of mucosal CD4+ T cells have been observed in simian immunodeficiency virus-infected monkeys (11, 12, 24, 25) and human immunodeficiency virus-infected humans (2, 5, 13). The acute depletion of mucosal CD4+ T cells and the disease outcome are correlated (1, 3, 21, 26). However, a decrease of mucosal CD4+ T cells has also been observed in the early phases of natural host infections, such as SIVagm infection in African green monkeys and SIVsmm infection in sooty mangabeys, which typically do not progress to AIDS (4, 14, 19). In addition, the levels of apoptosis and immune activation and the degrees of CD4+ T-cell restoration differ between progressors and nonprogressors in simian immunodeficiency virus models (4, 14, 19). Taken together, these results raise the possibility that the severe acute depletion of mucosal CD4+ T cells is not sufficient to induce AIDS. The restoration of CD4+ T cells and normal immune function after the severe acute depletion may define the eventual disease outcome (20). The abilities of KS661- and #64-infected monkeys to restore the immune system may be different, because KS661, but not #64, impairs thymic T-cell differentiation (18). Currently, we are focusing on the restoration of CD4+ T cells and the functional aspect of the immune cells in the small intestines of animals infected with KS661 and #64 to further clarify the determinant(s) of the disease outcome.

We are grateful to James Raymond for English editing of the manuscript and to Takahito Kazama for technical support.

This work was supported, in part, by Research on Human Immunodeficiency Virus/AIDS in Health and Labor Sciences research grants from the Ministry of Health, Labor and Welfare, Japan, a grant-in-aid for scientific research from the Ministry of Education and Science, Japan, a research grant for health sciences focusing on drug innovation for AIDS from the Japan Health Sciences Foundation, and a grant from the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

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JOURNAL OF VIROLOGY, Nov. 2008, p. 11054–11065 Vol. 82, No. 22
0022-538X/08/$08.00+0 doi:10.1128/JVI.01341-08
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Importance of the V1/V2 Loop Region of Simian-Human Immunodeficiency Virus Envelope Glycoprotein gp120 in Determining the Strain Specificity of the Neutralizing Antibody Response[▽]

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Received 26 June 2008/Accepted 28 August 2008

Plasma samples from individuals infected with human immunodeficiency virus type 1 (HIV-1) are known to be highly strain specific in their ability to neutralize HIV-1 infectivity. Such plasma samples exhibit significant neutralizing activity against autologous HIV-1 isolates but typically exhibit little or no activity against heterologous strains, although some cross-neutralizing activity can develop late in infection. Monkeys infected with the simian-human immunodeficiency virus (SHIV) clone DH12 generated antibodies that neutralized SHIV DH12, but not SHIV KB9. Conversely, antibodies from monkeys infected with the SHIV clone KB9 neutralized SHIV KB9, but not SHIV DH12. To investigate the role of the variable loops of the HIV-1 envelope glycoprotein gp120 in determining this strain specificity, variable loops 1 and 2 (V1/V2), V3, or V4 were exchanged individually or in combination between SHIV DH12 and SHIV KB9. Despite the fact that both parental viruses exhibited significant infectivity and good replication in the cell lines examined, 3 of the 10 variable-loop chimeras exhibited such poor infectivity that they could not be used further for neutralization assays. These results indicate that a variable loop that is functional in the context of one particular envelope background will not necessarily function within another. The remaining seven replication-competent chimeras allowed unambiguous assignment of the sequences principally responsible for the strain specificity of the neutralizing activity present in SHIV-positive plasma. Exchange of the V1/V2 loop sequences conferred a dominant loss of sensitivity to neutralization by autologous plasma and a gain of sensitivity to neutralization by heterologous plasma. Substitution of V3 or V4 had little or no effect on the sensitivity to neutralization. These data demonstrate that the V1/V2 region of HIV-1 gp120 is principally responsible for the strain specificity of the neutralizing antibody response in monkeys infected with these prototypic SHIVs.

