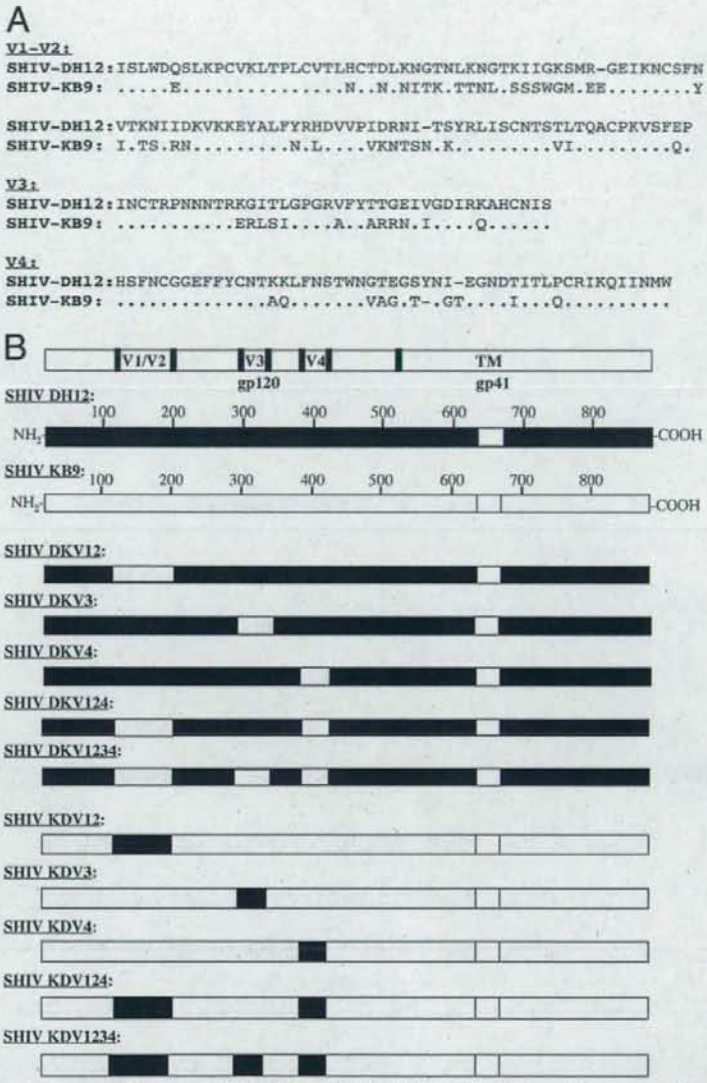

FIG. 2. Variable-loop exchanges made between SHIV DH12 and SHIV KB9. (A) Comparative amino acid alignment of variable-loop sequences between SHIV DH12 and SHIV KB9. (B) Schematic representation of a variable-loop exchange(s) within the SHIV DH12 and SHIV KB9 gp120 envelope glycoproteins.

Infectivities of the SHIV-derived variable-loop chimeras. At 3 days postinfection, three of the five SHIV KB9-derived variable-loop chimeras, SHIV KDV3, SHIV KDV4, and SHIV KDV1234, demonstrated substantial infectivity that was only slightly less than that of parental SHIV KB9 (Fig. 3A). Although quantifying SEAP at day 3 postinfection best approximated a single round of infection, additional assays were performed at day 5 postinfection to assess whether SHIV KDV12 and SHIV KDV124 required further rounds of replication to produce quantifiable SEAP, reflective of impaired infectivity of these particular variable-loop chimeras (Fig. 3B). However, at all time points examined, SHIV KDV12 and SHIV KDV124 produced little or no SEAP activity and were not used further for neutralization assays.

The infectivities of the SHIV DH12-derived variable-loop chimeras displayed a somewhat different pattern than those of the reciprocal SHIV KB9-derived chimeras in that all of the SHIV DH12-derived chimeras were substantially less infectious than the parental SHIV DH12 when assayed for SEAP production 3 days postinfection (Fig. 3C). At day 5 postinfection, four of the five SHIV DH12-derived chimeras (SHIV DKV12, SHIV DKV4, SHIV DKV124, and SHIV DKV1234)

