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Fundamental Difference in the Content of High-Mannose Carbohydrate in the HIV-1 and HIV-2 Lineages▽†

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The virus-encoded envelope proteins of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) typically contain 26 to 30 sites for N-linked carbohydrate attachment. N-linked carbohydrate can be of three major types: high mannose, complex, or hybrid. The lectin proteins from *Galanthus nivalis* (GNA) and *Hippeastrum* hybrid (HHA), which specifically bind high-mannose carbohydrate, were found to potently inhibit the replication of a pathogenic cloned SIV from rhesus macaques, SIVmac239. Passage of SIVmac239 in the presence of escalating concentrations of GNA and HHA yielded a lectin-resistant virus population that uniformly eliminated three sites (of 26 total) for N-linked carbohydrate attachment (Asn-X-Ser or Asn-X-Thr) in the envelope protein. Two of these sites were in the gp120 surface subunit of the envelope protein (Asn244 and Asn460), and one site was in the envelope gp41 transmembrane protein (Asn625). Maximal resistance to GNA and HHA in a spreading infection was conferred to cloned variants that lacked all three sites in combination. Variant SIV gp120s exhibited dramatically decreased capacity for binding GNA compared to SIVmac239 gp120 in an enzyme-linked immunosorbent assay (ELISA). Purified gp120s from six independent HIV type 1 (HIV-1) isolates and two SIV isolates from chimpanzees (SIVcpz) consistently bound GNA in ELISA at 3- to 10-fold-higher levels than gp120s from five SIV isolates from rhesus macaques or sooty mangabeys (SIVmac/sm) and four HIV-2 isolates. Thus, our data indicate that characteristic high-mannose carbohydrate contents have been retained in the cross-species transmission lineages for SIVcpz-HIV-1 (high), SIVsm-SIVmac (low), and SIVsm-HIV-2 (low).

The envelope proteins of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) are heavily glycosylated. N-linked carbohydrate is attached to the nascent protein at the asparagine of the consensus sequence N-X-S or N-X-T, where X is any amino acid except a proline (31, 52, 53). The number of potential N-linked carbohydrate attachment sites in the surface subunit of Env (gp120) ranges from 18 to 33, with a median of 25 (34, 65). There are typically 3 or 4 potential N-linked sites in the ectodomain of the Env transmembrane protein (gp41) (34).

N-linked glycosylation of a protein consists of the *en bloc* transfer of the carbohydrate core oligosaccharide (two *N*-acetylglucosamines, nine mannoses, and three glucoses) from dolichol to the asparagine of the N-linked attachment site (8, 60). Initially the attached carbohydrate is processed into the high-mannose type (8). In the Golgi complex, high-mannose carbohydrate may be further processed into complex or hybrid oligosaccharides (58). Incomplete processing of N-linked carbohydrate results in the production of high-mannose carbohydrate chains, which terminate in mannose (58). Fully processed complex carbohydrate chains terminate in galactose, *N*-acetylglucosamine, sialic acid, or glucose (33, 57). Hybrid carbohydrate chains have two branches from the core, one that terminates in mannose and one that terminates in a sugar of the complex type (63).

Glycoproteins exist as a heterogeneous population, exhibiting heterogeneity with respect to the proportion of potential glycosylation sites that are occupied and to the oligosaccharide structure observed at each site. Factors that influence the type of carbohydrate chain that is attached at any one N-linked site are the accessibility of the carbohydrate chain to processing enzymes (49), protein sequences surrounding the site (5, 40), and the type of cell from which the protein is produced (19).

The N-linked carbohydrate chains of HIV and SIV Env are critical for the proper folding and cleavage of the fusion-competent envelope spike (20, 59, 61). After Env is assembled, enzymatic removal of N-linked carbohydrate does not dramatically affect the functional conformation (2, 6, 7, 13, 24, 38). It is generally accepted that the carbohydrate attached to Env limits the ability of the underlying protein to be recognized by B cells (11, 48, 62). This carbohydrate also shields protein epitopes that would otherwise be the direct targets of antibodies that neutralize viral infection (41, 48, 62, 64). Furthermore, the high-mannose carbohydrates of HIV and SIV Env bind dynamically to an array of lectin proteins that are part of the host lymphoreticular system. The interaction of viral high-mannose carbohydrate with host lectin proteins has been associated with the enhancement (9, 16, 17, 43–45) or suppression (42, 56) of viral infection of CD4-positive T cells. The high-mannose carbohydrate of Env is also known to activate the release of immune-modulatory proteins from a subset of host antigen-presenting cells (12, 54).

The plant lectin proteins from *Galanthus nivalis* (GNA) and *Hippeastrum* hybrid (HHA) specifically bind terminal α-1,3-

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and/or α-1,6-mannose of high-mannose oligosaccharides but not hybrid oligosaccharides (28, 55). GNA and HHA inhibit the replication of HIV-1 and SIVmac251, and uncloned, resistant populations of virus have been selected (3, 14). In this report, we define two N-linked sites in the external surface glycoprotein gp120 and one in the transmembrane glycoprotein gp41 whose mutation imparts high-level resistance to the inhibitory effects of GNA and HHA to cloned SIVmac239. Furthermore, using a GNA-binding enzyme-linked immunosorbent assay (ELISA), we show that assorted HIV-1 and SIVcpz gp120s consistently are considerably higher in mannose content than assorted gp120s from SIVmac, SIVsm, and HIV-2. These results shed new light on the impact of virus-host evolutionary dynamics on viral carbohydrate composition, and they may have important implications for the mechanisms by which long-standing natural hosts such as sooty mangabeys can resist generalized lymphoid activation and disease despite high levels of SIV replication.

MATERIALS AND METHODS

***Macaca mulatta*.** Rhesus macaques (*Macaca mulatta*) were housed at the New England Primate Research Center in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. The Harvard Medical Area Standing Committee on Animals approved the animal experiments. Procurement of rhesus macaque blood was conducted according to the principles of relevant national and international guidelines. The animals used in this study were healthy at the time of blood draw.

Cell culture and antibodies. HEK293T and CEMx174 cells were obtained from the American Type Culture Collection. C8166 secreted alkaline phosphatase (C8166-SEAP) cells were generated previously (39). HEK293T cells were maintained in complete Dulbecco's modified Eagle medium (DMEM) (DMEM supplemented with 10% fetal bovine sera, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin; Gibco BRL, Rockville, MD). CEMx174 and C8166-SEAP cells were maintained in complete RPMI medium (RPMI medium supplemented with 10% fetal bovine sera, 100 U penicillin G sodium, and 100 μg/ml streptomycin; Gibco). Cell lines expressing each rhesus monoclonal antibody (RhMAb) were maintained in RPMI medium supplemented with 20% fetal bovine sera and 100 μg/ml Primocin (InvivoGen, San Diego, CA).

Virus production. Viral stocks were generated by transient transfection of a full-length SIV proviral vector in HEK293T cells. Briefly, 1.5×10^6 cells were plated in a T75 flask 24 h prior to transfection. For each viral stock produced, 5 μg of proviral DNA was transfected by the calcium phosphate method (Promega Corporation, Madison, WI). The medium was replaced 16 h after transfection. Two days later, the cell culture supernatant was passed through a 0.45-μm polyethersulfone syringe filter. Virus production was measured with a capsid protein (p27) antigen capture assay (Advanced Bioscience Laboratories, Inc., Kensington, MD).

Replication of SIVmac239 in the presence of HHA or GNA. CEMx174 cells at a density of 1×10^6 cells per milliliter were infected with 30 ng/ml capsid protein (p27) of SIVmac239. The cultures were divided 24 h after infection, and the medium was replaced with medium that contained GNA or HHA at 0, 0.1, 1, 10, 100, or 200 μg/ml. Every 3 to 4 days, the cell cultures were split one to two with medium that contained the same concentration of either GNA or HHA. SIV p27 released into the supernatant was measured by antigen capture (Advanced Bioscience Laboratories, Inc.).

GNA and HHA selection of SIVmac239. CEMx174 cells at a density of 1×10^6 cells per milliliter were infected with 30 ng/ml capsid protein (p27) of SIVmac239. The cultures were divided 24 h after infection, and the medium was replaced with medium that contained GNA or HHA at 0, 10, 100, or 200 μg/ml. Every 3 to 4 days, the cell cultures were split one to two with medium that contained the same concentration of either GNA or HHA. SIV p27 released into the supernatant was measured by antigen capture (Advanced Bioscience Laboratories, Inc.). For the second passage, CEMx174 cells were infected with 30 ng p27 of virus that grew from GNA at 100 μg/ml, GNA at 200 μg/ml, or HHA at 10 μg/ml. The medium was changed 24 h later to that which contained both GNA (100 μg/ml) and HHA (25 μg/ml). Every 3 to 4 days, the cell cultures were split one to two with medium that contained the same concentration of GNA and HHA. SIV p27 released into the supernatant was measured. Virus that grew from selection at passage 2 was used for a new infection (passage 3). The medium was changed 24 h after infection to medium that contained twice the concentration of GNA and HHA than that used in the previous passage. Virus was passaged in this manner until passage 6, when the selection medium used contained GNA at 1 mg/ml and HHA at 0.5 mg/ml. Virus was then passaged 3 more times in the presence of GNA (1 mg/ml) and HHA (0.5 mg/ml). The GNA/HHA-selected virus was passaged 9 times in the presence of lectin. As a control, SIVmac239 was passaged in CEMx174 cells concurrently with the lectin-selected population.

Isolation of RNA and *env* from the GNA/HHA-resistant population. Viral RNA was isolated from culture supernatant using the MagMAX viral RNA isolation kit (Applied Biosystems, Foster City, CA). Briefly, 400 μl of culture supernatant containing SIV was incubated with 800 μl of a guanidinium thiocyanate-based lysis/binding solution and 20 μl of paramagnetic beads with a nucleic acid binding surface. The beads were washed three times before viral RNA was eluted. The envelope gene (*env*) sequence was amplified from isolated viral RNA using the SuperScript III One-Step reverse transcription-PCR (RT-PCR) system (Invitrogen, Carlsbad, CA). Primers used to amplify *env* were as follows: forward primer, 5′-GACGAGCGCTCTTCATGCATTTCAGAGG-3′; reverse primer, 5′-AAGCTTGCATGCTATAACACATGC-3′. The PCR product was then cloned with the TOPO XL PCR cloning kit (Invitrogen). Twenty *env* clones were sequenced (Retrogen Inc., San Diego, CA) and then aligned with the SIVmac239 sequence.