In order to achieve complete and durable protection against human immunodeficiency virus (HIV), it has been suggested that a vaccine may have to elicit a potent, broadly neutralizing antibody response as well as a strong cell-mediated immune response (93). Multiple studies have shown that passive transfer of HIV-specific antibodies is able to provide sterile protection against HIV type 1 (HIV-1) or simian-HIV (SHIV) challenge, whether administered intravenously or mucosally, in various animal models of infection (3, 32, 57, 58, 70, 73, 85). Moreover, several unusual, well-characterized monoclonal antibodies that are broadly acting, relatively potent, and capable of neutralizing viruses in a cross-clade manner have been isolated from HIV-infected individuals (7, 9, 13, 34, 78, 88, 91). These studies demonstrate that neutralizing antibodies can, at least in theory, provide protection against HIV-1 infection. However, envelope-based immunogens developed to date have not been capable of eliciting a robust antibody response with the broad target specificity and high plasma concentrations that are likely to be necessary for protection (5, 12, 20, 22, 24–26, 31, 56, 59, 62, 92).

HIV-1 infection in humans typically elicits high levels of antibodies directed against the viral surface glycoprotein gp120. It has been estimated that as much as 5% of all immunoglobulin in some HIV-infected individuals may be directed to the virus-encoded surface glycoprotein (6). However, the abilities of these antibodies to neutralize HIV-1 infectivity are extremely limited in both potency and breadth. The neutralizing antibodies that are elicited upon HIV-1 infection tend to be highly strain specific; they neutralize autologous virus most effectively but exhibit little or no neutralizing activity against heterologous HIV-1 strains (10, 11, 61, 74, 81). Due to rapid sequence evolution within the envelope protein in response to immune pressure and selection, the original antibody population loses potency over the course of infection as persistently replicating viruses acquire resistance to antibody-mediated detection. There is a responsive shift in the production of neutralizing antibodies over time, apparently evolving new target specificities to counter newly emerged viral variants (1, 29, 33, 81, 95).

The HIV-1 envelope glycoprotein is the primary target for

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▽ Published ahead of print on 3 September 2008.

antibody-mediated neutralization (2, 4). In order to circumvent antibody recognition, HIV-1 has evolved a number of shielding strategies to occlude conserved epitopes and limit the accessibility of glycoprotein spikes on the surfaces of virions to antibody binding. The mature envelope spike exists as a trimer of gp120-gp41 noncovalently associated heterodimers. Each trimer is tightly packed in a closed conformation such that epitopes that otherwise might serve as targets for antibody binding and neutralization reside in the core of the protein and are not readily exposed (45, 66, 67, 77). Additionally, gp120 contains five distinct regions that are characterized by extensive sequence variation. Four of these hypervariable regions are thought to form loops through intrachain disulfide bonds that are exposed on the outer surface of the envelope protein, resulting in the occlusion of conserved core envelope epitopes from antibody recognition prior to viral entry (38, 40, 50, 54, 96).

A number of studies have been performed to explore the roles of the variable loops as immunological decoys or in shielding conserved elements within the core of the envelope. Surprisingly, the deletion of the entire variable loop 1 and 2 (V1/V2) complex, as many as 100 amino acids, can still yield replication-competent HIV-1 or simian immunodeficiency virus (SIV) (44, 97). These V1/V2 deletion mutants are substantially more sensitive to neutralization by a panel of monoclonal antibodies targeting multiple epitopes on gp120 (14, 44, 46, 52, 83, 84, 87, 97). Several highly strain-specific neutralizing antibodies targeting the V1 and/or V2 loop have been identified, although exact epitopes have not yet been defined (18, 27, 28, 30, 89). A considerable body of evidence suggests that the majority of anti-V3 antibodies are also strain specific and can efficiently neutralize tissue culture-adapted virus strains but not primary isolates (65). In SIV infection, the V4 loop contains a conformational, neutralization-sensitive epitope in which sequence changes emerge to escape antibody detection (43, 90).

