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Chimeric *gag*-V3 virus-like particles of human immunodeficiency virus induce virus-neutralizing antibodies

(AIDS vaccine/chimeric proteins/baculovirus expression system)

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ABSTRACT **A 41-kDa unprocessed human immunodeficiency virus 2 (HIV-2) *gag* precursor protein that has a deletion of a portion of the viral protease assembles as virus-like particles by budding through the cytoplasmic membrane of recombinant baculovirus-infected insect cells. We have constructed six different combinations of chimeric genes by coupling the truncated HIV-2 *gag* gene to the neutralizing domain (V3) or the neutralizing and the CD4 binding domains (V3+CD4BD) of gp120 *env* gene sequences from HIV-1 or HIV-2. The *env* gene sequences were inserted either into the middle of the *gag* gene or at the 3′ terminus of the *gag* gene. Virus-like particles were formed by chimeric gene products only when the *env* gene sequences were linked to the 3′ terminus of the *gag* gene. Insertion of *env* gene sequence in the middle of the *gag* gene resulted in high-level chimeric gene expression but without the formation of virus-like particles. Three different chimeric genes [*gag* gene with HIV-1 V3 (1V3), *gag* gene with HIV-2 V3 (2V3), and *gag* gene with HIV-2 V3+CD4BD (2V3+CD4BD)] formed virus-like particles that were secreted into the cell culture medium. In contrast, the HIV-1 V3+CD4BD/HIV-2 *gag* construct did not form virus-like particles. The chimeric *gag-env* particles had spherical morphology and the size was slightly larger than that of the *gag* particles, but the chimeric particles were similar to the mature HIV particles. Western blot analysis showed that the *gag-env* chimeric proteins were recognized by antibodies in HIV-positive human serum and rabbit anti-gp120 serum. Rabbit anti-*gag* 1V3 and anti-*gag* 2V3 sera reacted with authentic gp120 of HIV-1 and HIV-2, respectively, and neutralized homologous HIV infectivity. Our results show that precursor *gag* protein has potential as a carrier for the presentation of foreign epitopes in good immunological context. The *gag* protein is highly immunogenic and has the ability to carry large foreign inserts; as such, it offers an attractive approach for HIV vaccine development.**

The human immunodeficiency viruses (HIVs) are the etiologic agents for acquired immunodeficiency syndrome (AIDS). The envelope glycoprotein gp120 of HIV has been the major target for developing a candidate vaccine against AIDS (1, 2). gp120 recognizes the cellular receptor (CD4) on helper T lymphocytes (3) and carries the V3 loop domain that induces neutralizing antibodies (4, 5). The third hypervariable loop region (V3) of HIV-1 gp120 (amino acid residues 308–331) contains not only a major immunodominant neutralizing epitope but also the epitopes for antigen-dependent cellular cytotoxicity (6) and cytotoxic T-lymphocyte recognition (7, 8). Although the majority of the amino acids in the V3 loop are variable among different strains of HIV, a G-P-G-R motif at the tip of the loop is conserved (9). Huang *et al.* (10) and Björling *et al.* (11) demonstrated that the principal neutralization domain of the envelope glycoprotein of HIV-2 is also located in the region corresponding to the hypervariable motif in the V3 loop of HIV-1 gp120. The CD4 binding region, which is located within the C-terminal third of HIV-1 gp120 (amino acid residues 397–439), plays an essential role in infectivity of HIV (12). This region also seems to be weakly immunogenic because it forms a pocket that is not accessible to the immune system; thus high-titer neutralizing antibody against this region is not presently available (13). To overcome these obstacles, we chose HIV-2 *gag* particles as a carrier for the presentation of the V3 loop or the V3 loop plus the CD4 binding domain. The *gag* protein has the unique ability to form particles in the absence of all other components of the virus (14), and the chimeric *gag* particles are devoid of genomic RNA. Formation of chimeric *gag* particles containing the major neutralizing epitope (V3) and/or the CD4 binding domain (CD4BD) of gp120 may allow efficient generation of HIV-neutralizing antibodies; antigens presented in a particulate form may enhance immunogenicity of the epitopes and multiple copies of specific epitopes can be presented. Furthermore, secreted chimeric particles can be safely and easily collected and purified from cell culture medium by centrifugation.

