portion of the opsonizing antibody and FcγR on APC is considered to be the most effective mechanism by which APC identify and internalize antigens that should be targeted for an effective immune response (54). The same principle applies to vaccines that express α-gal epitopes and thus form immune complexes with anti-Gal antibodies which can target the vaccine to APC in any immunized individual. This anti-Gal-mediated targeting to APC is supported by previous in vitro studies of inactivated influenza virions that express α-gal epitopes and form immune complexes with anti-Gal. These virions displayed a 10-fold-higher uptake by APC and subsequent processing and presentation of envelope hemagglutinin peptides than virions lacking

α-gal epitopes and incubated with anti-Gal (17). It should be stressed that anti-Gal is the only antibody in humans that can serve for this purpose of targeting vaccines to APC. This is because anti-Gal is the only natural antibody known to be produced ubiquitously in humans as ~1% of IgG (16). Thus, any particulate or soluble vaccine that expresses α-gal epitopes will form immune complexes with anti-Gal and will be targeted for effective uptake by APC (13).

In view of the ability of gp120 to mutate during infection and evade the detrimental effect of neutralizing antibodies, vaccination only with gp120 may not suffice for conferring resistance to HIV infections in large populations (3–5, 22, 27, 31, 40, 41, 53, 58). Other viral proteins such as tat, rev, p17, and p24 may also be used as vaccines eliciting a cellular immune response for destruction of HIV-infected cells. However, because of poor targeting to APC, immunogenicity of these proteins also may be low. The effective anti-Gal-mediated targeting of $gp120_{\alpha gal}$ to APC may be further exploited for effective targeting of other HIV proteins to APC, in order to induce a protective cellular immune response. This can be achieved by fusion of tat, rev, p17, or p24 to gp120 and enzymatic conversion of the SA residues on the carbohydrate chains of gp120 into α-gal epitopes as in Fig. 1. Thus, vaccination with $gp120_{\alpha gal}$ that is fused to each of these proteins is likely to produce high titers of anti-gp120 antibodies, as well as high CTL activity against cells infected by HIV.

As indicated above, KO mice are the only nonprimate mammal that produces anti-Gal and thus can serve as a model for anti-Gal-mediated targeting of vaccines to APC. Anti-Gal production in these mice is achieved by immunization with PKMs. This mouse-produced anti-Gal is an elicited antibody, whereas anti-Gal in humans is a natural antibody. Nevertheless, they share similar characteristics in their class and subclass distribution (Fig. 4) and in biological activities. Both human and mouse anti-Gal mediate hyperacute xenograft rejection and induce antibody-dependent cell cytotoxicity and phagocytosis of the various antigens (9, 11, 20, 45). Despite these similarities, demonstration of primate anti-Gal ability to target $gp120_{\alpha gal}$ to APC will require studies in a monkey model. Since Old World monkeys (e.g., rhesus monkeys, cynomolgus monkeys, and baboons) naturally produce anti-Gal in titers comparable to those of humans (12), future studies of immunogenicity in monkeys immunized with SIV $gp120_{\alpha gal}$ and with $gp120_{\alpha gal}$ fused to other viral proteins will enable evaluation of the efficacy of these vaccines in eliciting a protective immune response against challenge with SIV.

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