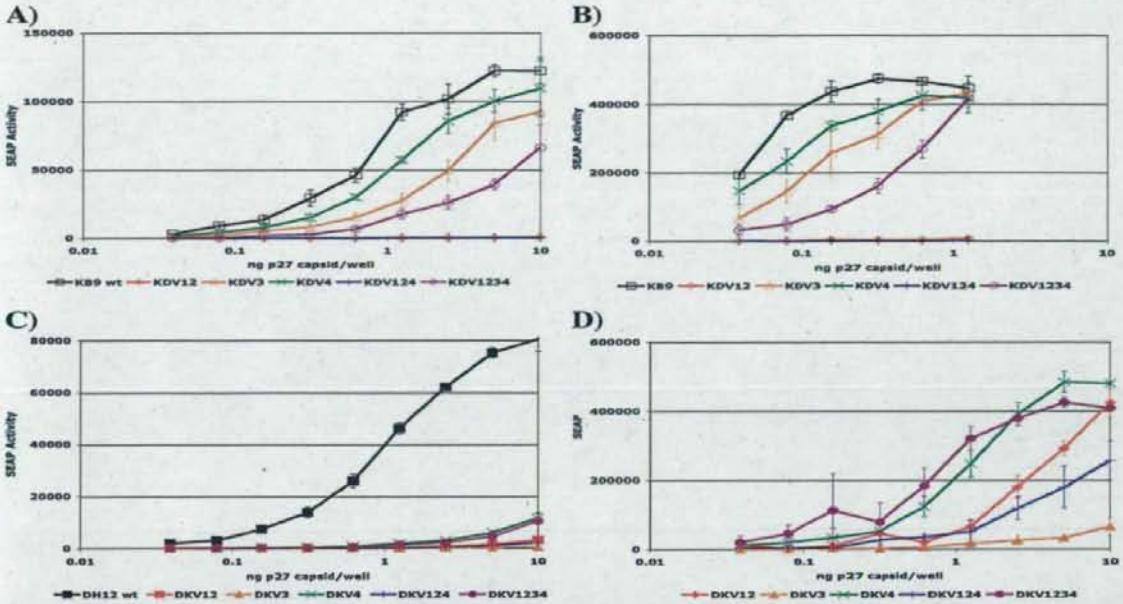

FIG. 3. Comparative infectivities of parental and SHIV-derived variable-loop chimeric viruses. Virus stocks were obtained from transfection of HEK 293T cells, normalized by the amount of p27, and used to infect C8166-45 LTR-SEAP cells. (A) SEAP activity was measured at 3 days postinfection with SHIV KB9 and SHIV KB9-derived variable-loop chimeras. (B) SEAP activity was measured at 5 days postinfection with SHIV KB9-derived chimeras. (C) SEAP activity was measured at 3 days postinfection with SHIV DH12 and SHIV DH12-derived variable-loop chimeras. (D) SEAP activity was measured at 5 days postinfection with SHIV DH12-derived chimeras. SEAP production of some viruses at high p27 concentrations was not included in this analysis because limited additional rounds of wild-type (wt) infection had lysed the majority of target SEAP-producing cells by day 5.

demonstrated substantial infectivity (Fig. 3D). SHIV DKV3 displayed low and inconsistent SEAP production at all time points postinfection examined and was also not included in further neutralization assays.

All SHIV-derived variable-loop chimeras were also assessed for infectivity in the $CD4^+$ $CCR5^+$ HeLa-derived TZM-bl cell line (23, 76, 94), the human B-cell/T-cell hybrid line LTR-SEAP-CEMx174 (60), and a newly derived LTR-SEAP-MT4 cell line. SHIV DKV3, SHIV KDV12, and SHIV KDV124 also demonstrated little or no infectivity in these cells, not allowing further assays in any of the cell lines examined (data not shown). Viral growth curves were performed in both C8166-45 and MT4 cells for all parental SHIVs and SHIV-derived chimeras. Replication of the SHIV-derived variable-loop chimeras in these two cell lines largely recapitulated and was consistent with the infectivity measurements described above (data not shown).

Comparative neutralization of the SHIV DH12-derived variable-loop chimeras by DH12-positive and KB9-positive monkey plasma. We next measured the neutralization sensitivities of the parental SHIV DH12 and the infectious SHIV DH12-derived variable-loop chimeras to plasma samples taken from monkeys infected with either SHIV DH12 or SHIV KB9 at multiple time points postinfection. Figure 4 shows two representative neutralization curves demonstrating the neutralization sensitivities of parental SHIV DH12 and the SHIV DH12-derived variable-loop chimeras when assayed with SHIV KB9-positive plasma from animal 477-99 (Fig. 4A) and SHIV DH12-positive plasma from animal Rh418 (Fig. 4B), both collected 12 weeks postinfection. Reflective of the neutralization sensitivities shown in Fig. 1, parental SHIV DH12 displayed complete resistance to neutralization by SHIV KB9-positive plasma and was extremely sensitive to neutralization by the matched SHIV DH12-positive plasma. Fifty percent neutralization of SHIV DH12 by the positive plasma from animal Rh418 was achieved at a dilution of 1:20,000. Moreover, the neutralization phenotype of SHIV DKV4 closely mirrored that of the parental SHIV DH12. SHIV DKV4 was also highly resistant to heterologous SHIV KB9-positive plasma and extremely sensitive to autologous SHIV DH12-positive plasma, achieving 50% neutralization at the same dilution, 1:20,000. However, when the SHIV KB9 V1/V2 loop was introduced into the SHIV DH12 envelope background, the neutralization pattern was reversed. The SHIV DKV12 variable-loop chimera demonstrated extreme sensitivity to neutralization by the SHIV KB9-positive plasma from animal 477-99, achieving 50% neutralization at a dilution of 1:15,000. Additionally, SHIV DKV12 had lost sensitivity to the SHIV DH12-positive plasma collected from animal Rh418; no neutralization was observed even at the lowest dilution of plasma tested, 1:40. This gain of sensitivity to heterologous plasma and loss of neutralization by autologous plasma was dominant, as the two SHIV DH12-derived chimeras that contained the SHIV KB9 V1/V2 loop complex in combination with other variable loops, SHIV

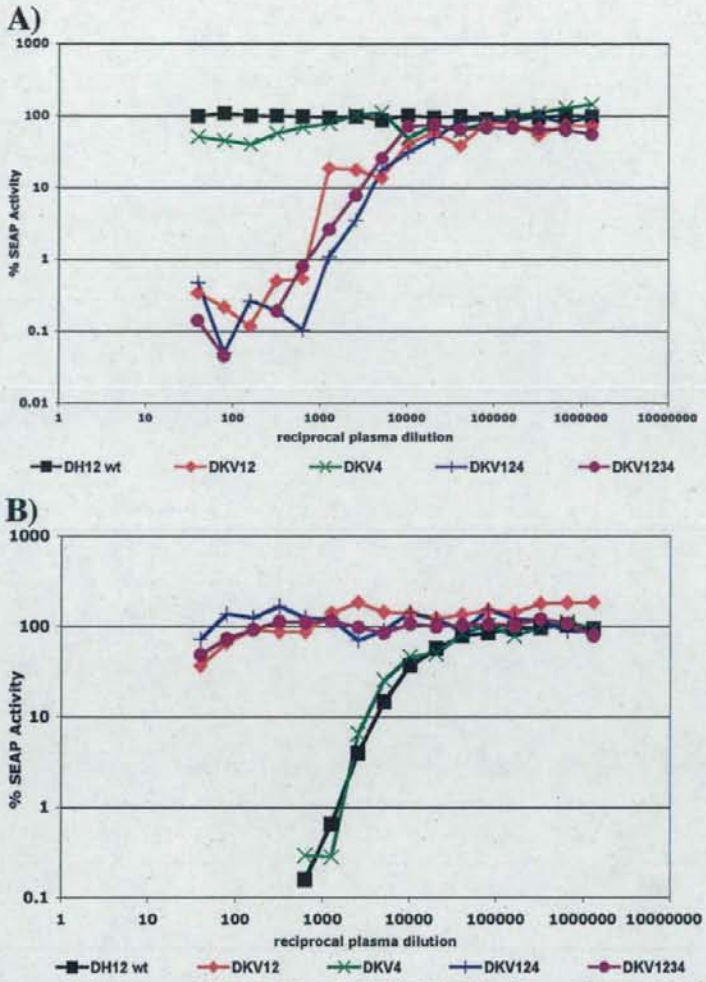

FIG. 4. Representative neutralization curves of SHIV DH12 and SHIV DH12-derived loop chimeras with plasma from SHIV-infected monkeys. (A) Neutralization of SHIV DH12 and SHIV DH12-derived loop chimeras by plasma from a SHIV DH12-infected monkey, Rh418, collected 13 weeks postinfection. (B) Neutralization of SHIV DH12 and SHIV DH12-derived loop chimeras by plasma from a SHIV KB9-infected monkey, 477-99, collected 12 weeks postinfection. wt, wild type.