In this report, we show construction of six different chimeric *gag* genes containing either the V3 loop (V3) or the V3 loop plus the CD4 binding domain (V3+CD4BD) of gp120 from HIV-1 or HIV-2. These constructs were expressed in insect cells using a baculovirus expression vector. Our data show that certain combinations of these fusion proteins are expressed, assembled as virus-like particles, and retain antigenicity and immunogenicity of *gag* and *env* epitopes.

MATERIALS AND METHODS

Cells and Viruses. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV were grown and assayed in *Spodoptera frugiperda* (SF9) cell monolayers using complete TNM-FH medium containing 10% fetal bovine serum at 27°C as described (14–16). Wild-type AcNPV DNA was purified by the method of Smith and Summers (17).

Clones. Plasmids pHXB-2D and p1BM containing the entire HIV-1_{HXB2D} and HIV-2_{NIHZ} genomes, respectively, were obtained from R. Gallo (National Institutes of Health, Bethesda, MD). The recombinant plasmids pUC19-gp120-NSS, pUC18-gp120, and pUC19-GAG containing full-length cDNA copies of the HIV-1 *env*, HIV-2 *env*, and a truncated *gag* gene of HIV-2, respectively, were subcloned from pHXB-2D and p1BM as described elsewhere (ref. 14; Y.L., L.L., D. Thomas, and C.Y.K., unpublished data).

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Abbreviations: HIV, human immunodeficiency virus; AcNPV, *Autographa californica* nuclear polyhedrosis virus; $TCID_{50}$, tissue culture 50% infective dose; RT, reverse transcriptase; p.i., postinfection.

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Neutralization Assay. Virus was prepared from H9 cells infected with HIV-1_{IIIB} or HIV-2_{ROD}. The infectivity [tissue culture 50% infective dose ($TCID_{50}$)] of the viral stocks was determined by measuring syncytium formation using the human T-lymphoid line C8166 (18). Virus neutralizations by immune rabbit sera were carried out by the standard methods for detection of reverse transcriptase (RT) (19) and *gag* proteins.

RESULTS

Construction and Expression of Chimeric Genes. To determine if it is possible to replace a portion of the *gag* protein with other sequences without destroying the ability of the *gag* protein to form particles and if the antigens presented in a particulate structure have enhanced immunogenicity, we used a *gag* gene lacking protease coding sequences from HIV-2 (14) to construct several different *gag* chimeras containing the gp120 V3 neutralizing epitope and/or V3 plus the CD4 binding domain. The basic strategy used to construct plasmids for expression of *gag-env* fusion proteins is illustrated in Fig. 1. PCR-amplified HIV-1 DNA fragment containing the V3 domain (91 amino acids, positions 273–363), HIV-2 DNA fragments containing the V3 equivalent domain (90 amino acids, positions 294–383), and V3+CD4BD (198 amino acids, positions 294–491) were inserted into *Bgl* II sites of pUC19-GAG of HIV-2 at nucleotide positions 345 and 1275, which were created by crossover linker mutagenesis (20, 21). One *Bgl* II site is downstream of the *Pst* I site and another is at the C terminus, just in front of the termination codon (Fig. 1). In addition, a 582-bp *Bgl* II fragment representing V3+CD4BD (194 amino acids, positions 273–466) of HIV-1 gp120 was isolated from pUC19-gp120-NSS and inserted into the C-terminal *Bgl* II site of pUC19-GAG (Fig. 1). The six different chimeric constructs were isolated after digestion with *Bam*HI and inserted into the baculovirus transfer vector pAcYM1 (22). Following cotransfection with wild-type AcNPV DNA (14), six corresponding recombinant baculoviruses were generated and designated as Ac-*gag*M-1V3, Ac-*gag*C-1V3, Ac-*gag*C-1V3+1CD4BD, Ac-*gag*M-2V3, Ac-*gag*C-2V3, and Ac-*gag*C-2V3+2CD4BD. The numbers 1 and 2 in front of V3 and CD4 denote genes from HIV-1 and HIV-2, respectively. To examine synthesis and immunogenicity of each chimeric protein, SF9 cells were infected and whole cell lysates were prepared at day 3 postinfection (p.i.) and analyzed by SDS/PAGE and Western blot. Fig. 2 shows the proteins produced by recombinant baculoviruses Ac-*gag*M-1V3, Ac-*gag*C-1V3, and Ac-*gag*C-1V3+1CD4BD. Since the original HIV-2 *gag* protein has 93 amino acids deleted from the C terminus (14), an insertion of the V3 (91 amino acids) or V3+CD4BD (194 amino acids) of HIV-1 gp120 into the *gag* gene would be expected to produce proteins of 55 kDa and 66 kDa, respectively. As shown in Fig. 2*A*, strongly stained protein bands migrating at either 55 kDa or 66 kDa were observed in the lysates of SF9 cells infected with Ac-*gag*M-1V3 (lane 2), Ac-*gag*C-1V3 (lane 3), and Ac-*gag*C-1V3+1CD4BD (lane 4) but were not present in lysates of mock- or wild-type baculovirus-infected cells. Western blot analysis revealed that the p55 and p66 fusion proteins were recognized by pooled HIV-1-positive human sera (Fig. 2*B*, lanes 2–4) (24). The results clearly demonstrate that insertion of either V3 or V3+CD4BD of HIV-1 and HIV-2 into the *gag* protein resulted in expression of proteins at levels as high as that of recombinant AcNPV-HIV-2*gag* alone (Fig. 2*A*, lane 1). Similar results were obtained when three other recombinant baculoviruses containing the V3 and V3+CD4BD genes from HIV-2 gp120, Ac-*gag*M-2V3, Ac-*gag*C-2V3, and Ac-*gag*C-2V3+2CD4BD were analyzed (data not shown).