Construction of GNA/HHA-based SIV variant clones. Mutant derivatives of the SIVmac239 proviral vector that introduced a glutamine codon in place of an asparagine codon were constructed in the following manner: (i) the codon at nucleotides (nt) 7333 to 7335 was changed from AAT to CAG, (ii) the codon at nt 7981 to 7983 was changed from AAC to CAA, (iii) the codon at nt 8029 to 8031 was changed from AAC to CAG, and (iv) the codon at nt 8476 to 8478 was changed from AAT to CAA. In addition, the codon that encoded asparagine at nt 7981 to 7983 was changed from AAC to TCA to encode serine and the glycine codon (GGA) at nt 8026 to 8028 was changed to GAG to encode glutamic acid. The nucleotide number corresponds to the original published sequence of SIVmac239 (47). To introduce changes to the desired codons, the 3′ half of the SIVmac239 genome was used as a template for PCR site-directed mutagenesis. For each single mutant, complementary and reverse-oriented mutagenic primers containing one of the above-listed nucleotide changes compared to the SIVmac239 sequence were designed. Multiple-round PCR using PfuUltra II Fusion HS DNA polymerase (Stratagene, Cedar Creek, TX) incorporated the mutation from the primers into the 3′ SIVmac239 plasmid. Double mutants were generated by sequential PCRs. Following amplification, the entire coding region of the 3′ half of the SIVmac239 genome was sequenced to confirm that only the intended changes were introduced. Sequence-confirmed variants were then digested with SphI and XhoI and ligated to the 5′ half of SIVmac239. Full-length clones were confirmed by restriction digestion.

Infectivity assay. SIVmac239 and each GNA/HHA-based virus stock were used to infect an immortalized human T-cell line (C8166-45) with a stably integrated, Tat-inducible SEAP gene as described previously (39). Briefly, 25 ng p27 for each virus was serially diluted 2-fold in RPMI complete medium. Virus from each dilution (100 μl) was added to 5,000 C8166-SEAP cells in 100 μl medium. Three days later, SIV infection of C8166-SEAP cells was determined by measuring the production of SEAP in the culture medium. SEAP activity in the supernatant was measured using a Phospha-Light assay system (Applied Biosystems).

Replication of SIV in rhesus PBMCs. Rhesus macaque peripheral blood mononuclear cells (PBMCs) were isolated from fresh citrate-containing blood of a rhesus macaque using lymphocyte separation medium (LSM; MP Biomedicals, LCC, Solon, OH). PBMCs were resuspended at 1×10^6 cells per milliliter in RPMI medium supplemented with 20% fetal bovine serum and were activated for 72 h with 1 μg/ml of phytohemagglutinin (Sigma, St. Louis, MO). For analysis of viral replication in culture, activated PMBCs were washed two times with RPMI medium supplemented with 20% fetal bovine serum (FBS) and 10% interleukin-2. Cells at a density of 1×10^6 cells per milliliter were infected with SIVmac239 containing 30 ng/ml capsid protein (p27). The culture was divided in half 24 h later. PBMCs from one half of the culture were resuspended with RPMI medium supplemented with 20% FBS and 10% interleukin-2. PBMCs from the other half of the culture were resuspended with RPMI medium supplemented with 20% FBS, 10% interleukin-2, 1 mg/ml GNA, and 0.5 mg/ml HHA. One-half of the culture supernatant was replaced every 3 to 4 days, and the concentration of p27 capsid protein in the supernatant was measured using a SIV p27 antigen capture assay (Advanced BioScience Laboratories, Inc.).

c.o. SIV gp120 expression vectors. The codon-optimized (c.o.) SIVmac239 Env expression vector (50) was modified using PCR mutagenesis to introduce two stop codons after the arginine codon at nt 8176 to 8178 in the SIVmac239 genome. These changes were made in the c.o. expression vector with the forward primer 5′-GAACAAGCGGTGATAATTCGTCCTGG-3′ together with a reverse-oriented mutagenic primer (c.o.SIVmac239 gp120). In addition the SIVN2 variant was made using PCR mutagenesis in the context of the c.o.SIVmac239 gp120 expression vector. c.o.PBj14 clone 6.6 was synthesized (GenScript, Piscataway, NJ) based on the genomic sequence (accession number L09212.1). SIVsmE543.3 gp120 was cloned from the c.o.SIVsmE543.3 expression vector, provided by Vanessa Hirsch. For both SIVsmPBj14 6.6 and SIVsmE543.3, *env* was amplified with primers that contained the appropriate restriction site. *env* was then cloned via the NheI and ApaI cut sites into the cytomegalovirus (CMV)-driven mammalian expression vector pcDNA3.1(+) (Invitrogen).

ELISA protein production. The c.o. SIV gp120 constructs and the c.o. HIV-2 gp120 constructs were used to transfect HEK293T cells using the GenJet method (SignaGen Laboratories, Ijamsville, MD). The medium was replaced 24 h later with serum-free DMEM. Two days later, culture supernatant was passed through a 0.45-μm polyethersulfone syringe filter. Filtered supernatant was incubated with concanavalin A-agarose beads (Sigma) for 24 h at 4°C. The beads were washed sequentially with 10 ml phosphate-buffered saline (PBS). The last wash was determined when the eluent reached an optical density less than 0.03. Then, gp120 was eluted from the beads with 18 ml of 0.75 M methyl *manno*-pyranoside in PBS. The samples were concentrated using Vivaspin20 columns with a 50-kDa cutoff (Fisher, Pittsburgh, PA). Each sample was dialyzed against PBS. Following dialysis, protein was recovered and total protein was determined with a bicinchoninic acid assay (Pierce, Rockford, IL). Recombinant histidine-tagged SIVmac239 gp120, SIVmac239 gp140, SIVsmE660 gp120, SIVsmE660 gp140, HIV-1 Bal gp120, HIV-1 JRCSF gp120, HIV-1 p1006_11.C3.1601 gp120, HIV-1 consensus B gp120, HIV-1 consensus A2 gp120, HIV-1 consensus C gp120, SIVcpzEK505 gp120, and SIVcpzMB66 gp120 produced from HEK293 cells were purchased from Immune Technology Corp., New York, NY.

GNA ELISA. A normalized amount of total protein was loaded into six wells of a high-protein-binding 96-well plate (Fisher). After two washes with 0.05% Tween 20 in PBS, the wells were blocked for 1 h at 37°C with carbohydrate-free blocking reagent (Vector Laboratories, Burlingame, CA). Wells were washed 5 times with 0.05% Tween 20 in PBS. Then, 50 μl of GNA-horseradish peroxidase (HRP) (US Biological, Swapscott, MA) diluted 1,000-fold in carbohydrate-free blocking buffer was added to four of the test wells. For the case of SIV and HIV proteins purchased from Immune Technology Corp., 50 μl of His-HRP antibody (Abcam, Cambridge, MA) was diluted 1:100 and added to duplicate wells of each sample as a normalization control. SIVgp120 proteins that did not include the His tag were normalized with polyclonal sera from SIVmac239-positive rhesus macaques. Serum IgGs were detected with anti-rhesus-HRP antibody (Southern Biotech, Birmingham, AL). Following a 1-hour incubation with the secondary antibody, wells were washed 10 times with 0.05% Tween 20 in PBS, and then 50 μl of fresh soluble TMB (3,3′,5,5′-tetramethylbenzidine; EMD Chemicals, Gibbstown, NJ) was added to each well. Wells were allowed to develop for 30 min before the reaction was stopped. Plates were read at 450 nm on an MRX Revelation (Dynex Technologies, Chantilly, VA).

Statistical significance of GNA binding. Prism software (GraphPad Software, Inc., La Jolla, CA) was used to run an unpaired *t* test to calculate the significance of the difference in GNA binding capacities between SIVmac/sm and HIV-1. The unpaired *t* test method tests the null hypothesis that the population means relating to two independent random samples from an approximately normal distribution are equal.

Neutralization assay. Sensitivity of the infection of GNA/HHA-based viral variants SIVmac239 and SIV316 to neutralization by sera from SIVmac239-infected rhesus macaques, soluble CD4 (sCD4), and monoclonal antibodies that bind SIV Env was measured using C8166-SEAP cells as previously described (39). Briefly, virus equivalent to 2 ng capsid protein (p27) for SIVmac239 and each GNA/HHA-based SIV variant and 5 ng of SIV316 was used to infect C8166-SEAP cells in the absence and presence of one of the following neutralizing agents: a pool of sera collected from four rhesus macaques infected with SIVmac239 for greater than 6 months, sCD4, and monoclonal antibodies that bind SIV Env. To perform neutralization assays, each virus was set up in a 96-well plate as follows: three wells in the second column contained 100 μl of RPMI complete medium, three wells in the third column contained 25 μl of complete medium, and three wells in each of columns 4 through 11 contained 25 μl of each of 8 dilutions of the neutralizing agent. Sera from SIVmac239-infected rhesus macaques were pooled and then heat inactivated at 56°C for 30 min before use in neutralization assays. Each virus in a total volume of 75 μl was added to each of the wells containing 25 μl of either medium or diluted sera, and the plate was incubated in 5% CO_2 at 37°C for 1 h. After the 1-h incubation, 5,000 C8166-SEAP cells in a volume of 100 μl were added to each well in columns 2 through 11 of the 96-well plate. SEAP activity in the culture supernatant was measured 3 and 5 days later using the chemiluminescent Phospha-Light assay system (Applied Biosystems) and detected using a Victor V multilabel counter (Perkin-Elmer; Waltham, MA). Neutralization activity for all antibodies and serum samples was reported as a percentage of SEAP activity. The percentage of SEAP activity was calculated as follows: SEAP activity from cells alone (column 2) was averaged and subtracted from that for each of the wells in columns 3 through 11. Then the counts from each column were divided by those for column 3 and multiplied by 100.

RESULTS

***Galanthus nivalis* agglutinin and *Hippeastrum* hybrid agglutinin inhibit the replication of SIVmac239.** Lectin proteins from *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA) bind carbohydrate and can inhibit the replication of HIV-1 (3, 4). Specifically, GNA binds α-1,3-linked mannose on high-mannose carbohydrate chains and HHA binds mannose sugars attached via both an α-1,3 and an α-1,6 linkage to high-mannose carbohydrate chains (28, 55). To determine if SIVmac239 is sensitive to GNA or HHA, replication of SIVmac239 was assayed at a low multiplicity of infection under conditions of a spreading infection. In the absence of lectin, SIVmac239 had a peak height of virus production 8 days after infection as determined by quantitation of the capsid protein (p27) from the culture supernatant. Similar replication kinetics were observed for SIVmac239 in the presence of 0.1 μg/ml GNA (Fig. 1A) or 0.1 μg/ml HHA (Fig. 1B). Progressively lower levels of viral replication were observed with increasing concentrations of lectin. The peak of virus replication was delayed 8 days for SIVmac239 in the presence of 1, 10, and 100 μg/ml GNA (Fig. 1A) and in the presence of 1 and 10 μg/ml HHA (Fig. 1B). In the presence of 200 μg/ml GNA, production of SIVmac239 remained at low levels (8 to 10 ng/ml p27) for up to 21 days after infection (Fig. 1A). Low SIVmac239 p27 production was also detected in the presence of 100 and 200 μg/ml HHA (Fig. 1B), consistent with inefficient spread through the culture at these concentrations of lectin.