DKV124 and SHIV DKV1234, both maintained complete resistance to SHIV DH12-positive plasma and substantial sensitivity to neutralization by SHIV KB9-positive plasma.

To ensure that the distinctive neutralization sensitivities of the SHIV DH12-derived variable-loop chimeras were not specific to a particular plasma sample or time point postinfection, we performed more extensive analyses using several additional plasmas from independent experiments to extend the results (Table 1). All plasma samples examined confirmed the neutralization phenotypes demonstrated by Fig. 4. Specifically, four plasma samples collected from two monkeys infected with SHIV KB9 (477-99 and 481-99) at 12 and 24 weeks postinfection were able to effectively neutralize all of the SHIV DH12-derived variable-loop chimeras that contained the SHIV KB9 V1/V2 region, to which the parental SHIV DH12 and SHIV DKV4 remained completely resistant. Moreover, these V1/V2 loop chimeras acquired total resistance to neutralization by five SHIV DH12-positive plasmas, taken from two infected animals (RHTPP and RH418) at 10, 12, and 24 weeks postinfection, all of which efficiently neutralized the parental SHIV DH12 and SHIV DKV4.

Comparative neutralization of the SHIV KB9-derived variable-loop chimeras by DH12-positive and KB9-positive monkey plasmas. One of the benefits of the design of these experiments was our ability to measure both gain and loss of function in both directions with the two sets of reciprocal chimeras and the two sets of plasma specific for one SHIV clone or the other. We thus performed neutralization assays

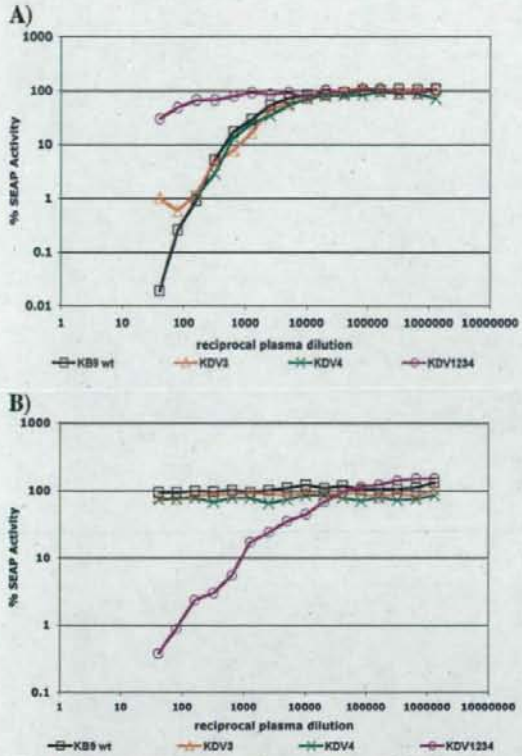

FIG. 5. Representative neutralization curves of SHIV KB9 and SHIV KB9-derived loop chimeras with plasma from SHIV-infected monkeys. (A) Neutralization of SHIV KB9 and SHIV KB9-derived loop chimeras by plasma from a SHIV DH12-infected monkey, Rh418, collected 13 weeks postinfection. (B) Neutralization of SHIV KB9 and SHIV KB9-derived loop chimeras by plasma from a SHIV KB9-infected monkey, 477-99, collected 12 weeks postinfection. wt, wild type.

with the reciprocal SHIV KB9-derived variable-loop chimeras, using SHIV KB9-positive plasma from animal 477-99 collected 12 weeks postinfection (Fig. 5A) and SHIV DH12-positive plasma from animal Rh418 collected 12 weeks postinfection (Fig. 5B). As previously observed (Fig. 1), the parental SHIV KB9 was sensitive to neutralization by the SHIV KB9-positive plasma from animal 477-99, with 50% neutralization occurring at a dilution of 1:2,500. Furthermore, SHIV KB9 was completely resistant to neutralization by the SHIV DH12-positive plasma from animal Rh418. Both SHIV KDV3 and SHIV KDV4 demonstrated neutralization phenotypes similar to that of the parental SHIV KB9. Fifty percent neutralization of SHIV DKV3 and SHIV DKV4 was achieved by the autologous, SHIV KB9-positive plasma at a dilution of 1:5,000, and both chimeras were completely resistant to the heterologous SHIV DH12-positive plasma at all dilutions tested. The exchange of the DH12 V1/V2 loop complex in SHIV KDV1234, however, conveyed the same reversal of neutralization sensitivity observed with the reciprocal exchange of the V1/V2 loop sequences described above. SHIV KDV1234 demonstrated almost complete resistance to SHIV KB9-positive plasma, only reaching 50% neutralization at the very low plasma dilution of 1:80, and acquired specific sensitivity to SHIV DH12-positive plasma, achieving 50% neutralization at a dilution of 1:10,500.

We again performed more extensive analyses using several SHIV-positive plasmas to assess the neutralization sensitivities of the SHIV KB9-derived variable-loop chimeras across independent experiments and multiple time points postinfection (Table 2). Similar to the previous experiment, the trends observed (Fig. 5) were consistent with the results observed using plasmas from four animals at several time points and from independent experiments. In all SHIV KB9-positive plasmas examined, parental SHIV KB9, SHIV KDV3, and SHIV KDV4 demonstrated similar, high-titer neutralization sensitivities, whereas SHIV KDV1234 showed little to no sensitivity, even at the lowest dilutions of plasma tested. Furthermore, when screened for sensitivity to SHIV DH12-positive plasma, parental SHIV KB9, SHIV KDV3, and SHIV KDV4 were completely resistant; however, SHIV KDV1234 was extremely sensitive to all SHIV DH12-positive heterologous plasma samples, achieving 50% neutralization at high dilutions of plasma, varying between 1:3,000 and 1:10,500 (Table 2).