The present study examines the role of variable-loop sequences in the strain specificity of antibody-mediated neutralization. To investigate if certain variable loops might be responsible for the strain-specific neutralizing activity characteristic of HIV-1-positive plasma, we constructed a panel of chimeric viruses between SHIV DH12 and SHIV KB9, exchanging each variable loop between both SHIV envelopes individually and in combination. We then examined the effects of these variable-loop exchanges on virus infectivity and sensitivity to highly strain-specific plasma samples collected from SHIV DH12- and SHIV KB9-infected monkeys. Seven of the 10 SHIV-derived variable-loop chimeras were replication competent and capable of inducing robust secreted alkaline phosphatase (SEAP) production in the C8166-45 LTR-SEAP cell line. We demonstrate that the exchange of the V1/V2 loop complex between SHIV DH12 and SHIV KB9 is consistently associated with a dramatic loss of sensitivity to autologous plasma and a gain of sensitivity to heterologous plasma. Our results demonstrate that the V1/V2 loop is the principal determinant of the strain specificity of the neutralizing-antibody response in SHIV-infected monkeys.

MATERIALS AND METHODS

PCR mutagenesis and plasmid construction. Mutations within *env* were generated by a two-step PCR method that combined the technique of splicing by overlap extension PCR (69) to generate a primary product with site-directed PCR mutagenesis to incorporate the mutations into the hemigenomic plasmids. For SHIV KB9, initial mutagenesis was performed on the 3′ half of the genome, which was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH) (47). The full-length proviral plasmid DNA of SHIV DH12 was kindly provided by T. Igarashi and M. A. Martin (86). To facilitate optimal mutagenesis efficiency for the SHIV DH12 envelope sequence, the envelope gene was subcloned from the full-length genome.

For the splicing by overlap extension step of our mutagenesis protocol, two overlapping PCR products were generated, with the desired substitution incorporated in the region of overlap. Specifically, the amplified and incorporated fragment was the complete variable-loop sequence under investigation. Each primer included, in its 5′ portion, nucleotides complementary to the backbone envelope sequence and, in its 3′ half, nucleotides complementary to the variable-loop sequence of interest. These primers were combined with the plasmid DNA containing the variable-loop sequence. The result was a pool of PCR products containing the amplified variable-loop sequence flanked by single-stranded overhangs of sequence complementary to the reciprocal envelope sequence. These purified variable-loop amplicons were used in lieu of primers for site-directed mutagenesis (Stratagene, La Jolla, CA) reactions using the envelope subclones as template DNA.

The following paired primers were used to generate the amplicons necessary for creating the variable-loop chimeric envelopes (the first capitalized letter indicates the SHIV envelope background, and the SHIV strain that is the source of the variable loop exchanged is indicated by the lowercase abbreviation prior to the variable-loop identification; within the primer sequence, the loop sequences that are being introduced are underlined): K.dV1V2F, 5′ CC CCA CTC TGT GTT ACT TTA AAT TGC ACT GAT TTG AAG AAT GG 3′; K.dV1V2R, 5′ GGC CTG TGT AAT GAC TGA GGT GTT ACA ACT TAT CAA CCT ATA GC 3′; K.dV3F, 5′ CAG CTA AAT GAA TCT GTA GTA ATT AAT TGT ACA AGA CCC AAC AAC AAT ACA AGA AAA GGG 3′; K.dV3R, 5′ CCA TTT TGC TCT ACT AAT GTT ACA ATG TGC TTG TCT TAT ATC TCC 3′; K.dV4F, 5′ GGA GGG GAA TTT TTC TAC TGT AAT ACA AAA AAA CTG 3′; K.dV4R, 5′ CC TAC TTT CTG CCA CAT ATT TAT AAT TTG TTT TAT TCT GCA TGG GAG TGT GAT AGT GTC ATT TCC 3′; D.kV1V2F, 5′ CC CCA CTC TGT GTT ACT TTA CAT TGC ACT AAT TTG AAT ATC AC 3′; D.kV1V2R, 5′ GG ACA GGC CTG TGT AAG GGT TGA GGT GTT ACA ACT TAT TAA CCT ATA CTT AG 3′; D.kV3F, 5′ CAG CTG AAT GAA ACT GTA GAA ATT AAT TGT ACA AGA CCC AAC AAC AAT ACA AGA GAA AGG 3′; D.kV3R, 5′ G CCA TTT TAC TTT ACT AAT GTT ACA ATG TGC TTG TCT TAT ATC TCC 3′; D.kV4F, 5′ GT GGA GGG GAA TTT TTC TAC TGT AAT ACA GCA CAA CTG TTT AAT AGT ACT TGG 3′; D.kV4R, 5′ C CTG CCA CAT GTT TAT AAT TTG TTT TAT TCT GCA TTG GAG TGT GAT TAT GTC ATT TCC 3′ (Sigma Genosys, The Woodlands, TX).