Selection of an SIV population that replicates efficiently in the presence of GNA and HHA. To select for a population of SIV that replicated in the presence of both GNA and HHA, SIVmac239 was passaged in CEMx174 cells in the presence of escalating concentrations of both lectin proteins. CEMx174 cells were infected with SIVmac239, and 24 h later the medium was replaced with medium containing no lectin proteins or with medium containing 100 μg/ml GNA and separately 10 μg/ml HHA. Production of p27 in the culture supernatant was measured over the course of infection. When the p27 value increased significantly, at day 16 for 100 μg/ml GNA (Fig. 1A) and 10 μg/ml HHA (Fig. 1B), a new infection was started using virus normalized for the amount of SIV capsid protein (p27) from the selected population. At passage 2, the selection medium contained 200 μg/ml GNA and 25 μg/ml HHA. For each successive passage, the lectin concentration was doubled until the concentration of lectin in the medium reached 1 mg/ml GNA and 0.5 mg/ml HHA, at which point virus was selected for three more passages at a constant lectin concentration. After passage 9, the lectin-selected virus population replicated similarly to a parallel, unselected virus population when as-

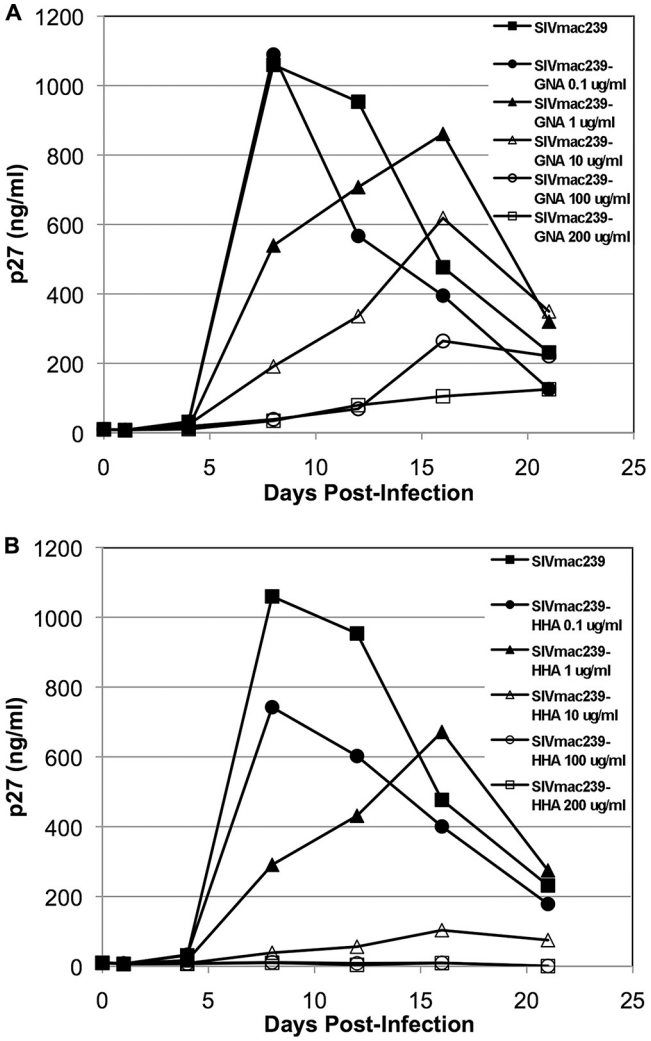

FIG. 1. The lectins GNA and HHA inhibit the replication of SIVmac239. (A) Representative replication of SIVmac239 in the absence of GNA and in the presence of GNA at 0.1 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml, and 200 μg/ml. (B) Representative replication of SIVmac239 in the absence of HHA and in the presence of HHA at 0.1 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml, and 200 μg/ml. CEMx174 cells were infected with SIVmac239 containing 5 ng of p27. The medium was changed 24 h later to contain the indicated concentrations of lectin. SIV capsid protein (p27) that was released into the culture medium was measured on the days indicated to follow virus production.

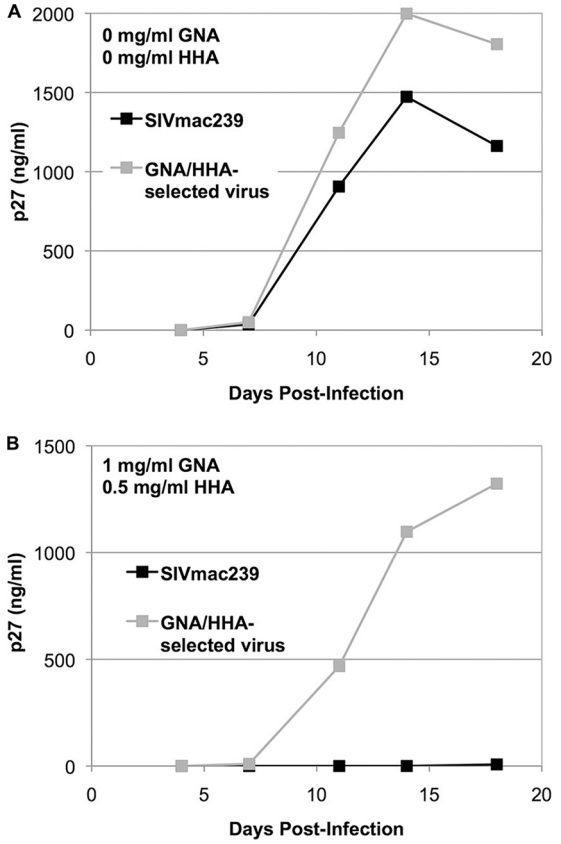

FIG. 2. Replication of the GNA/HHA-selected virus population in the absence and presence of the lectins GNA and HHA. (A) Replication of SIVmac239 and the GNA/HHA-selected population in the absence of lectin. (B) Replication of SIVmac239 and the GNA/HHA-selected population in the presence of GNA (1 mg/ml) and HHA (0.5 mg/ml). CEMx174 cells were infected with SIVmac239 or the GNA/HHA-selected virus, each containing 5 ng of p27. The culture was divided in half 24 h later, and the medium was exchanged either for that containing no lectin or for medium containing 1 mg/ml GNA and 0.5 mg/ml HHA. SIV capsid protein (p27) that was released into the culture medium was measured on the days indicated to follow virus production.

sayed in the absence of lectin (Fig. 2A). However, in the presence of 1 mg/ml GNA and 0.5 mg/ml HHA, only the lectin-selected virus had a resistant phenotype (Fig. 2B).

SIVmac239 variants cloned from the GNA/HHA-selected, lectin-resistant virus population. *env* sequences were amplified from the viral genomes of the SIV population that replicated in the presence of 1 mg/ml GNA and 0.5 mg/ml HHA, and 20 clones were sequenced. Six of the sequences contained at least one stop codon in the coding region of gp120 and were excluded from the analysis. Sequences of the GNA/HHA-selected envelope protein (Env) for the remaining 14 clones were aligned to the parental SIVmac239 Env (Fig. 3). Eleven Env sequences were full length. Three *env* sequences had truncations due to stop codons that truncated SIV gp41.

Truncation of the cytoplasmic tail of SIV gp41 has been frequently noted when SIV has been serially passaged in human cells (22, 32). Truncation occurred at glutamine at position 739 in clones 15 and 17 (Fig. 3). In both cases, the CAG codon (nt 8818 to 8820) was changed to TAG. This change was silent for the region overlapping the *tat* reading frame. Codon ACA (nt 8817 to 8819; threonine) was changed to ATA (isoleucine) in *rev*. GNA/HHA clone 19 was truncated at tryptophan at position 782. In this case the TGG codon (nt 8947 to 8949) was changed to TGA. In the *rev* reading frame, codon GCT (nt 8949 to 8951; alanine) was changed to ACT (threonine).

All 14 clones from the GNA/HHA-selected population had amino acid substitutions at the asparagine in the N-linked consensus sequence (Asn-X-Ser/Thr) at three of the N-linked sites in Env. Asn244 was replaced with Asp in the second conserved region (C2) and Asn460 was replaced with Ser in the