DISCUSSION

Studies that use SHIVs benefit from the combination of an established animal model for HIV infection and access to an extensive array of HIV-1 reagents, including stored plasma samples and genetically modifiable molecular clones. Using two well-characterized SHIV clones and plasma collected from animals infected with the virus matched to each clone, we designed a straightforward panel of variable-loop chimeras in each SHIV background to evaluate the role of each loop sequence in determining the strain specificity of antibody-mediated neutralization. The beauty of these experiments was that, by using two sets of reciprocal chimeric constructs and matched SHIV-positive plasma samples, we were able to measure both gain and loss of sensitivity to strain-specific neutralization based on the presence or absence of a particular variable-loop sequence. All SHIV-derived variable-loop chimeras that included an exchanged V1/V2 variable loop lost sensitivity to positive plasma samples from monkeys infected with the autologous virus and acquired specific sensitivity to positive plasma from monkeys infected with the SHIV strain containing that V1/V2 sequence originally. The plasma dilutions at which the V1/V2 variable-loop chimeras were 50% neutralized were quite similar to the dilutions needed to neutralize the viruses from which the V1/V2 sequences were derived. The presence of these exchanged V1/V2 loop complexes was the dominant factor in establishing a neutralization phenotype for each variable-loop chimera. These results demonstrate unambiguously that the V1/V2 variable-loop complex is principally responsible for the strain-specific neutralizing activity observed in plasma from monkeys infected with these prototypic SHIVs.

The V3 loop of gp120 is often viewed as a major target of anti-HIV antibody responses and an important immunogen to be considered in vaccine design (8, 37, 42, 53, 64, 71, 72, 82). Arguments for the importance of V3 include the targeting of V3 by antibodies from the majority of HIV-1-infected individuals (15, 92), the ability of some anti-V3 monoclonal antibodies to potently neutralize HIV-1 infectivity (19, 35, 36, 48), and

V3's structural conservation (21, 41, 51, 98). Even though much effort has gone into defining the nature and characteristics of anti-V3 antibody responses, others have pointed out that most anti-V3 antibodies are actually quite limited in their abilities to neutralize primary isolates of HIV-1 (7, 39, 55, 63). Our results indicate that HIV-1 V3 is not a target of the neutralizing-antibody response to any appreciable extent in monkeys infected with the prototypic SHIV strains DH12 and KB9. It is, of course, possible that V3 may be an important target for neutralization in the context of R5-only HIV-1 infection or at later time points in the course of monkey infection with these same SHIV strains (49). However, the results reported here provide strong evidence for the predominance of the V1/V2 loop complex in determining the strain-specific neutralizing-antibody response that characterizes both HIV-1 and SHIV infections.

Using very different approaches and reagent sets, Pinter et al. and Ching et al. have also recently concluded that the V1/V2 region is the dominant determinant of HIV-1 neutralization sensitivity (16, 75). In contrast to their studies, our studies employed plasma matched to the cloned virus with which the monkeys were infected, did not use viruses that were globally sensitive to antibody-mediated neutralization, and employed variable-loop swaps in both directions. Nonetheless, all three studies similarly found a dominant role for V1/V2 in determining sensitivity to antibody-mediated neutralization. It will be important in the future to perform analogous experiments with CCR5-using clade B HIV-1 isolates and matched plasma collected from HIV-1-infected individuals.

Others have found more complex determinants for the strain specificity of the neutralizing-antibody response (17, 68). The study by Moore et al. published in 2008 (68) is the only study in addition to our own that performed reciprocal exchanges of variable loops in both directions. Although Moore et al. found a substantial role for V1/V2 in determining the strain specificity of the neutralizing-antibody response to clade C HIV-1 infection, the C3-V4 region also contributed importantly (68). It is possible that clade, tropism, and individual-to-individual variation could contribute to the degree of dominance of the V1/V2 region.

There are multiple mechanisms by which the V1/V2 loop complex may be acting in order to dramatically alter the neutralization sensitivities of the SHIV-derived V1/V2 variable-loop chimeras. Most directly, the V1/V2 loop sequence may contain the epitope targets of neutralizing antibodies in the plasma from monkeys infected with either SHIV DH12 or SHIV KB9. In this scenario, exchanging the V1/V2 loop complex between the two SHIVs will concomitantly switch the targets for antibody recognition and neutralization. Alternatively, the V1/V2 variable-loop complex might shield particular epitopes from antibody recognition while allowing others to be bound and neutralized by circulating antibody. The conformational change in envelope following a V1/V2 loop exchange may shift this shielding to occlude previously exposed epitopes and expose previously shielded epitopes to antibody recognition, leading to reciprocal gains and losses of neutralization sensitivity. Lastly, the V1/V2 loop complex might be critically involved in the formation of complex, conformational neutralizing determinants. Consequently, the V1/V2 loop exchange would disrupt such epitopes and render the resultant virus refractory to neutralization by autologous SHIV-positive plasma while creating a conformational structure that could be recognized and neutralized by heterologous SHIV-positive plasma. Extensive epitope mapping will be necessary to discern which of these potential mechanisms is principally responsible for the V1/V2-dependent determination of the strain-specific neutralizing activity described by this study.

Despite robust replication and infectivity of both parental viruses in C8166-45 LTR-SEAP cells, three of the SHIV-derived variable-loop chimeras (SHIV DKV3, SHIV KDV12, and SHIV KDV124) exhibited poor infectivity and were not included in further studies. Our inability to obtain infectious recombinant viruses with these chimeras strongly suggests that although one variable loop is able to function within the context of its parental envelope spike, this same loop was nonfunctional when introduced into a different envelope context. The reduced infectivity resulting from the variable-loop exchange does not appear to be an inherent characteristic of the amino acid sequence, as infectivity was not impaired in the reciprocal exchange. These differences are most likely based upon inherent differences in the abilities of the envelope complexes to tolerate a heterologous variable-loop exchange. gp120 is thought to be stabilized in its tight, compact conformation within the envelope spike by intratrimeric interactions between monomers and by an extensive glycosylation network, the pattern of which differs between envelope species (80, 95). Mismatched variable-loop sequences and/or differential N-linked glycosylation patterns of the SHIV-derived variable-loop chimeras may destabilize the trimeric envelope framework such that specific loop exchanges (DKV3, KDV12, and KDV124) result in viruses with severely reduced infectivity.