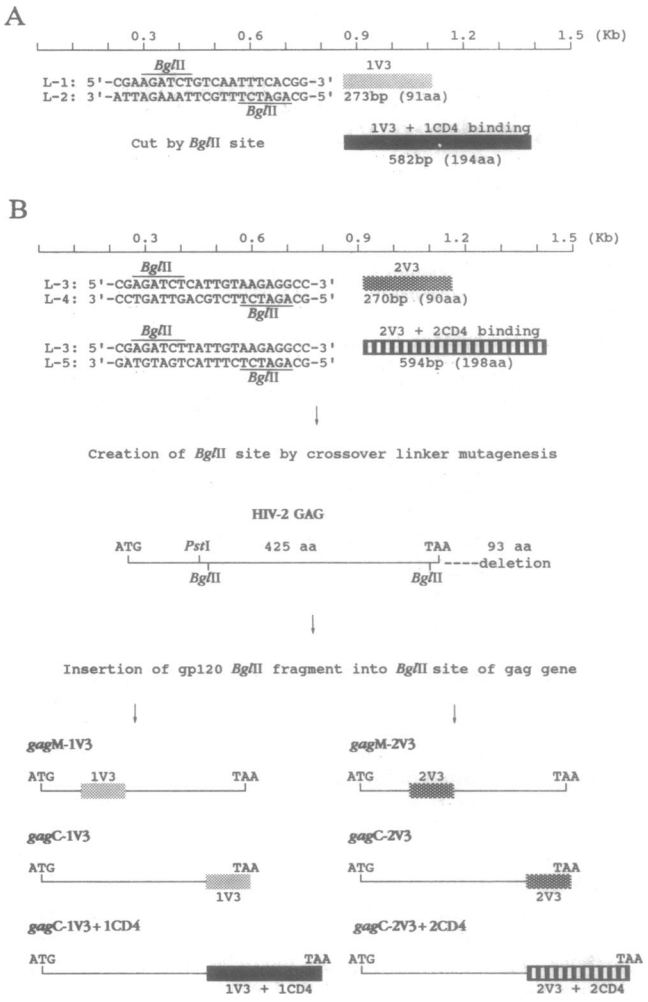

FIG. 1. Construction of chimeric *gag-env* genes. *Bgl* II fragments containing the V3 (amino acid positions 273–363) or V3+CD4BD (amino acid positions 273–466) from HIV-1 gp120 and V3 (amino acid positions 294–383) or V3+CD4BD (amino acid positions 294–491) from HIV-2 gp120 were amplified by PCR. All primers were designed to create a *Bgl* II (underlined) restriction site at the 5′ end so that gp120 DNA fragments could be inserted into the *Bgl* II site of pUC19-GAG. bp, Base pairs; kb, kilobases. (*A*) The HIV-1 V3 DNA fragment was amplified using L-1 and L-2 primers, which are complementary to nucleotides 816–836 and 1075–1089, respectively. The V3+CD4BD DNA fragment of HIV-1 was generated from pUC19-gp120-NSS *Bgl* II digestion at nucleotide positions 816 and 1398 of gp120. (*B*) Primers L-3 and L-4, complementary to nucleotide positions 879–893 and 1135–1149 of HIV-2 gp120, respectively, were used to synthesize the HIV-2 V3 DNA fragment. Primers L-3 and L-5, which are complementary to nucleotides 1459–1473 of HIV-2 gp120, were used to synthesize the HIV-2 V3+CD4BD DNA fragment. The V3+CD4BD sequences of HIV-1 were directly isolated from pUC-gp120-NSS by *Bgl* II digestion. A total of six constructs was made. The dot-hatched box and solid black box represent the V3 and V3+CD4BD of HIV-1 gp120, respectively. The checkered box and the box with vertical lines represent the V3 and V3+CD4BD of HIV-2 gp120, respectively. The number of amino acids presented in each fragment is indicated.

Some Combinations of *gag-env* Chimeric Particles Assemble into Spherical Virus-Like Particles. To examine which of the chimeric *gag* proteins are capable of assembling into virus-like particles and being secreted into cell culture medium, each of the six recombinant baculovirus-infected cell culture supernatants and AcNPV-HIV-2 *gag* (positive control) were analyzed 3 days p.i. We found that only three recombinant baculoviruses, Ac-*gag*C-1V3, Ac-*gag*C-2V3, and Ac-*gag*C-2V3+2CD4BD, produced virus-like particles that could be isolated from the sucrose gradients. *gag*C-1V3 and *gag*C-2V3