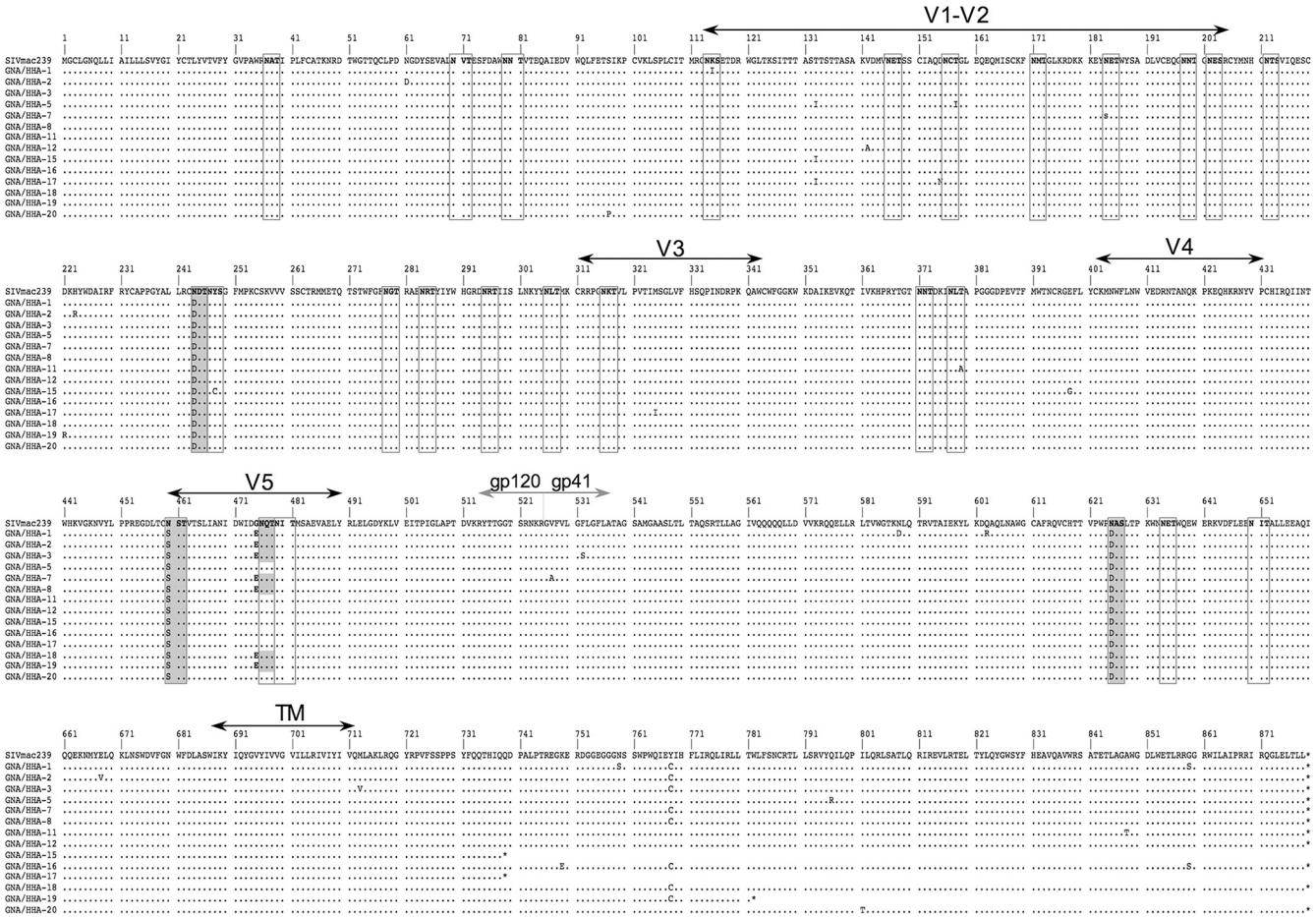

FIG. 3. SIV variants from the GNA/HHA-selected virus population consistently lack three potential N-linked glycosylation sites. Envelope sequences were amplified from viral RNA, and 20 clones were sequenced. Clones in which the gp120 coding sequence did not include a stop codon (14 out of 20) are shown. Predicted N-linked glycosylation sites (NXS/T) in gp120 and gp41 are boxed. Potential high-mannose carbohydrate attachment sites are highlighted in gray. The gp120 variable regions (V1 to V5), the junction between gp120 and gp41, and the transmembrane domain (TM) are also denoted. Amino acids in the GNA/HHA-selected virus population that are the same as those in the parental SIVmac239 are shown as periods. A stop codon is shown as an asterisk.

fifth variable region (V5) in gp120. In the ectodomain of gp41, Asn625 was replaced with Asp (Fig. 3). The N-linked consensus sequence that begins with Asn476 in gp120 was not directly altered; however, 50% of the clones from the GNA/HHA-selected population contained a Gly475-to-Glu substitution immediately preceding this attachment site (Fig. 3).

Construction of SIV site-specific variants based on the GNA/HHA-selected virus population. To investigate the functional contribution of amino acid changes observed in the GNA/HHA-selected population, cloned SIV variants based on the GNA/HHA-selected population were constructed in the context of the SIVmac239 proviral genome (Fig. 4). Asparagine at positions 244, 460, 476, and 625 was replaced with glutamine. This amino acid substitution was chosen because a two-nucleotide change would be required for the codons that encode glutamine to revert to the codons that encode asparagine. Asparagine and glutamine are also similar in structure, differing only by a single CH_2 group. Additionally, asparagine at position 460 was replaced with serine and glycine at position 475 was replaced with glu-

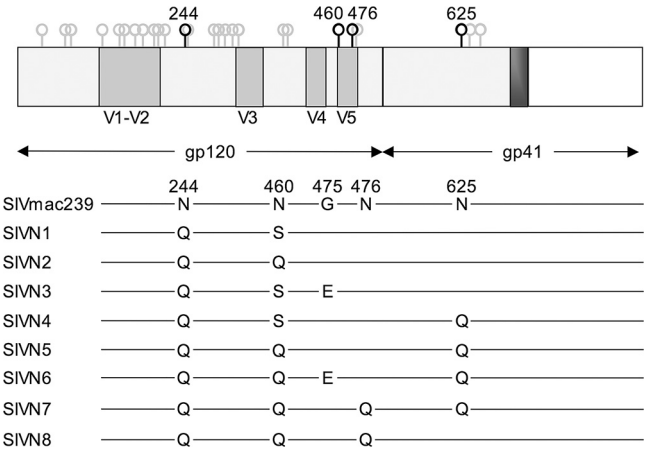

FIG. 4. Schematic representation of cloned SIV variants. Combinations of amino acid substitutions in and adjacent to canonical N-linked attachment sites (NXS/T) that were consistently changed in the GNA/HHA-selected virus population were introduced into the SIVmac239 proviral backbone. Asparagine at positions 244, 460, and 476 in gp120 and at position 625 in gp41 was replaced with glutamine. Glycine at position 475 in gp120 was replaced with glutamic acid.

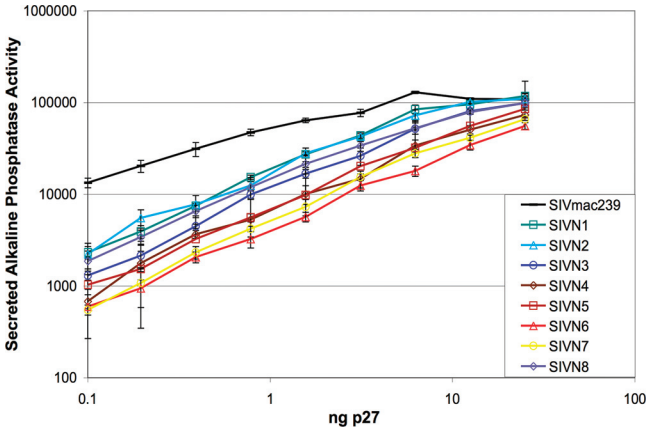

FIG. 5. Comparative infectivity of SIV variants that lack N-linked carbohydrate attachment sites. Virus stocks for each cloned SIV variant and the cloned SIVmac239 were produced by transient transfection of HEK293T cells. Virus containing normalized amounts of p27 was used to infect C8166-SEAP cells, which contain a stably integrated, Tat-inducible secreted alkaline phosphatase (SEAP) reporter gene. Viral infectivity is directly correlated to SEAP released into the culture supernatant. At 72 h after infection, SEAP activity was measured in triplicate with a Tropix Phospha-Light kit. The mean SEAP activity (± standard deviation) is shown for each viral input indicated.

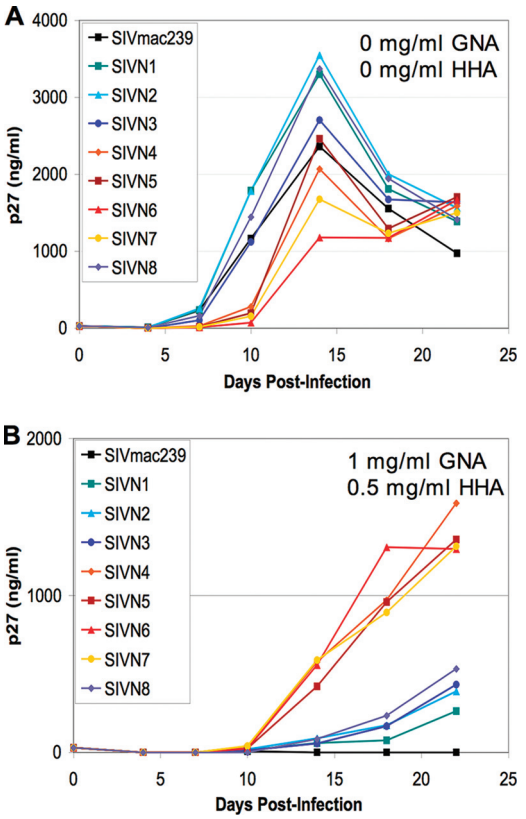

FIG. 6. Maximal resistance to GNA and HHA was conferred in variants that lacked Asn244, Asn460, and Asn625 in combination. (A) SIV variants deficient in potential N-linked glycosylation sites replicate similarly to the parental SIVmac239 in the absence of GNA and HHA. (B) SIV variants that lack N-linked sites are more resistant to GNA and HHA than the sensitive SIVmac239. Virus stocks for each cloned SIV variant and the cloned SIVmac239 were produced by transient transfection of HEK293T cells. CEMx174 cells were infected with SIV variant virus or SIVmac239, each containing 5 ng p27. One day following infection, the culture was split and the medium was changed to medium that did not contain lectin or to medium that contained GNA (1 mg/ml) and HHA (0.5 mg/ml). SIV capsid protein (p27) that was released into the culture medium was measured on the days indicated to follow virus production.

tamic acid to reflect the amino acid substitutions present in the lectin-selected SIV population.

SIV variants based on the GNA/HHA-selected virus population that are deficient for N-linked carbohydrate attachment sites are infectious. To determine if cloned SIV site-specific variants based on the GNA/HHA-selected virus population were infectious in the context of the SIVmac239 genome, virus stocks for SIVmac239 and for each of the SIV variants (SIVN1 to SIVN8) were produced by transient transfection of HEK293T cells. C8166-secreted alkaline phosphatase (C8166-SEAP) cells were infected with input virus normalized for the amount of SIV capsid protein (p27). Upon infection, C8166-SEAP cells secrete alkaline phosphatase into the culture medium such that the levels of phosphatase activity correlate directly with the amount of input p27. SEAP activity in the cell-free supernatant was measured 3 days after infection, a time that primarily reflects the first round of infection prior to appreciable spread of secondary progeny virions. The infectivity of each of the cloned SIV variants was 0.8 to 1.4 logs lower per ng of input p27 than that of SIVmac239 (Fig. 5).

Replication of the cloned SIV variants determined in the absence and presence of GNA and HHA. Spread of virus through culture was determined for each cloned SIV variant and the parental cloned SIVmac239. CEMx174 cells were infected with normalized amounts of p27-containing virus. Virus, as determined from p27, released into the culture supernatant was measured throughout the course of infection. In the absence of lectin, similar levels of virus production were observed for SIVmac239 and each cloned SIV variant (Fig. 6A). In the presence of GNA and HHA, SIVmac239 replication remained at background levels, indicative of an infection that could not overcome the inhibition of 1 mg/ml GNA and 0.5 mg/ml HHA (Fig. 6B). Maximal resistance to the inhibitory effects of GNA and HHA was conferred for variants SIVN4 and SIVN5, which lack potential N-linked carbohydrate attachment sites at positions 244, 460, and 625. Further replacement with glycine at position 475 or asparagine at position 476 (SIVN6 and SIVN7) did not impart additional lectin resistance (Fig. 6B). Thus, complete resistance to GNA and HHA in a spreading infection was conferred by alterations in three, and only three, sites for N-linked carbohydrate attachment.