One of the most daunting challenges for HIV-1 vaccine strategies aimed at eliciting a protective neutralizing antibody response is overcoming the enormous sequence variability that is a hallmark of the envelope protein. Thus far, such attempts have demonstrated little success, as the neutralizing activities elicited by the particular envelope immunogens tested have characteristically displayed low potency and/or high strain-specific neutralizing activity. An ideal immunogen would elicit potent neutralizing antibodies that were capable of neutralizing a broad range of diverse primary HIV-1 isolates and would avoid inducing antibodies that were weak and strain specific. There are at least two distinct approaches to achieve this. The first is to design an envelope-based immunogen that will elicit antibodies focused on conserved elements within gp120 that are able to access these epitopes in the context of the mature trimer spike on the surface of the virion. An alternative approach is to include a mixture of envelope sequences in an immunogen pool that can cover as broad a range of sequence variation as possible, thus inducing antibodies capable of neutralizing a large spectrum of primary isolates. This approach seems daunting if one is trying to adequately represent the sequence diversity of the entire envelope protein. However, if the sequence variation within the V1/V2 variable loop is the principal determinant of antibody-mediated neutralization, as indicated by the present study, this would considerably limit the range of sequences that would need to be included in such an envelope immunogen pool.

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Trans-species activation of human T cells by rhesus macaque CD1b molecules

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ABSTRACT

Despite crucial importance of non-human primates as a model of human infectious diseases, group 1 CD1 genes and proteins have been poorly characterized in these species. Here, we isolated *CD1A*, *CD1B*, and *CD1C* cDNAs from rhesus macaque lymph nodes that encoded full-length CD1 proteins recognized specifically by monoclonal antibodies to human CD1a, CD1b, and CD1c molecules, respectively. The monkey group 1 CD1 isoforms contained amino acid residues and motifs known to be critical for intramolecular disulfide bond formation, N-linked glycosylation, and endosomal trafficking as in human group 1 CD1 molecules. Notably, monkey CD1b molecules were capable of presenting a mycobacterial glycolipid to human CD1b-restricted T cells, providing direct evidence for their antigen presentation function. This also detects for the first time a trans-species crossreaction mediated by group 1 CD1 molecules. Taken together, these results underscore substantial conservation of the group 1 CD1 system between humans and rhesus macaque monkeys.

Besides MHC class I- and II-restricted αβ T cells that recognize protein antigens (Ags), discrete subsets of T cells exist in humans that specifically recognize non-protein Ags in a T-cell receptor (TCR)-dependent manner. These include αβ T cells that recognize lipid, glycolipid, and lipopeptide Ags in the context of group 1 CD1 molecules (CD1a, CD1b, and CD1c) as well as $V\gamma2^+V\delta2^+$ γδ T cells that recognize pyrophosphorylated isoprenoid intermediates [1,2]. Both T cell subsets have been implicated in host defense against mycobacterial infection [3], and therefore, animal species that have evolved these T cells in addition to MHC-restricted T cells would serve as an ideal animal model of human tuberculosis. The murine model has long been studied extensively, and by taking advantage of versatile genetic manipulation and a fine array of reagents, many important aspects of host defense against tuberculosis have been demonstrated explicitly, that include a critical role for MHC-restricted T cells [4]. However, a significant difference in pathology has been noted between the two species [3], and the lack of T cells in mice that correspond to human group 1 CD1-resricted T cells and $V\gamma2^+V\delta2^+$ γδ T cells makes the animals less useful particularly in an attempt to develop a new chemical class of non-protein vaccines against tuberculosis. In contrast to mice and rats, guinea pigs exhibit pathology that is comparable, if not identical, to that in human tuberculosis, and recent studies have shown that they contain four *CD1B* genes and three *CD1C* genes [5,6]. Nevertheless, CD1a-restricted T cells as well as CD1d-restricted NKT cells may not exist in guinea pigs. These and other significant differences in the organization and function of the immune system between humans and rodents often make it difficult to translate the results obtained from rodent models to humans. Further, certain human pathogens, such as HIV-1, exhibit highly limited host selectivity, and are unable to infect into rodents and other commonly used laboratory animals.

Recently, the value of non-human primates as a model of human infectious diseases has been appreciated greatly for elucidating pathogenesis and for developing vaccines and therapies against microbial infections, such as AIDS and tuberculosis [7,8]. Nevertheless, little has been defined about the genes, proteins, and function of the group 1 CD1 molecules in non-human primates, and therefore, the present study was aimed at identifying the rhesus macaque group 1 CD1 system. We, found it highly comparable to that in humans, and rhesus macaque CD1b molecules were indeed able to present a human CD1b-presented mycobacterial glycolipid Ag to specific human T cells.

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Materials and methods

Isolation of rhesus macaque group 1 CD1 cDNAs. Rhesus monkeys (*Macaca mulatta*) were used in accordance with the institutional regulations approved by the Committee for Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University, Kyoto, Japan. Total RNA was extracted from rhesus macaque lymph nodes using the RNeasy mini kit (Qiagen, Hilden, Germany), and the first-strand cDNA was synthesized from 0.5 mg of the total RNA using oligo(dT) and PrimeScript reverse transcriptase (Takara Bio, Inc., Otsu, Japan). To amplify specific transcripts, the samples were subjected to PCR amplification with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) for 35 cycles of 30 s at 94 °C, 1 min at 55 °C (for *CD1A*) or 60 °C (for *CD1B* and *CD1C*), 2 min at 72 °C, and a final cycle of 10 min at 72 °C. The primers used were: 5′-GCG GTA CCA AAT AAC ATC TGC AAA TGA C-3′ (sense) and 5′-GCC TCG AGA AGG AGG ATC ATG GTG TAT C-3′ (anti-sense) for *CD1A*; 5′-GCG GTA CCA GTA AGA AGT TGC ATC TCC C -3′ (sense) and 5′-GCC TCG AGG GAG CAG ACA TGG TGA GGG C-3′ (anti-sense) for *CD1B*; 5′-GCG GGT ACC ACC ATG CTG TTT CTG CAG TTT-3′ (sense) and 5′-GCG GCG GCC GCA TTG TAC TAG GCT CCT GG-3′ (anti-sense) for *CD1C*. The PCR products were purified and cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA), and DNA sequencing was done in both directions. This procedure was repeated twice to confirm that no PCR-associated errors were introduced.