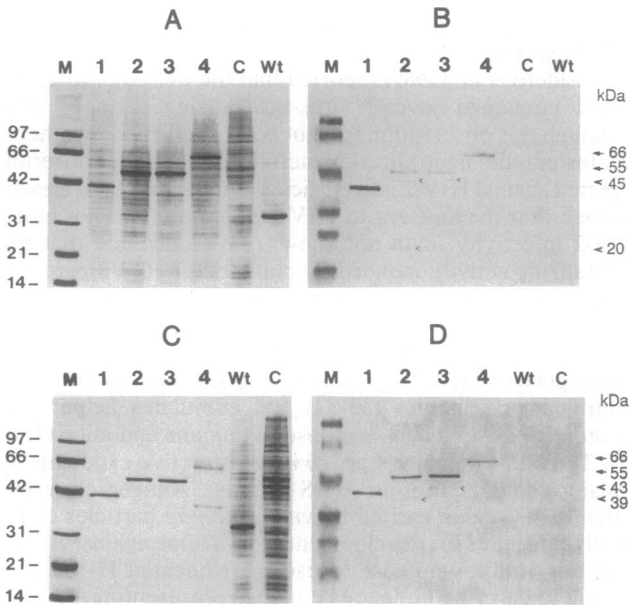

FIG. 2. Expression of chimeric *gag-env* proteins in SF9 cells infected by recombinant baculoviruses. Recombinant baculovirus-infected SF9 cell lysates were prepared by resuspending the cell pellet in water and adding an equal volume of 2× dissociation buffer (10% 2-mercaptoethanol/10% SDS/25% glycerol/100 mM Tris·HCl, pH 7.0/0.04% bromophenol blue). (*A*) Cell lysates were analyzed by electrophoresis in 12% polyacrylamide gels containing SDS (SDS/PAGE) and the protein bands were visualized by staining with Coomassie blue (23). (*B*) Western blot analyses were then performed using human HIV-1 immunoglobulin obtained from the Acquired Immunodeficiency Syndrome Research and Reference Reagent Program, Division of Acquired Immunodeficiency Syndrome, National Institute of Allergy and Infectious Diseases (24). Lanes 1–4, recombinant viruses AcNPV-HIV-2*gag*, Ac-*gag*M-1V3, Ac-*gag*C-1V3, and Ac-*gag*C-1V3+1CD4. The chimeric *gag-env* particles released into the culture supernatant were purified by centrifugation in 20–60% discontinuous sucrose density gradients, subjected to SDS/PAGE, and detected by Coomassie blue staining (*C*) and Western blot (*D*) using human HIV-1 immunoglobulin (24). Lanes 1, purified *gag* particles; lanes 2–4, purified *gag*C-1V3, *gag*C-2V3, and *gag*C-2V3+2CD4 chimeric particles, respectively; lanes M, marker proteins; lanes C, uninfected cell control; lanes Wt, wild-type AcNPV-infected cells. The major fusion proteins p55 and p66 are indicated by arrows and possible cleavage products are indicated by open arrows.

particles were recovered from the 50% sucrose cushion, whereas *gag*C-2V3+2CD4BD chimeric particles banded on top of the 60% sucrose cushion. These partially purified chimeric *gag* particles were analyzed by SDS/PAGE (Fig. 2 *C* and *D*). The major fusion protein bands of 55 kDa and 66 kDa were visualized