Replication of SIV in rhesus macaque PBMCs in the absence and presence of GNA and HHA. The potential influence of species on carbohydrate composition was investigated by analyzing the replication of SIV variants in rhesus macaque peripheral blood mononuclear cells (PBMCs) in the presence and absence of lectin. Rhesus PBMCs were isolated and infected with normalized amounts of p27-containing virus. Virus, as determined by p27, released into the culture supernatant was measured throughout the course of infection. In the ab-

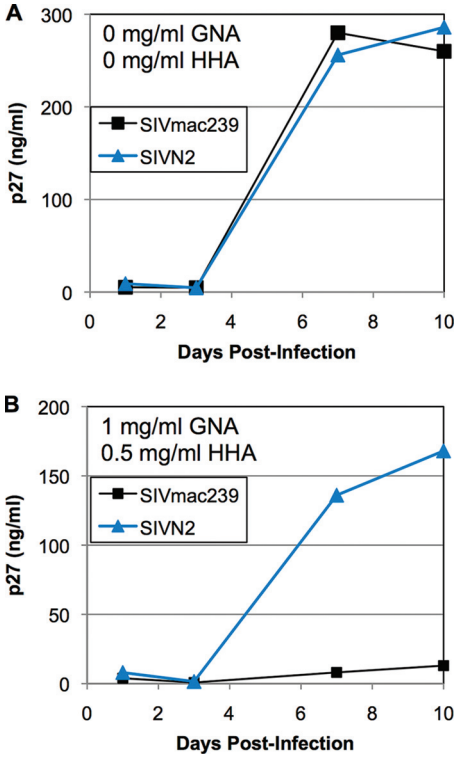

FIG. 7. Replication of the GNA/HHA-sensitive parental SIVmac239 and the GNA/HHA-resistant SIVN2 in rhesus macaque peripheral blood mononuclear cells (PBMCs). (A) Replication of SIVmac239 and SIVN2 in rhesus macaque PBMCs in the absence of lectin. (B) Replication of SIVmac239 and SIVN2 in rhesus macaque PBMCs in the presence of 1 mg/ml GNA and 0.5 mg/ml HHA. Rhesus macaque PBMCs were infected with HEK293T cell-produced SIVmac239 or SIVN2 that was normalized to contain equal amounts of p27 capsid protein. One day after infection, the cells were washed prior to being resuspended in medium with no lectin or with medium that contained 1 mg/ml GNA and 0.5 mg/ml HHA. SIV capsid protein (p27) that was released into the culture medium was measured on the days indicated to follow virus production.

sence of lectin, similar levels of virus production were observed for SIVmac239 and SIVN2 (Fig. 7A). In the presence of high concentrations of GNA and HHA, SIVN2 replicated well (Fig. 7B). The replication of SIVmac239 in rhesus PBMCs remained at background levels in the presence of GNA and HHA (Fig. 7B). These findings demonstrate that the loss of carbohydrate at positions 244 and 460 also confers relative resistance to GNA and HHA in primary rhesus cells. Consistent with a previous publication (64), replication of variants lacking the Asn625 site (SIVN5 and SIVN7) was sufficiently impaired in rhesus PBMCs, even in the absence of lectin, to preclude further analysis.

High-mannose carbohydrate attached to SIVmac239 Env at positions Asn244, Asn460, and Asn625 is accessible to GNA. The GNA binding capacity of SIV Env was measured in a newly developed ELISA. SIV gp120 proteins purified from HEK293T cell supernatant were normalized for total protein bound to each well. The SIV gp120 proteins were probed in quadruplicate with horseradish peroxidase (HRP)-conjugated GNA or in duplicate with sera from SIVmac239-positive macaques (Fig. 8A, black and gray bars, respectively). The gp120s

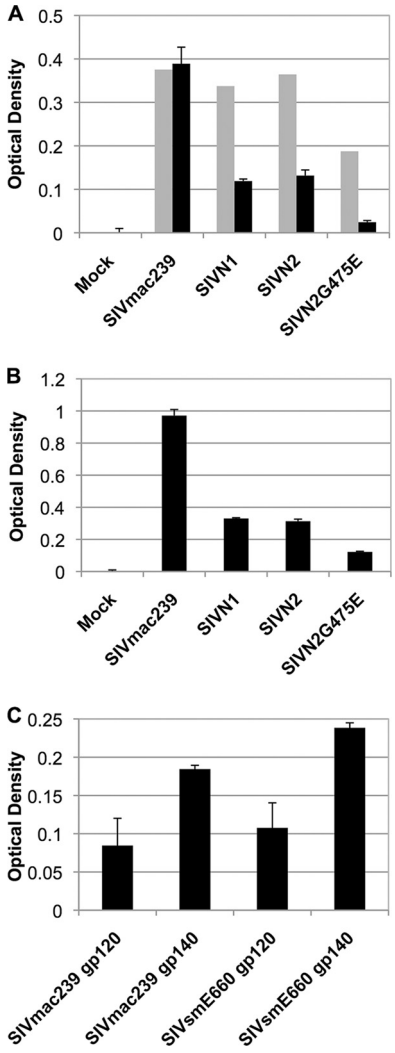

FIG. 8. GNA binds carbohydrate attached to Asn244, Asn460, Asn476, and Asn625 in SIVmac239. (A) SIVN1, SIVN2, and SIVN2G475E gp120 proteins have a markedly decreased GNA binding capacity compared to SIVmac239 gp120. HEK293T cells were transfected with no DNA (mock) or with the gp120 eukaryotic expression vector for SIVmac239, SIVN1, SIVN2, or SIVN2G475E. Protein released into serum-free medium was purified with the concanavalin A lectin. Equivalent amounts of protein from each sample were loaded into six wells of a high-protein-binding plate. GNA-HRP was used to probe each sample in quadruplicate. The mean GNA signal (±standard deviation) is shown in black. Sera from SIV-positive rhesus macaques were used to probe each sample in duplicate. The signal from serum binding gp120 is shown in gray. (B) Normalized GNA binding to gp120 proteins from SIVN1, SIVN2, and SIVN2G475E gp120 proteins. The SIVN2G475E variant is the SIVN2 variant with an additional replacement of glycine with glutamic acid at position 475 (see Fig. 3 and 4). GNA-HRP was used to probe each sample in quadruplicate. Sera from SIV-positive rhesus macaques were used to probe each sample in duplicate. The mean GNA signal was normalized to the mean signal from sera. The normalized GNA signal (±standard deviation) is shown. (C) SIVmac239 and SIVsmE660 gp140 proteins have a greater GNA binding capacity than SIVmac239 and SIVsmE660 gp120 proteins. Equivalent amounts of histidine-tagged SIVmac239 or SIVsmE660 gp120 or gp140 proteins were each loaded into six wells of a high-protein-binding plate. GNA-HRP was used to probe each sample in quadruplicate. A histidine-HRP antibody was used to probe each sample in duplicate. The mean GNA signal was normalized to the mean signal from the histidine tag. The normalized GNA signal (±standard deviation) is shown.

of SIVN1 and SIVN2, two variants that lack carbohydrate at Asn244 and Asn460, exhibited a GNA binding capacity that was 70% lower than that of gp120 of the parental SIVmac239 (Fig. 8B). In independent experiments, SIV variant gp120 that lacked carbohydrate at Asn244 and Asn460 exhibited 70- to 80%-decreased GNA binding capacity compared to gp120 of the parental SIVmac239. The variant gp120 of SIVN2G475E had a 90% lower GNA binding capacity than the gp120 of SIVmac239 (Fig. 8B).

To compare the GNA binding capacities of SIVmac239 and SIVsmE660 gp120 and gp140 proteins, commercially available recombinant SIV gp120 and gp140 histidine (His)-tagged proteins produced from HEK293 cells were probed with GNA-HRP and an anti-His-HRP antibody. The GNA signal was normalized to the signal from the His tag. The gp140 GNA binding capacity is greater than that for gp120 for both SIVmac239 and the unrelated SIVsmE660 (Fig. 8C). The data are thus consistent with high-mannose carbohydrate modification in the gp41 ectodomain of SIVsmE660. These data also further support high-mannose carbohydrate attachment at Asn625 in the gp41 of SIVmac239.

HIV-1 gp120 has a significantly higher content of high-mannose carbohydrate than SIVmac/sm gp120. Biochemical analysis of gp120 from HIV-1 strain SF2 identified 12 high-mannose carbohydrates (66). The biochemical analysis of the carbohydrate content of HIV-1 IIIB identified 10 high-mannose or hybrid carbohydrates in gp120 (35). Consistent with the high-mannose type at the majority of these sites, the GNA/HHA-selected population of HIV-1 IIIB lacked 8 N-linked sites in gp120 (3). In contrast, resistance to GNA and HHA was conferred in SIVmac239 variants that lacked only three N-linked sites in Env, only two of which were in gp120. Selection using the SIVmac239-related, uncloned, lab-adapted SIVmac251 with HHA resulted in the elimination of three N-linked carbohydrate attachment sites in gp120 (14). To determine if differences in the content of high-mannose carbohydrate can be consistently demonstrated in head-to-head comparisons, the GNA binding capacities of recombinant HIV-1 and SIVmac/sm gp120 proteins produced from HEK293 cells were determined. Six independent, commercially available HIV-1 His-tagged gp120 proteins and 3 SIVmac/sm His-tagged gp120 proteins were bound to a high-protein-binding plate. Subsequently, the GNA signal was normalized to the average signal from the His tag (Fig. 9A). The gp120s of two additional SIVsm isolates were purified from HEK293T cell supernatant (see Materials and Methods). The six HIV-1 isolates had 3- to 10-fold-higher GNA binding capacities than the five SIVmac/sm isolates (Fig. 9A and B). The difference in the GNA binding capacities of six unrelated isolates of HIV-1 and the five SIVmac/sm isolates was statistically significant ($P = 0.0022$) in an unpaired t test.

Since HIV-1 is thought to have arisen by the transmission of SIVcpz to humans (25, 29) and HIV-2 is thought to have arisen by the transmission of SIVsm to humans (18, 23, 51), we investigated the GNA binding capacity of SIVcpz and HIV-2 gp120 proteins. Similar to that of HIV-1, the SIVcpz gp120 had a high GNA binding capacity (Fig. 9A). The GNA binding capacity of HIV-2 gp120 was like that of SIVmac/sm gp120 (Fig. 9B). Thus, all nine SIVmac/sm and HIV-2 strains were consistently lower than all 8 HIV-1 and SIVcpz strains in their gp120 GNA binding capacities.