Transfection. A rhesus macaque kidney epithelial cell line, LLC-MK2 [9], was obtained from ATCC (Manassas, VA). The cells were transfected with pcDNA3.1(+) containing either rhesus macaque *CD1A*, *CD1B*, or *CD1C* by a calcium phosphate precipitation method, using the mammalian transfection kit (Stratagene). The transfected cells were then cultured in DMEM media (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and G418 (0.5 mg/ml) (Invitrogen), and the CD1-expressing cells were then enriched by labeling with specific antibodies (Abs), followed by positive selection with magnetic beads coated with goat anti-mouse IgG Abs (Invitrogen). A human lymphoblastoid cell line, T2 [10], was transfected with pCEP4 (Invitrogen) containing *CD1A* or *CD1B* of either human or rhesus macaque origin by electroporation as described [11], followed by selection in RPMI1640 media (Invitrogen) containing 0.2 mg/ml hygromycin B (Invitrogen). A human cervical epithelial cell line, HeLa [12], was transfected with rhesus macaque *CD1C* in pcDNA3.1(+) by a calcium phosphate precipitation method, and selection was performed as described above. These stably transfected cells were used as Ag-presenting cells (APCs) in T cell transfectants stimulation assays.

Flow cytometry. The expression of CD1 proteins on the surface of the LLC-MK2 cell transfectants as well as rhesus macaque thymocytes were analyzed by flow cytometry as described [13,14], using the BD FACSCanto II flow cytometer. The mouse monoclonal Abs (mAbs) used were 10H3 (anti-human CD1a) [15], SN13 (anti-human CD1b) (Ancell, Bayport, MN), M241 (anti-human CD1c) (Ancell), and SP34 (anti-monkey CD3) (BD Biosciences, Franklin Lakes, NJ). MAbs MOPC-31C (BD Biosciences) and RPC5.4 (ATCC) were used as negative controls.

T cell transfectants stimulation assays. TCR-deficient Jurkat cells, J.RT3, reconstituted with either the dideoxymycobactin-specific, CD1a-restricted TCR (J.RT3/CD8-2), the glucose monomycolate (GMM)-specific, CD1b-restricted TCR (J.RT3/LDN5) or the mannosyl phosphomycoketide-specific, CD1c-restricted TCR (J.RT3/CD8-1) have been described previously [16]. The TCR-reconstituted cells (5×10^4/well) were cultured with irradiated APCs expressing a relevant CD1 isoform (1×10^5/well) in wells of 96-well, flat-bottomed microtiter plates (200 μl media/well) in the presence of 10 ng/ml phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) and either the organic extract of *Mycobacterium tuberculosis* H37Ra (for J.RT3/CD8-2 and J.RT3/CD8-1) or *Rhodococcus equi* GMM (for J.RT3/LDN5) at indicated concentrations. After 20 h, aliquots of the culture supernatants were collected, and the amount of interleukin-2 (IL-2) released into the supernatants was measured by the IL-2 ELISA kit (BD Biosciences).

Molecular modeling of rhesus macaque CD1b proteins. Molecular modeling of the rhesus macaque CD1b molecule was performed, using the homology modeling software PDFAMS (Protein Discovery Full Automatic Modeling System; In-Silico Sciences, Inc., Tokyo, Japan) as described [17]. Briefly, the primary sequence of the rhesus macaque CD1b molecule was aligned with the sequence of the human CD1b molecule available from the Protein Data Bank (1UQS), using RPS-BLAST. Amino acid residues differing between the two molecules were mutated, and the obtained 3-dimensional structure was optimized by the simulated annealing method. Subsequently, the molecular model was subjected to energy minimization, using the SYBYL software. The overall structure and the cavity surface of the modeled rhesus macaque CD1b molecule were depicted in association with GMM from *Nocardia farcinica* by utilizing the MOLCAD module of SYBYL.

Results and discussion

Identification of rhesus macaque group 1 CD1 cDNAs

To isolate full-length cDNAs encoding rhesus macaque CD1a and CD1b, the first strand cDNA was synthesized from lymph node total RNA by reverse transcription, and then, PCR was carried out with specific pairs of 5′-end and 3′-end primers that were designed based on the rhesus macaque genomic *CD1A* and *CD1B* sequences. The rhesus macaque genomic *CD1C* sequence was only partially available, and the 3′-end sequence was undermined. Therefore, rhesus macaque *CD1C* cDNA was amplified by PCR using a specific 5′-end primer and a 3′-end primer that was designed based on the sequence of 3′-untranslated region of the human *CD1C* genome. The PCR products thus obtained were of expected size (approximately 1 kb) and the identity of the products was determined by DNA sequences. Identical nucleotide sequences were obtained after two independent PCR amplifications, ruling out the possibility for PCR-associated errors.

Alignment of the deduced amino acid sequences of the putative rhesus macaque *CD1A*, *CD1B*, and *CD1C* genes with the corresponding human CD1 proteins revealed a high-degree homology between the two species (85.6% for CD1a, 94.6% for CD1b, 90.4% for CD1c) (Fig. 1). The cysteine residues (indicated with triangles) involved in the intrachain disulfide bond formation in the α2 and the α3 domains as well as the putative N-linked glycosylation sites (indicated with asterisks) in the α1 and the α2 domains were totally conserved [2]. Further, the cytoplasmic tyrosine-based motif (YXXZ where Y is tyrosine, X is any amino acid, and Z is a hydrophobic amino acid) and its flanking sequences that are known to regulate differential early endosomal and lysosomal trafficking of CD1b and CD1c proteins [12,18,19] were identical between the two species (Fig. 1).