by Coomassie blue staining (Fig. 2*C*) and by Western blot using HIV-1-positive human sera (Fig. 2*D*). We examined the morphology of sucrose gradient-purified particles by transmission electron microscopy using uranyl acetate staining (Fig. 3). HIV-2 *gag* particles were spherical (Fig. 3*A*), had diameters of ≈100 nm, and were similar to those of mature HIV-1 particles budding from HIV-1-infected cells (25, 26). The chimeric particles *gag*C-1V3 (Fig. 3*B*), *gag*C-2V3 (Fig. 3*C*), and *gag*C-2V3+2CD4BD (Fig. 3*D*) exhibited morphologies similar to that of the *gag* particles. The size of chimeric *gag* particles was slightly larger than that of the *gag* particle alone, with approximate diameters of 130 nm, similar to the diameter of mature HIV-1 particles (27, 28).

No virus-like particles were detected after infection with Ac-*gag*M-1V3, Ac-*gag*M-2V3, or Ac-*gag*C-1V3+1CD4BD recombinant baculoviruses, although the level of expression was high (data not shown), as previously reported (29). Our results demonstrate that insertion of V3 into the middle of the

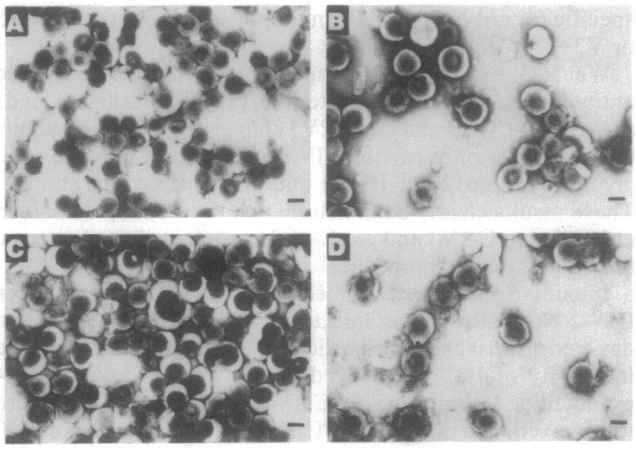

FIG. 3. Electron micrographs of sucrose gradient-purified *gag-env* chimeric particles. (*A*) HIV-2 *gag* particles produced by SF9 cells infected with recombinant AcNPV-HIV-2 *gag*. (*B*) Chimeric *gag-env* particles produced by recombinant Ac-*gag*C-1V3. (*C*) Chimeric *gag* particles produced by recombinant Ac-*gag*C-2V3. (*D*) Chimeric *gag* particles produced by recombinant Ac-*gag*C-2V3+2CD4. Samples were stained with uranyl acetate. (Bars = 100 nm.)

gag gene destroys the ability to form particles. This provides evidence that a large undisrupted N-terminal portion of the *gag* protein is essential for particle formation. Furthermore, there are likely to be size and sequence restrictions on the foreign protein that can be packaged into virus-like *gag* particles (30).