Neutralization of SIV variants based on the GNA/HHA-selected virus population by soluble CD4, SIVmac239-positive macaque sera, and a panel of anti-SIV monoclonal antibodies. The sensitivity of SIVN2, SIVN5, SIVN7, and SIVN8 to neutralization by soluble CD4 (sCD4), by pooled SIV-positive rhesus macaque sera, and by a panel of monoclonal antibodies directed to SIV Env was examined. SIVN2, SIVN5, SIVN7, and SIVN8 were equally sensitive to neutralization by sCD4 (Fig. 10A). The concentrations of sCD4 needed to reach 50% inhibition (IC_{50}) of infection for SIVN2, SIVN5, SIVN7, and SIVN8 were 3- to 5-fold less than the concentration of sCD4 needed to neutralize 50% of the infection of SIVmac239 (Table 1). Neutralization of infection by pooled SIV-positive rhesus macaque sera was more potent for SIVN2, SIVN5, SIVN7, and SIVN8 than for SIVmac239 (Fig. 10B). SIVN2 and SIVN8 were 2- to 3-fold more sensitive than SIVmac239 to neutralization by pooled SIV-positive rhesus macaque sera. SIVN5 and SIVN7 were 6- and 8-fold, respectively, more sensitive to neutralization by pooled sera than SIVmac239 (Table 1). SIV variants SIVN2, SIVN5, SIVN7, and SIVN8 were similar to SIVmac239 in resistance to neutralization by rhesus macaque-derived monoclonal antibodies that bind to both discontinuous and linear epitopes covering the majority of gp120 (10, 27) (Fig. 10C; see Fig. S1 in the supplemental material). These antibodies have been shown to neutralize SIV316 (27), a neutralization-sensitive derivative of SIVmac239 (46). Here, SIV316 was included as a positive control in the neutralization assay (Fig. 10C). The mouse monoclonal antibody KK41, which binds the ectodomain of gp41

TABLE 1. Sensitivity of SIV to neutralization by sCD4, SIV-positive rhesus macaque sera, and a panel of rhesus monoclonal anti-SIVmac239 envelope protein antibodies

Viral strain	IC_{50}[a]										
	sCD4	3.10A	3.11H	1.11A	3.11E	3.5F	1.9C	1.10A	C26	Sera	KK41
SIVmac239	1	>10	>10	>10	>10	>10	>10	>10	>10	32	<80
SIV316	0.01	>10	0.31	0.31	0.16	0.1	<0.08	<0.08	<0.08	>1,024	ND[b]
SIVN2	0.25	>10	>10	>10	>10	>10	>10	>10	>10	80	ND
SIVN5	0.2	>10	>10	>10	>10	>10	>10	>10	>10	200	<80
SIVN7	0.25	>10	>10	>10	>10	>10	>10	>10	>10	256	<80
SIVN8	0.25	>10	>10	>10	>10	>10	>10	>10	>10	100	ND

[a] Units are μg/ml except for sera and KK41, for which the units are inverse reciprocal dilutions.
[b] ND, not done.

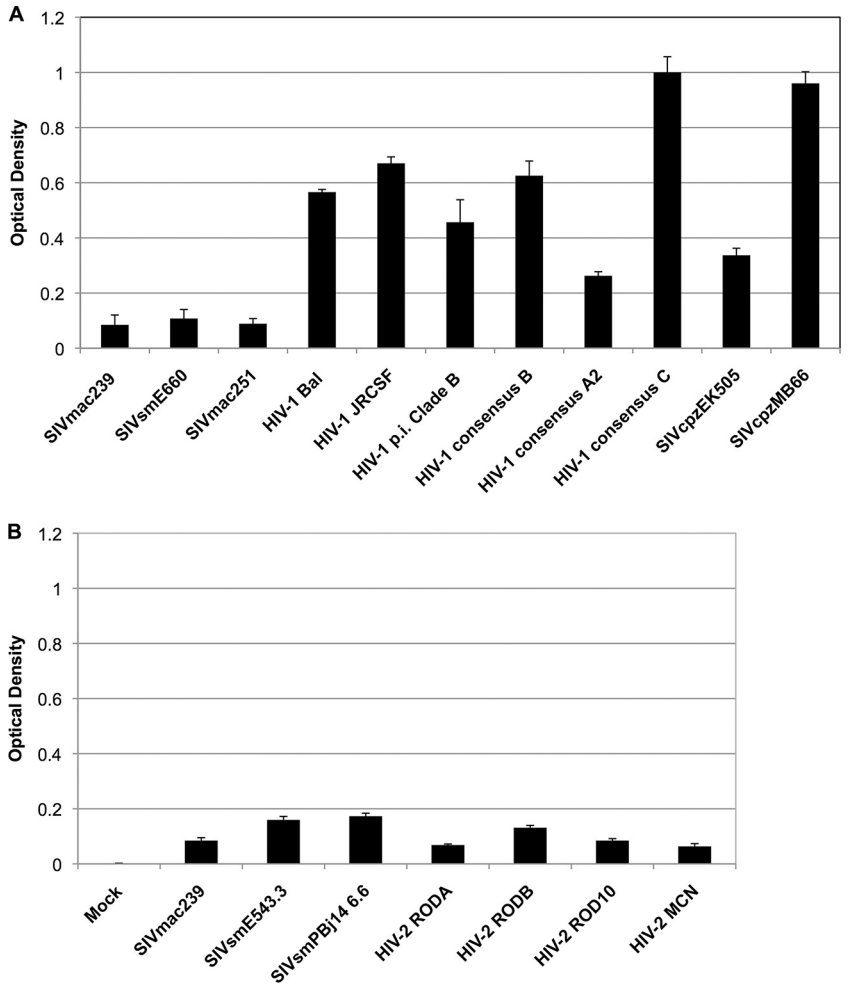

FIG. 9. HIV-1 and SIVcpz gp120 proteins have a higher GNA binding capacity than SIVmac/sm and HIV-2 gp120 proteins. (A) GNA binding capacity of SIVmac, SIVsm, HIV-1, and SIVcpz gp120 proteins. Equivalent amounts of histidine-tagged gp120 proteins were loaded into six wells of a high-protein-binding plate. GNA-HRP was used to probe each sample in quadruplicate. A histidine-HRP antibody was used to probe each sample in duplicate. The mean GNA signal was normalized to the mean signal from the histidine tag. The normalized GNA signal (±standard deviation) is shown. (B) Comparison of the SIVmac, SIVsm, and HIV-2 gp120 GNA binding capacities. HEK293T cells were transiently transfected with no DNA (mock) or a gp120 eukaryotic expression vector for SIVmac, SIVsm, and HIV-2. Env was purified from cell culture supernatant with the concanavalin A lectin. Equivalent amounts of total protein were loaded into the wells of a high-protein-binding plate. GNA-HRP was used to probe each sample in quadruplicate. The mean GNA signal (±standard deviation) is shown.

(30), did not result in neutralization of infection by SIVmac239 or the two SIV variants (SIVN5 and SIVN7) that lack carbohydrate at Asn625 (see Fig. S1 in the supplemental material).

DISCUSSION

The ease with which GNA/HHA-resistant variants of SIVmac239 were selected was somewhat surprising. Consistent with the ease of selection, mutations at only three sites were sufficient to confer high-level resistance of SIVmac239 to GNA and HHA. Two of these three sites were in gp120 (Asn244 and Asn460), and one was in gp41 (Asn625). Use of a newly developed ELISA showed that SIVmac239 gp120 that lacked these two N-linked sites exhibited 70- to 80%-decreased binding of GNA, indicative of a decreased content of high-mannose carbohydrate. Interestingly, additional replacement of a glycine with a glutamic acid just prior to the N-linked site at position 476 decreased GNA binding by 90%. One possible explanation for this observation is that the N-linked site at position 476 contains high-mannose carbohydrate some but not all of the time. It is possible that this G-to-E substitution at position 475 altogether inhibits carbohydrate attachment to Asn476 or that this change may facilitate the processing of carbohydrate to a complex or hybrid chain.

While our work was in progress, Francois et al. published the sequence of two gp120 clones from a lab-adapted, HHA-selected SIVmac251 population (14). Both sequences lacked three N-linked carbohydrate attachment sites in gp120, two of which correspond to the sites described here. Our findings described here extend these observations in several important ways. We show that a presumed high-mannose carbohydrate site in gp41 contributes importantly to GNA/HHA resistance,

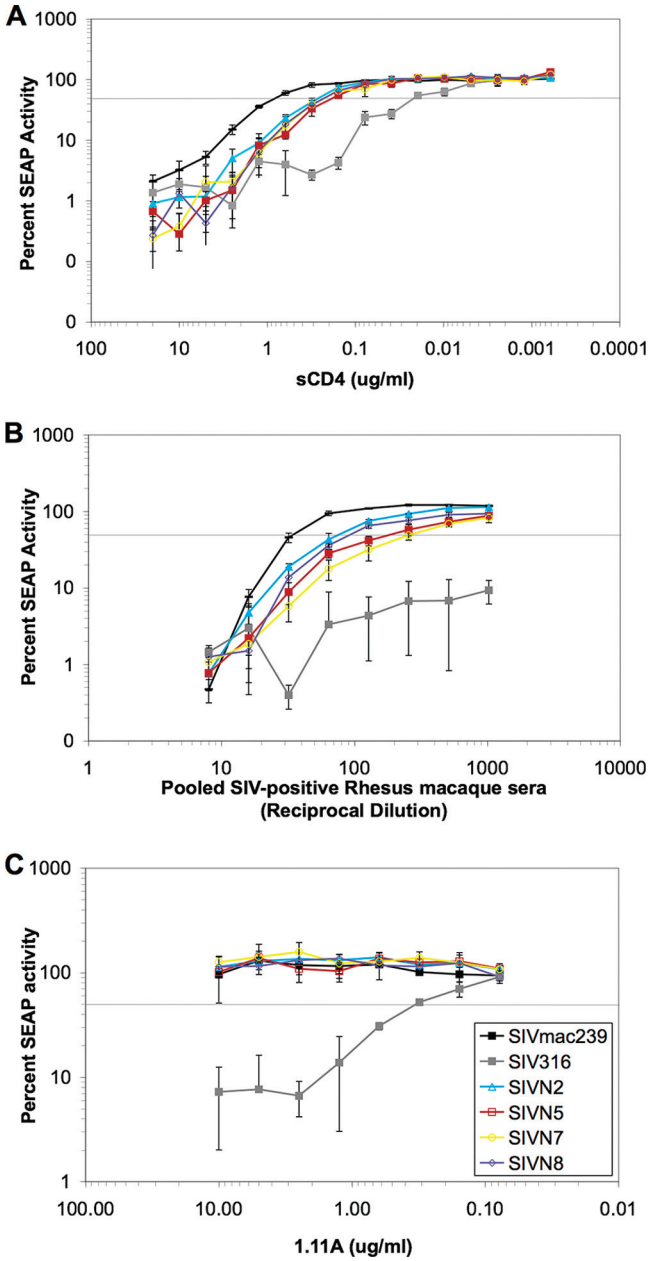

FIG. 10. Comparative neutralization of SIVmac239 and four SIV variants that lack N-linked carbohydrate attachment sites. (A) Neutralization of infectivity with soluble CD4 (sCD4). (B) Neutralization of infectivity by pooled SIV-positive sera. (C) Neutralization of viral infectivity by the rhesus anti-gp120 monoclonal antibody 1.11A. HEK293T-produced virus for each cloned SIV variant and, separately, the parental SIVmac239 were incubated for 1 h with either sCD4, pooled sera from SIVmac239-positive rhesus macaques, or the rhesus monoclonal anti-gp120 antibody 1.11A. Then, C8166-SEAP cells, which contain a stably integrated, Tat-inducible secreted alkaline phosphatase (SEAP) reporter gene, were added. SEAP activity was measured 72 h later with a Tropix Phospha-Light kit. A lower percentage of SEAP activity is indicative of neutralization, while 100% SEAP activity indicates the lack of neutralization of virus infectivity.

at least for SIVmac239. Significantly, we used cloned SIV to demonstrate the replication competence of defined sequence variants and to demonstrate that changes at three sites were necessary and sufficient to confer high-level resistance to GNA and HHA. The use of the SIVmac239 clone will allow meaningful investigation of the impact of these selective N-linked high-mannose carbohydrate knockouts upon experimental rhesus macaque infection in an informative animal model for AIDS. Finally, and perhaps most importantly, we have employed a GNA-binding ELISA to expand information on the high-mannose carbohydrate contents of five SIVmac/sm strains, four HIV-2 strains, two SIVcpz strains, and six HIV-1 strains.