The variable-loop-exchanged envelopes within the envelope subclones were sequenced to verify accuracy. In the constructs that contained variable loops exchanged in combination, this process was repeated sequentially. Sequencing of these combinatorial constructs was performed subsequent to each mutagenesis step. Full-length proviral DNA clones of each mutant were constructed.

DNA sequencing. Cloned plasmids containing selected mutations were sequenced with a CEQ8000 genetic-analysis system, using a dye terminator cycle-sequencing chemistry kit as specified by the manufacturer (Beckman-Coulter, Fullerton, CA).

Virus stocks and cell culture. To generate virus stocks, 5 μg of the appropriate full-length proviral DNA plasmid was transfected into 1.5×10^6 HEK 293T cells using the calcium phosphate method (Promega, Madison, WI). The medium was changed 24 h posttransfection. Supernatants were harvested on day 3 posttransfection. Virus concentrations in the supernatant were quantified by determining the concentration of p27 capsid protein using an antigen capture assay, according to the manufacturer's instructions (Advanced Bioscience Laboratories, Inc., Kensington, MD). HEK 293T, MT4, C8166, and C8166-45 LTR-SEAP cells were cultured and maintained as described previously (10, 60).

Infectivity assay. Viral infectivity was quantified using the C8166-45 LTR-SEAP indicator cell line (60). C8166-45 LTR-SEAP cells contain a Tat-inducible SEAP reporter construct allowing SIV infection of the cells to be measured by SEAP concentrations in the cell-free supernatant. Nine serial twofold dilutions were made, starting from 10 ng of p27 capsid equivalents of HEK 293T cell-produced stocks. C8166-45 LTR-SEAP cells (8,000) were added to each