To monitor protein expression of these rhesus macaque *CD1* genes, we first screened mAbs against human CD1 proteins for their cross-reactivity to rhesus macaque thymocytes, a cell type that is presumed to express all forms of group 1 CD1 molecules. As shown in Fig. 2A, mAb clones 10H3 (anti-human CD1a), SN13 (anti-human CD1b), and M241 (anti-human CD1c) labeled a significant fraction of $CD3^{dim}$ thymocytes in a pattern comparable to that for human thymocytes [20]. We then stably transfected each

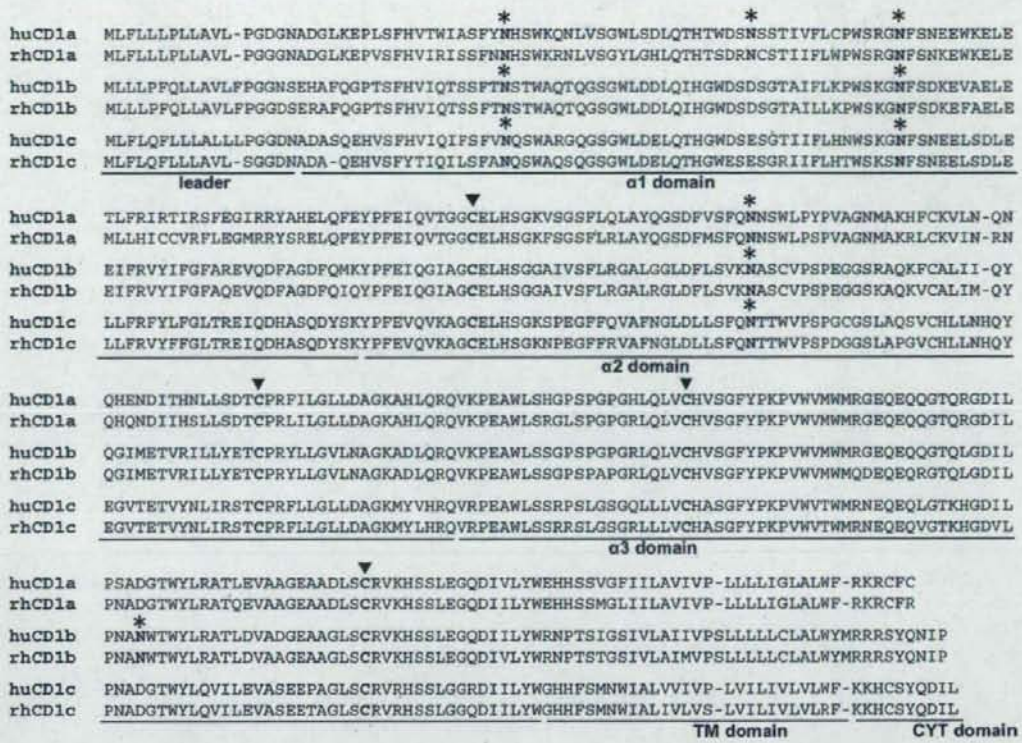

Fig. 1. Alignment of deduced amino acid sequences of human (hu) and rhesus macaque (rh) group 1 CD1 proteins. Residues conserved between the two species are shaded in light gray. Solid triangles denote cysteines conserved in all the group 1 CD1 proteins of both species that are presumed to be involved in intradomain disulfide bond formation. Asterisks indicate potential N-linked glycosylation sites. Dashes represent gaps that have been introduced to maximize alignment. TM domain, transmembrane domain; CYT domain, cytoplasmic domain.

of the putative rhesus macaque *CD1A*, *CD1B*, and *CD1C* genes into a rhesus macaque kidney epithelial cell line, LLC-MK2, and their protein expression was monitored by flow cytometry using the cross-reactive mAbs (Fig. 2B). The 10H3 anti-human CD1a mAb recognized only *CD1A* transfected cells, but not those transfected with the other genes. Similarly, the SN13 anti-human CD1b mAb and the M241 anti-human CD1c mAb showed specific reactivity to cells transfected with the *CD1B* and the *CD1C* genes, respectively. These results provided both evidence for protein expression of the isolated genes and further support for their identity, and therefore, the nucleotide sequences of the putative *CD1A*, *CD1B*, and *CD1C* cDNAs were deposited to the DDBJ/GenBank/EMBL databases as those of rhesus macaque *CD1A* (Accession Nos: AB458511), *CD1B* (AB458512), and *CD1C* (AB458513), respectively.

Trans-species activation of human T cells by rhesus macaque CD1b molecules

With the exception of mice and rats, group 1 CD1 genes have been identified in virtually all mammalian animals so far analyzed, but the Ag presentation function of their products has not been demonstrated so explicitly as in humans [21]. This is partly due to difficulties in obtaining specific T cell lines and clones that recognize lipid Ags in the context of CD1 molecules of a given animal species. Because of the highly conserved amino acid sequences of human and rhesus macaque group 1 CD1 proteins, we considered the possibility that rhesus macaque CD1 molecules might bind lipid Ags that were known to be presented by human CD1 molecules, and interact with specific human TCRs. To address this, human TCRs derived either from a dideoxymycobactin-specific, CD1a-restricted T cell line (CD8-2), from a GMM-specific, CD1b-restricted T cell line (LDN5) or from a mannosyl phosphomycoketide-specific, CD1c-restricted T cell line (CD8-1) were reconstituted in TCR-deficient Jurkat cells (J.RT3) by gene transfer, and the T cell reactivity to specific Ag in the presence of cell transfectants expressing a relevant CD1 isoform of either human or rhesus macaque origin was assessed by measuring IL-2 released from the T cells. J.RT3/CD8-2 cells responded to dideoxymycobactin in the presence of APCs expressing human CD1a molecules, but not those expressing rhesus macaque CD1a molecules (Fig. 3, top panel). Similarly, J.RT3/CD8-1 cells responded to mannosyl phosphomycoketide in the presence of APCs expressing human CD1c molecules, but not those expressing rhesus macaque CD1c molecules (bottom panel). Strikingly, however, APCs expressing rhesus macaque CD1b molecules were capable of presenting GMM efficiently to J.RT3/LDN5 cells (middle panel), providing evidence for their Ag presentation function. The apparently more efficient Ag presentation function for rhesus macaque CD1b molecules as compared with human CD1b molecules could be accounted for by the slightly higher expression on rhesus macaque CD1b transfectants than on human CD1b transfectants (data not shown).