How do our findings with SIVmac compare with what is known for HIV-1? Using a biochemical approach, Leonard et al. reported that 11 of the N-linked sites in gp120 of HIV-1 strain IIIB were either high-mannose or hybrid carbohydrate sites (35). While this study did not discriminate between high-mannose and hybrid carbohydrate sites, the expectation would be that most would be high-mannose carbohydrate sites. Consistent with this expectation, Balzarini et al. found substitutions that eliminated 8 N-linked sites in a GNA/HHA-selected population of HIV-1 strain IIIB (3). Also using biochemical analyses and mass spectrometry, Zhu et al. reported that carbohydrates attached at 12 N-linked sites in HIV-1 strain SF2 gp120 were purely high mannose (66). These studies would suggest these two HIV-1 strains contain considerably more high-mannose carbohydrate than SIVmac239. Here, we have used a GNA binding assay to gain information on the high-mannose carbohydrate content of gp120s from six HIV-1 strains and five SIVmac/sm strains. All six HIV-1 strains were epidemiologically unrelated, and most of the SIVmac/sm strains were epidemiologically unrelated. The gp120s of all six HIV-1 strains exhibited considerably higher high-mannose carbohydrate content than the gp120s of all five SIVmac/sm strains ($P = 0.0022$). The gp120s of the three natural HIV-1 isolates (Bal, JRCSF, and p.i.) exhibited 4- to 6-fold-higher levels of GNA binding than the gp120s of SIVmac239, SIVsmE543.3, and SIVsmPBj14 6.6, three epidemiologically unlinked SIVmac/sm isolates. Thus, SIVmac/sm appears to differ fundamentally from HIV-1 in the content of high-mannose carbohydrate.

Might there be marked differences in the content or location of high-mannose carbohydrate depending on the species from which gp120 was produced? The number and location of high mannose carbohydrate sites in HIV-1 IIIB determined biochemically by Leonard et al. (35) agree very well with the number and location of presumed high-mannose carbohydrate sites determined by GNA/HHA selection of HIV-1 IIIB (3). The former study produced gp120 from Chinese hamster ovary cells, and the latter study used viral replication in human cells. In addition, the two mutations in gp120 that confer relative resistance to GNA/HHA for viral replication of SIVmac239 in human cells also confer relative lectin resistance to replication in rhesus monkey PBMC cultures. Thus, marked variation in the content or location of high-mannose carbohydrate sites is not apparent among these three species.

Since HIV-1 in humans is believed to have arisen by cross-species transmission of SIVcpz from chimpanzees (25, 29) and HIV-2 in humans is believed to have arisen by cross-species transmission of SIVsm from sooty mangabey monkeys (18, 23, 51), we also investigated the high-mannose

carbohydrate content in a limited number of gp120s from SIVcpz and HIV-2. The high-mannose carbohydrate content of the HIV-2 strains reflected that of SIVmac/sm, and the high-mannose carbohydrate content of the SIVcpz strains reflected that of HIV-1. Thus, the two distinct HIVs in the human population appear to have high-mannose carbohydrate contents that reflect the SIVs in the species from which each was derived. Since sooty mangabey monkeys are also believed to be the source of cross-species transmission of SIV to macaques in captivity (1, 37), the low content of high-mannose carbohydrate in SIVsm appears to have been preserved following transmission and establishment in two different species: humans and macaques.

While it is possible that these fundamental differences in high-mannose carbohydrate content have no functional or mechanistic consequences, it is also possible that the difference in HIV-1 versus SIVmac/sm in high-mannose carbohydrate content may reflect the evolution of virus and host adaptation for properties such as viral adhesion to cells, trafficking, and cellular activation. High-mannose carbohydrate has been implicated in the binding of Env to DC-SIGN and to the macrophage mannose receptor (16, 17, 43, 44), and these interactions are thought to facilitate the infection of natural target cells (17, 43). The binding of HIV-1 high-mannose carbohydrate to DC-SIGN and to the soluble mannose binding lectin induces the release of immune-modulatory cytokines (21, 54). A few published reports have related polymorphisms in the human mannose binding lectin to the course of HIV-1 disease and ability to control infection (15, 26, 36). To determine if the differences in carbohydrate composition reflect virus and host adaptation, further studies will need to be directed at variations in carbohydrate-sensing proteins among host species in the context of the difference in HIV and SIV carbohydrate composition reported here.

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REFERENCES

1. **Apetrei, C., N. W. Lerche, I. Pandrea, B. Gormus, G. Silvestri, A. Kaur, D. L. Robertson, J. Hardcastle, A. A. Lackner, and P. A. Marx.** 2006. Kuru experiments triggered the emergence of pathogenic SIVmac. AIDS **20:**317–321.
2. **Bahraoui, E., A. Benjouad, D. Guetard, H. Kolbe, J. C. Gluckman, and L. Montagnier.** 1992. Study of the interaction of HIV-1 and HIV-2 envelope glycoproteins with the CD4 receptor and role of N-glycans. AIDS Res. Hum. Retroviruses **8:**565–573.
3. **Balzarini, J., K. Van Laethem, S. Hatse, M. Froeyen, E. Van Damme, A. Bolmstedt, W. Peumans, E. De Clercq, and D. Schols.** 2005. Marked depletion of glycosylation sites in HIV-1 gp120 under selection pressure by the mannose-specific plant lectins of Hippeastrum hybrid and Galanthus nivalis. Mol. Pharmacol. **67:**1556–1565.
4. **Balzarini, J., K. Van Laethem, S. Hatse, K. Vermeire, E. De Clercq, W. Peumans, E. Van Damme, A. M. Vandamme, A. Bolmstedt, and D. Schols.** 2004. Profile of resistance of human immunodeficiency virus to mannose-specific plant lectins. J. Virol. **78:**10617–10627.
5. **Bause, E., and G. Legler.** 1981. The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the N-glycosylation step during glycoprotein biosynthesis. Biochem. J. **195:**639–644.
6. **Benjouad, A., T. Babas, L. Montagnier, and E. Bahraoui.** 1993. N-linked oligosaccharides of simian immunodeficiency virus envelope glycoproteins are dispensable for the interaction with the CD4 receptor. Biochem. Biophys. Res. Commun. **190:**311–319.
7. **Binley, J. M., R. Wyatt, E. Desjardins, P. D. Kwong, W. Hendrickson, J. P. Moore, and J. Sodroski.** 1998. Analysis of the interaction of antibodies with a conserved enzymatically deglycosylated core of the HIV type 1 envelope glycoprotein 120. AIDS Res. Hum. Retroviruses **14:**191–198.
8. **Caccam, J. F., J. J. Jackson, and E. H. Eylar.** 1969. The biosynthesis of mannose-containing glycoproteins: a possible lipid intermediate. Biochem. Biophys. Res. Commun. **35:**505–511.
9. **Cannon, G., Y. Yi, H. Ni, E. Stoddard, D. A. Scales, D. I. Van Ryk, I. Chaiken, D. Malamud, and D. Weissman.** 2008. HIV envelope binding by macrophage-expressed gp340 promotes HIV-1 infection. J. Immunol. **181:**2065–2070.
10. **Cole, K. S., M. Alvarez, D. H. Elliott, H. Lam, E. Martin, T. Chau, K. Micken, J. L. Rowles, J. E. Clements, M. Murphey-Corb, R. C. Montelaro, and J. E. Robinson.** 2001. Characterization of neutralization epitopes of simian immunodeficiency virus (SIV) recognized by rhesus monoclonal antibodies derived from monkeys infected with an attenuated SIV strain. Virology **290:**59–73.
11. **Cole, K. S., J. D. Steckbeck, J. L. Rowles, R. C. Desrosiers, and R. C. Montelaro.** 2004. Removal of N-linked glycosylation sites in the V1 region of simian immunodeficiency virus gp120 results in redirection of B-cell responses to V3. J. Virol. **78:**1525–1539.
12. **den Dunnen, J., S. I. Gringhuis, and T. B. Geijtenbeek.** 2009. Innate signaling by the C-type lectin DC-SIGN dictates immune responses. Cancer Immunol. Immunother. **58:**1149–1157.
13. **Fenouillet, E., B. Clerget-Raslain, J. C. Gluckman, D. Guetard, L. Montagnier, and E. Bahraoui.** 1989. Role of N-linked glycans in the interaction between the envelope glycoprotein of human immunodeficiency virus and its CD4 cellular receptor. Structural enzymatic analysis. J. Exp. Med. **169:**807–822.
14. **Francois, K. O., J. Auwerx, D. Schols, and J. Balzarini.** 2008. Simian immunodeficiency virus is susceptible to inhibition by carbohydrate-binding agents in a manner similar to that of HIV: implications for further preclinical drug development. Mol. Pharmacol. **74:**330–337.
15. **Garred, P., H. O. Madsen, U. Balslev, B. Hofmann, C. Pedersen, J. Gerstoft, and A. Svejgaard.** 1997. Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. Lancet **349:**236–240.
16. **Geijtenbeek, T. B., G. Koopman, G. C. van Duijnhoven, S. J. van Vliet, A. C. van Schijndel, A. Engering, J. L. Heeney, and Y. van Kooyk.** 2001. Rhesus macaque and chimpanzee DC-SIGN act as HIV/SIV gp120 trans-receptors, similar to human DC-SIGN. Immunol. Lett. **79:**101–107.
17. **Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk.** 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **100:**587–597.
18. **Gojobori, T., E. N. Moriyama, Y. Ina, K. Ikeo, T. Miura, H. Tsujimoto, M. Hayami, and S. Yokoyama.** 1990. Evolutionary origin of human and simian immunodeficiency viruses. Proc. Natl. Acad. Sci. U. S. A. **87:**4108–4111.
19. **Gottlieb, C., A. M. Skinner, and S. Kornfeld.** 1974. Isolation of a clone of Chinese hamster ovary cells deficient in plant lectin-binding sites. Proc. Natl. Acad. Sci. U. S. A. **71:**1078–1082.
20. **Gruters, R. A., J. J. Neefjes, M. Tersmette, R. E. de Goede, A. Tulp, H. G. Huisman, F. Miedema, and H. L. Ploegh.** 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature **330:**74–77.
21. **Heggelund, L., T. E. Mollnes, T. Espevik, F. Muller, K. I. Kristiansen, P. Aukrust, and S. S. Froland.** 2005. Modulatory effect of mannose-binding lectin on cytokine responses: possible roles in HIV infection. Eur. J. Clin. Invest. **35:**765–770.
22. **Hirsch, V. M., P. Edmondson, M. Murphey-Corb, B. Arbeille, P. R. Johnson, and J. I. Mullins.** 1989. SIV adaptation to human cells. Nature **341:**573–574.
23. **Hirsch, V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson.** 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature **339:**389–392.
24. **Hu, H., T. Shioda, C. Moriya, X. Xin, M. K. Hasan, K. Miyake, T. Shimada, and Y. Nagai.** 1996. Infectivities of human and other primate lentiviruses are activated by desialylation of the virion surface. J. Virol. **70:**7462–7470.
25. **Huet, T., R. Cheynier, A. Meyerhans, G. Roelants, and S. Wain-Hobson.** 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature **345:**356–359.
26. **Hundt, M., H. Heiken, and R. E. Schmidt.** 2000. Low mannose-binding lectin serum concentrations in HIV long-term nonprogressors? AIDS Res. Hum. Retroviruses **16:**1927.
27. **Johnson, W. E., H. Sanford, L. Schwall, D. R. Burton, P. W. Parren, J. E.**

Robinson, and R. C. Desrosiers. 2003. Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. J. Virol. **77:**9993–10003.