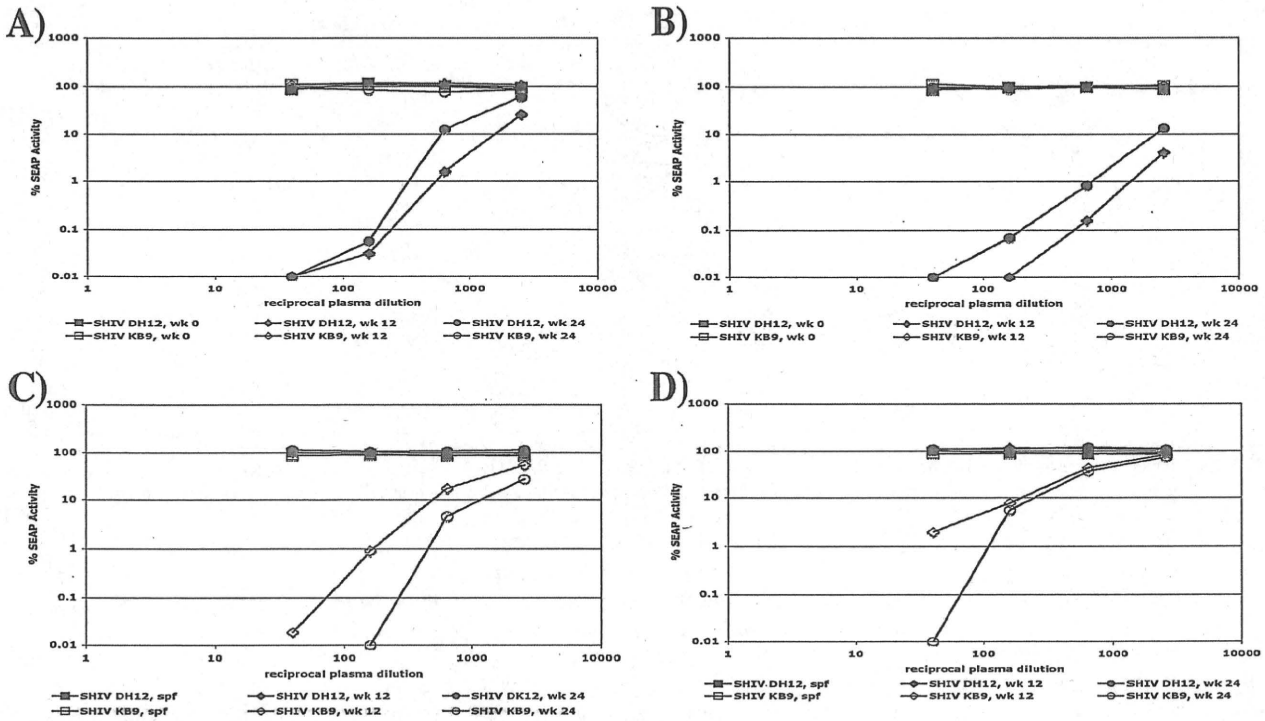

FIG. 1. Strain-specific neutralizing activity of positive plasmas when assayed with SHIV DH12 and SHIV KB9. (A) Neutralization of SHIV DH12 and SHIV KB9 with positive plasma from a SHIV DH12-infected monkey, RHTPP, collected at 0, 12, and 24 weeks (wk) postinfection. (B) Neutralization of SHIV DH12 and SHIV KB9 with positive plasma from a SHIV DH12-infected monkey, Rh418, collected at 0, 12, and 24 weeks postinfection. (C) Neutralization of SHIV DH12 and SHIV KB9 with positive plasma from a SHIV KB9-infected monkey, 477-99 collected 12 and 24 weeks postinfection, as well as with plasma from a SHIV-negative, specific-pathogen-free (SPF) monkey. (D) Neutralization of SHIV DH12 and SHIV KB9 with positive plasma from a SHIV KB9-infected monkey, 481-99, collected 12 and 24 weeks postinfection, as well as with plasma from an SPF monkey.

well. The cells were incubated at 37°C in a humidified CO_2 incubator. Three days postinfection, SEAP activity was measured in the cell-free supernatant (Applied Biosystems, Foster City, CA).

SHIV-positive plasmas. Five plasma samples taken at selected time points postinfection from rhesus macaques infected with SHIV DH12 were obtained from Malcolm Martin (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). Four plasma samples taken at selected time points postinfection from rhesus macaques infected with SHIV KB9 were available from a previous study performed at the New England Primate Research Center.

Neutralization. The neutralization sensitivity of each virus to both SHIV-positive monkey plasmas was assayed using the SEAP reporter cell assay as described previously (60). Virus equivalent to 2.5 ng of p27 capsid protein was determined to be the lowest level of virus input sufficient to give a reliable SEAP signal within the linear range of the assay for all of the virus strains used. SEAP activity was quantified on the earliest day postinfection when levels were sufficiently high over background to give consistent measurements (at least 10-fold), ranging from 3 days for SHIV DH12, SHIV KB9, SHIV KDV3, and SHIV KDV4 to 5 days for SHIV DKV12, SHIV DKV4, SHIV DKV1234, and SHIV KDV1234 and 7 days for SHIV DKV124.