Antigenicity and Immunogenicity of Chimeric *gag* Particles. Antigenicity of chimeric *gag* particles was investigated by immunoblot analysis. Purified chimeric *gag* particles were subjected to SDS/PAGE and analyzed by Western blotting with rabbit antisera directed against HIV-1 and HIV-2 gp120 and ^{125}I-labeled protein A. As expected, the HIV-2 *gag* protein was not recognized by anti-gp120 sera (Fig. 4, lane 1), whereas the nonglycosylated forms of gp120 of HIV-1 and HIV-2 showed strong reactivity with their corresponding antisera (Fig. 4, lanes 2). The 55-kDa and 66-kDa fusion proteins were specifically recognized by rabbit antisera against HIV-1 or HIV-2 gp120s (Fig. 4, lanes 3 and 4). The intensity of the band was proportional to the size of inserted heterologous polypeptide. Our results clearly demonstrate that the chimeric proteins can be detected by antiserum

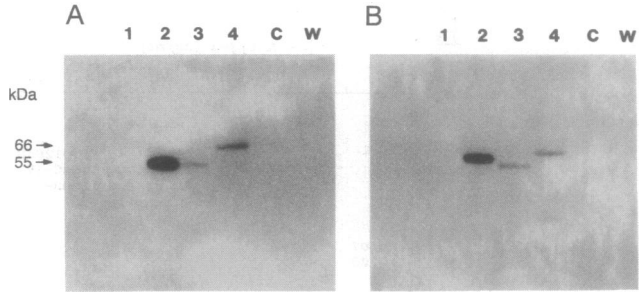

FIG. 4. Immunoblot analysis of chimeric *gag-env* proteins. The chimeric *gag-env* proteins and gp120 of HIV-1 and HIV-2 were subjected to SDS/PAGE and electrotransferred to nitrocellulose filters. Filters were incubated with rabbit antisera specific for HIV-1 gp120 (*A*) and HIV-2 gp120 (*B*) and with ^{125}I-labeled protein A. Lanes 1, HIV-2 *gag* protein; lanes 2, gp120 protein; lanes 3, chimeric *gag*C-1V3 protein; lanes 4, chimeric *gag*C-1V3+1CD4 protein; lanes C, cell control; lanes W, wild-type AcNPV-infected cell control. The rabbit antisera against HIV-1 and HIV-2 gp120 will be described elsewhere (Y.L., L.L., D. Thomas, and C.Y.K., unpublished data).

specific for gp120 and reaffirm that inserted sequences of V3 or V3+CD4BD are antigenic.

We also examined the capacity of particles to induce antibodies to *gag* and *env* proteins of HIV. The antisera made against *gag*C-1V3 and *gag*C-2V3 chimeric particles in rabbits recognize not only carrier HIV-2 *gag* protein but also nonglycosylated gp120 of HIV-1 and HIV-2 (data not shown). These results clearly demonstrate that the V3 loop domain in chimeric *gag*C-1V3 and *gag*C-2V3 particles retains antigenic and immunogenic properties.

Immune Sera Against Chimeric *gag*-V3 Particles of HIV-1 or HIV-2 Neutralize Virus Infectivity *in Vitro*. Rabbit antisera directed against chimeric particles were used to neutralize the infectivity of HIV as assayed by RT activity and *gag* p24 production. Rabbit anti-*gag*C-1V3 and anti-*gag*C-2V3 sera were mixed with either 5000 $TCID_{50}$ units of $HIV\text{-}1_{IIIB}$ or 8000 $TCID_{50}$ units of $HIV\text{-}2_{ROD}$, respectively, and the mixtures were used to infect H9 cells. The amount of p24 *gag* protein and RT activity in the culture medium were assayed as quantitation of virus production on different days after infection (days 1–16), and the levels were compared with those of control samples in which virus was incubated with preimmune serum or rabbit anti-gp120 serum.