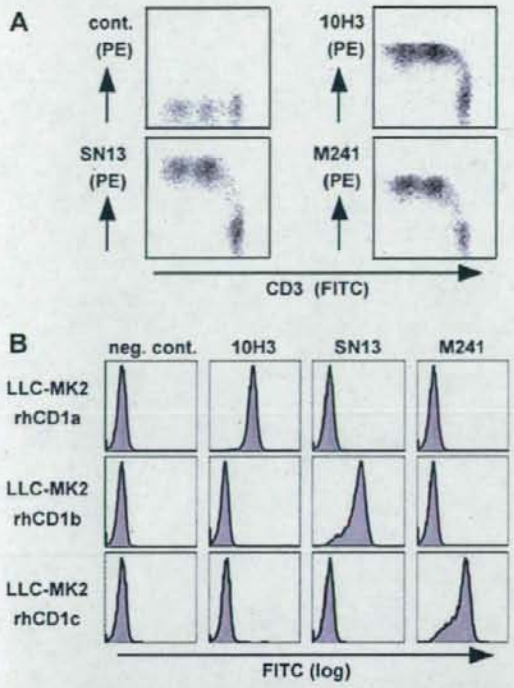

Fig. 2. Cross-reactivity of anti-human CD1 mAbs to rhesus macaque group 1 CD1 proteins. (A) Rhesus macaque thymocytes were double-labeled with the SP34 anti-CD3 mAb and either the 10H3 anti-human CD1a mAb, the SN13 anti-human CD1b mAb, the M241 anti-human CD1c mAb, or negative control Abs, followed by analysis by flow cytometry. (B) A rhesus macaque kidney cell line, LLC-MK2, that stably transfected with either rhesus macaque *CD1A* (LLC-MK2 rhCD1a), *CD1B* (LLC-MK2 rhCD1b), or *CD1C* (LLC-MK2 rhCD1c) were labeled with indicated mAbs and analyzed by flow cytometry.

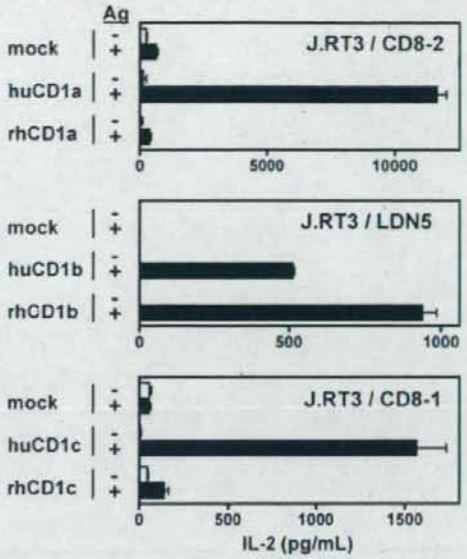

Fig. 3. Ag presentation function of rhesus macaque CD1b molecules. The J.RT3/CD8-2 cells were cultured in the presence or absence of the organic extract of *M. tuberculosis* (50 mg/ml) with T2 cells expressing either human CD1a (huCD1a) or rhesus macaque CD1a (rhCD1a) or those that were mock-transfected (top panel). The J.RT3/LDN5 cells were cultured in the presence or absence of purified GMM (5 mg/ml) with T2 cells expressing either human CD1b (huCD1b) or rhesus macaque CD1b (rhCD1b) or those that were mock transfected (middle panel). The J.RT3/CD8-1 cells were cultured in the presence or absence of the organic extract of *M. tuberculosis* (1.56 mg/ml) with HeLa cells expressing either human CD1c (huCD1c) or rhesus macaque CD1c (rhCD1c) or those that were mock transfected (bottom panel). After 20 h, the culture supernatants were harvested and the amount of IL-2 secreted into the supernatants were measured.

Trans-species crossreaction has never been observed previously for any of the group 1 CD1 molecules. Nevertheless, a molecular model of the rhesus macaque CD1b molecule has detected the α1 and α2 helix structure as well as intramolecular pockets (A′, C′, and F′) and a tunnel (T′) virtually identical to those for human CD1b molecules [22,23], allowing stable interaction with a human CD1b-presented mycobacterial Ag, GMM (Fig. 4). Further, amino acid residues, such as E80 and D83 in the α1 domain and T157 and T165 in the α2 domain, that are proposed to be critical for interaction with specific TCRs [24] are shared between rhesus macaque and human CD1b molecules, suggesting a conserved function for CD1b in these two species. The extent of amino acid sequence conservation is higher in CD1b than in CD1a and CD1c (Fig. 1), which may imply that immune responses to mycolic acid-containing glycolipds are critical for host defense against tuberculosis. So far, no experimental animals have proved extremely useful as a model for studying the group 1 CD1-mediated immunity in human infectious diseases. The present study underscores that monkeys are indispensable for a variety of challenges, including development of a new type of lipid-based vaccines against tuberculosis.

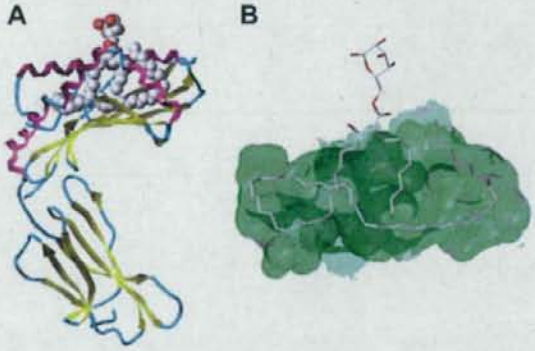

Fig. 4. A molecular model of rhesus macaque CD1b proteins. The rhesus macaque CD1b structure was constructed, based on the crystal structure of the human CD1b-GMM complex. (A) The overall structure of the rhesus macaque CD1b-GMM complex is shown, in which the CD1b heavy chain is depicted in ribbon diagram and the non-hydrogen atoms of GMM are drawn as van der Waals spheres (carbon in gray; oxygen in red). The associated β2-microglobulin is not depicted for simplicity purposes. (B) The binding surface of the Ag-binding groove is drawn in green with the bound GMM in stick (carbon in gray; oxygen in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper).

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