28. **Kaku, H., E. J. Van Damme, W. J. Peumans, and I. J. Goldstein.** 1990. Carbohydrate-binding specificity of the daffodil (Narcissus pseudonarcissus) and amaryllis (Hippeastrum hybr.) bulb lectins. Arch. Biochem. Biophys. **279:**298–304.
29. **Keele, B. F., F. Van Heuverswyn, Y. Li, E. Bailes, J. Takehisa, M. L. Santiago, F. Bibollet-Ruche, Y. Chen, L. V. Wain, F. Liegeois, S. Loul, E. M. Ngole, Y. Bienvenue, E. Delaporte, J. F. Brookfield, P. M. Sharp, G. M. Shaw, M. Peeters, and B. H. Hahn.** 2006. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science **313:**523–526.
30. **Kent, K. A., L. Gritz, G. Stallard, M. P. Cranage, C. Collignon, C. Thiriart, T. Corcoran, P. Silvera, and E. J. Stott.** 1991. Production and of monoclonal antibodies to simian immunodeficiency virus envelope glycoproteins. AIDS **5:**829–836.
31. **Kiely, M. L., G. S. McKnight, and R. T. Schimke.** 1976. Studies on the attachment of carbohydrate to ovalbumin nascent chains in hen oviduct. J. Biol. Chem. **251:**5490–5495.
32. **Kodama, T., D. P. Wooley, Y. M. Naidu, H. W. Kestler III, M. D. Daniel, Y. Li, and R. C. Desrosiers.** 1989. Significance of premature stop codons in *env* of simian immunodeficiency virus. J. Virol. **63:**4709–4714.
33. **Kornfeld, S., E. Li, and I. Tabas.** 1978. The synthesis of complex-type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein. J. Biol. Chem. **253:**7771–7778.
34. **Kuiken, C., T. Leitner, B. Foley, B. Hahn, P. Marx, F. McCutchan, S. Wolinsky, and B. Korber.** 2009. HIV sequence compendium. Los Alamos National Laboratory, Los Alamos, NM.
35. **Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**10373–10382.
36. **Mangano, A., C. Rocco, S. M. Marino, D. Mecikovsky, F. Genre, P. Aulicino, R. Bologna, and L. Sen.** 2008. Detrimental effects of mannose-binding lectin (MBL2) promoter genotype XA/XA on HIV-1 vertical transmission and AIDS progression. J. Infect. Dis. **198:**694–700.
37. **Mansfield, K. G., N. W. Lerch, M. B. Gardner, and A. A. Lackner.** 1995. Origins of simian immunodeficiency virus infection in macaques at the New England Regional Primate Research Center. J. Med. Primatol. **24:**116–122.
38. **Means, R. E., and R. C. Desrosiers.** 2000. Resistance of native, oligomeric envelope on simian immunodeficiency virus to digestion by glycosidases. J. Virol. **74:**11181–11190.
39. **Means, R. E., T. Greenough, and R. C. Desrosiers.** 1997. Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J. Virol. **71:**7895–7902.
40. **Miller, E., D. Fiete, N. M. Blake, M. Beranek, E. L. Oates, Y. Mi, D. S. Roseman, and J. U. Baenziger.** 2008. A necessary and sufficient determinant for protein-selective glycosylation in vivo. J. Biol. Chem. **283:**1985–1991.
41. **Mori, K., Y. Yasutomi, S. Ohgimoto, T. Nakasone, S. Takamura, T. Shioda, and Y. Nagai.** 2001. Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. J. Virol. **75:**4023–4028.
42. **Naarding, M. A., A. M. Dirac, I. S. Ludwig, D. Speijer, S. Lindquist, E. L. Vestman, M. J. Stax, T. B. Geijtenbeek, G. Pollakis, O. Hernell, and W. A. Paxton.** 2006. Bile salt-stimulated lipase from human milk binds DC-SIGN and inhibits human immunodeficiency virus type 1 transfer to $CD4^+$ T cells. Antimicrob. Agents Chemother. **50:**3367–3374.
43. **Nguyen, D. G., and J. E. Hildreth.** 2003. Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages. Eur. J. Immunol. **33:**483–493.
44. **Pohlmann, S., F. Baribaud, B. Lee, G. J. Leslie, M. D. Sanchez, K. Hiebenthal-Millow, J. Munch, F. Kirchhoff, and R. W. Doms.** 2001. DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus. J. Virol. **75:**4664–4672.
45. **Pohlmann, S., E. J. Soilleux, F. Baribaud, G. J. Leslie, L. S. Morris, J. Trowsdale, B. Lee, N. Coleman, and R. W. Doms.** 2001. DC-SIGNR, a DC-SIGN homologue expressed in endothelial cells, binds to human and simian immunodeficiency viruses and activates infection in trans. Proc. Natl. Acad. Sci. U. S. A. **98:**2670–2675.
46. **Puffer, B. A., S. Pohlmann, A. L. Edinger, D. Carlin, M. D. Sanchez, J. Reitter, D. D. Watry, H. S. Fox, R. C. Desrosiers, and R. W. Doms.** 2002. CD4 independence of simian immunodeficiency virus Envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity. J. Virol. **76:**2595–2605.
47. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses **6:**1221–1231.
48. **Reitter, J. N., R. E. Means, and R. C. Desrosiers.** 1998. A role for carbohydrates in immune evasion in AIDS. Nat. Med. **4:**679–684.
49. **Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth.** 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. Cell **12:**893–900.
50. **Rosati, M., A. von Gegerfelt, P. Roth, C. Alicea, A. Valentin, M. Robert-Guroff, D. Venzon, D. C. Montefiori, P. Markham, B. K. Felber, and G. N. Pavlakis.** 2005. DNA vaccines expressing different forms of simian immunodeficiency virus antigens decrease viremia upon SIVmac251 challenge. J. Virol. **79:**8480–8492.
51. **Santiago, M. L., F. Range, B. F. Keele, Y. Li, E. Bailes, F. Bibollet-Ruche, C. Fruteau, R. Noe, M. Peeters, J. F. Brookfield, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 2005. Simian immunodeficiency virus infection in free-ranging sooty mangabeys (Cercocebus atys atys) from the Tai Forest, Cote d'Ivoire: implications for the origin of epidemic human immunodeficiency virus type 2. J. Virol. **79:**12515–12527.
52. **Sefton, B. M.** 1977. Immediate glycosylation of Sindbis virus membrane proteins. Cell **10:**659–668.
53. **Shakin-Eshleman, S. H., S. L. Spitalnik, and L. Kasturi.** 1996. The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. J. Biol. Chem. **271:**6363–6366.
54. **Shan, M., P. J. Klasse, K. Banerjee, A. K. Dey, S. P. Iyer, R. Dionisio, D. Charles, L. Campbell-Gardener, W. C. Olson, R. W. Sanders, and J. P. Moore.** 2007. HIV-1 gp120 mannoses induce immunosuppressive responses from dendritic cells. PLoS Pathog. **3:**e169.
55. **Shibuya, N., I. J. Goldstein, E. J. Van Damme, and W. J. Peumans.** 1988. Binding properties of a mannose-specific lectin from the snowdrop (Galanthus nivalis) bulb. J. Biol. Chem. **263:**728–734.
56. **Spear, G. T., M. R. Zariffard, J. Xin, and M. Saifuddin.** 2003. Inhibition of DC-SIGN-mediated trans infection of T cells by mannose-binding lectin. Immunology **110:**80–85.
57. **Tabas, I., and S. Kornfeld.** 1978. The synthesis of complex-type oligosaccharides. III. Identification of an alpha-D-mannosidase activity involved in a late stage of processing of complex-type oligosaccharides. J. Biol. Chem. **253:**7779–7786.
58. **Tabas, I., S. Schlesinger, and S. Kornfeld.** 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. J. Biol. Chem. **253:**716–722.
59. **Tyms, A. S., E. M. Berrie, T. A. Ryder, R. J. Nash, M. P. Hegarty, D. L. Taylor, M. A. Mobberley, J. M. Davis, E. A. Bell, and D. J. Jeffries.** 1987. Castanospermine and other plant alkaloid inhibitors of glucosidase activity block the growth of HIV. Lancet **ii:**1025–1026.
60. **Waechter, C. J., and W. J. Lennarz.** 1976. The role of polyprenol-linked sugars in glycoprotein synthesis. Annu. Rev. Biochem. **45:**95–112.
61. **Walker, B. D., M. Kowalski, W. C. Goh, K. Kozarsky, M. Krieger, C. Rosen, L. Rohrschneider, W. A. Haseltine, and J. Sodroski.** 1987. Inhibition of human immunodeficiency virus syncytium formation and virus replication by castanospermine. Proc. Natl. Acad. Sci. U. S. A. **84:**8120–8124.
62. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.
63. **Yamashita, K., Y. Tachibana, and A. Kobata.** 1978. The structures of the galactose-containing sugar chains of ovalbumin. J. Biol. Chem. **253:**3862–3869.
64. **Yuste, E., J. Bixby, J. Lifson, S. Sato, W. Johnson, and R. Desrosiers.** 2008. Glycosylation of gp41 of simian immunodeficiency virus shields epitopes that can be targets for neutralizing antibodies. J. Virol. **82:**12472–12486.
65. **Zhang, M., B. Gaschen, W. Blay, B. Foley, N. Haigwood, C. Kuiken, and B. Korber.** 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology **14:**1229–1246.
66. **Zhu, X., C. Borchers, R. J. Bienstock, and K. B. Tomer.** 2000. Mass spectrometric characterization of the glycosylation pattern of HIV-gp120 expressed in CHO cells. Biochemistry **39:**11194–11204.