Fig. 5 shows that rabbit anti-*gag*C-1V3 and anti-*gag*C-2V3 sera contained antibodies capable of neutralizing HIV infection of H9 cells. By day 9 p.i., antisera to *gag*C-1V3 and *gag*C-2V3 chimeric particles completely blocked the production of HIV-1 and HIV-2, respectively (Fig. 5). However, at day 12 p.i., a small amount of HIV-1 was detected in cultures treated with anti-*gag*C-1V3 serum (Fig. 5 *A* and *B*). In contrast, the antisera to *gag*C-2V3 chimeric particles completely neutralized HIV-2 infectivity (Fig. 5 *C* and *D*). Rabbit anti-gp120 sera of HIV-2 showed stronger neutralizing activity of $HIV\text{-}2_{ROD}$ than rabbit anti-gp120 sera of HIV-1 against $HIV\text{-}1_{IIIB}$ (Fig. 5).

Papsidero *et al.* (1989) reported that the p17 *gag* protein of HIV-1 contained several virus-neutralizing epitopes (31). Although this observation has not been confirmed by others, we tested the neutralization activity of rabbit antiserum directed against HIV-2 *gag* particles alone. Our result clearly showed that the antisera to HIV-2 *gag* alone did not block HIV-2 infectivity (data not shown). We conclude that the neutralizing activity induced by chimeric *gag*C-V3 particles is directed against the V3 domain but not to *gag* p17.

DISCUSSION

The hypervariable V3 region of HIV gp120 induces the major neutralizing antibodies (32–34) and stimulates helper and cytotoxic T-lymphocyte response in humans and mice (7, 8, 35). Thus, this domain of gp120 is an attractive candidate as a major component of an AIDS vaccine. Antigenic presentation of V3 region carried by virus-like *gag* particles could be advantageous for development of a vaccine against AIDS.

In this study, we chose to use the truncated HIV-2 *gag* protein to carry heterologous epitopes representing V3 loop (V3) or V3 plus CD4 binding domain (CD4BD) of gp120 from HIV-1 and HIV-2. We found 91 amino acids representing HIV-1 V3 alone and 90 amino acids representing HIV-2 V3 alone were efficiently packaged into virus-like particles, and virus-neutralizing antibodies were generated in rabbits by these particles. Furthermore, 198 amino acids representing HIV-2 V3+CD4BD were also packaged into the chimeric *gag* particles. In contrast, 194 amino acids representing the V3+CD4BD of HIV-1 failed to be packaged into the *gag* particles. Our results suggested that the homotypic proteins can interact with each other to form particles, whereas

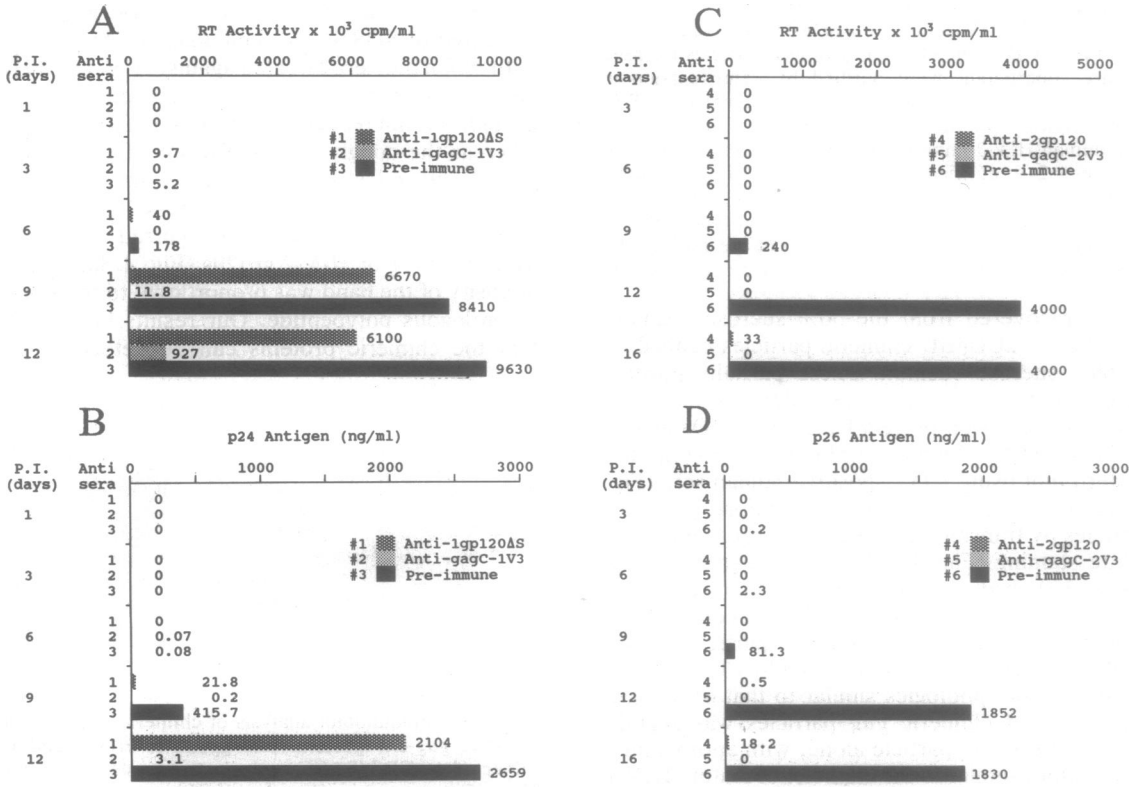

FIG. 5. Neutralization of $HIV\text{-}1_{IIIB}$ and $HIV\text{-}2_{ROD}$ infection with immune rabbit sera. Antisera against *gag*C-1V3 (HIV-1) and *gag*C-2V3 (HIV-2) chimeric particles were prepared by the procedure of Luo *et al.* (14) and tested for neutralization of virus using RT and viral p24 or p26 assays. (*A* and *B*) HIV-1. (*C* and *D*) HIV-2. The neutralizing activities of anti-*gag*C-1V3 and anti-*gag*C-2V3 sera were determined by incubation of serum (1:5 dilution) with a stock virus preparation of $HIV\text{-}1_{IIIB}$ (5000 $TCID_{50}$ units) or $HIV\text{-}2_{ROD}$ (8000 $TCID_{50}$ units) at 37°C for 1 hr before infecting H9 cells. Viral infection was monitored by RT activity (*A* and *C*) and the production of HIV-1 p24 (*B*) or HIV-2 p26 (*D*) *gag* proteins at 1–16 days p.i.

oversized heterotypic proteins cannot interact properly to form a particle. Insertion of V3 sequences in the middle of the *gag* gene prevented particle assembly, which suggests that an uninterrupted *gag* protein is necessary for particle assembly.

The chimeric *gag* particles elicit neutralizing antibodies in rabbits that completely block HIV infection but *gag* particles alone do not. This result demonstrates that linear epitopes of V3 are properly presented by the chimeric particles. Due to the low yield of particle production, we did not prepare antiserum against the *gag*C-2V3+2CD4BD particles to determine whether or not these particles can induce neutralizing antibodies and/or blocking antibodies against CD4 binding.

Because the conserved domain of the envelope proteins of HIV-1 and HIV-2 are weakly immunogenic in native envelope configuration (36) and viral antigens presented as components of membrane structures are more immunogenic than their soluble counterparts (37), chimeric particles of viral or subviral components of polio virus, adenovirus, human hepatitis B virus, etc., containing HIV epitopes, have been evaluated. The major disadvantage of these approaches is the limitation of maximal acceptable size of inserted epitopes, the level of expression, and the complexity of purification method. In our study, although we do not know yet the maximum size limitation of polypeptide extension that can be accommodated in the virus-like *gag* particles, the largest of them, *gag*C-2V3+2CD4, has 198 amino acids attached to the C terminus, which makes a total of 623 amino acids as compared with 518 amino acids of the normal *gag* precursor polypeptide. So far, this represents the largest size of foreign polypeptide packaged into virus-like particles compared with other systems in which the capacity was limited to about 20–100 foreign amino acids.

Considering possible immune enhancement phenomena by antibodies against various epitopes of HIV envelope glycoproteins (38, 39) and the superantigen and autoimmunity theories to explain the depletion of uninfected CD4-positive T lymphocytes (40, 41), the chimeric *gag* virus-like particles provide an attractive option to develop a recombinant subunit vaccine using defined epitopes that can induce humoral and cell-mediated immunity against HIV infection.

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