To perform neutralization assays, 96-well plates were organized as follows. Twenty-five microliters of medium (RPMI 1640 supplemented with 10% fetal bovine serum, penicillin/streptomycin, L-glutamine, and HEPES buffer [R10]) was added to each well in the first three columns. To the wells in the remaining columns (columns 4 to 12), 25-μl aliquots of successive twofold dilutions of test plasma or antibody, resuspended in medium, were added. All plasmas were heat inactivated at 56°C for 30 min before use in neutralization assays. For monkey plasma, 25 μl of a 1:10 dilution of plasma was mixed with 75 μl of the test virus. This constituted a 1:40 dilution in the representative graph. Virus equivalent to 2.5 ng of p27 capsid protein in a total volume of 75 μl of R10 was then added to each well in columns 3 to 12. Virus-free medium was added to each well in columns 1 and 2 (mock infection). The plate was incubated for 1 h at 37°C. Following this incubation, 8,000 C8166-45 LTR-SEAP cells resuspended in 100 μl of R10 were added to each well. The plate was placed in a humidified chamber within a CO_2 incubator at 37°C for 3 to 7 days. SEAP activity was measured. Neutralizing activities for all antibodies and plasma samples were measured in triplicate using a Victor V multilabel counter (Perkin-Elmer, Norwalk, CT) and reported as the average percent SEAP activity.

RESULTS

Stringent strain-specific neutralization of SHIV DH12 and SHIV KB9. The choice of SHIV DH12 and SHIV KB9 as representative viruses in this study was based on several factors: (i) both SHIVs are well-characterized, genetically modifiable molecular clones; (ii) positive plasmas from monkeys infected with the matched molecular clones over several time points postinfection were available; and (iii) positive plasmas from infected animals displayed extremely stringent strain-specific neutralizing activity.

To assess the specificity of the neutralizing activity present in monkeys infected with SHIV DH12 or SHIV KB9, neutralization assays were performed using plasma samples from animals RHTPP and Rh418, collected 0, 12, and 24 weeks postinfection with SHIV DH12 and plasma samples from animals 477-99 and 481-99, collected 0, 12, and 24 weeks postinfection, with SHIV KB9 (Fig. 1). Assays performed with plasma collected prior to infection (week zero) demonstrated no preexisting nonspecific neutralizing activity for either SHIV DH12 or SHIV KB9. Highly strain-specific neutralizing activity was ob-

TABLE 1. Neutralization of SHIV-DH12 chimeras with positive plasmas

Animal	Infecting SHIV	Time point of sera (wk)	KB9 wt[a,b]	Neutralization activity for SHIV-DH12 and DH12-derived chimeric virus[b]					
				DH12 wt	DKV12	DKV3	DKV4	DKV124	DKV1234
RHTPP	DH12	10	–	9,000	–	ND	12,000	–	–
		12	–	7,000	–	ND	15,000	–	–
		24	–	2,000	–	ND	7,500	–	–
Rh418	DH12	12	–	20,000	–	ND	20,000	–	40
		24	–	10,000	–	ND	10,000	–	–
477-99	KB9	12	2,500	–	15,000	ND	40	20,000	8,000
		24	5,000	–	8,000	ND	–	9,000	7,000
481-99	KB9	12	1,000	–	130	ND	–	300	420
		24	1,100	–	650	ND	40	1,100	800

[a] wt, wild type.
[b] The numbers indicate the reciprocals of the dilutions of plasma required to reduce the infectivity of the indicated viruses by 50%. –, 50% neutralization was not achieved, even at the lowest dilution (highest concentration) of plasma tested. ND, not determined due to inadequate infectivity of designated viruses.

served in plasma samples collected from all four experimentally infected monkeys at 12 and 24 weeks postinfection. Plasma from animals RHTPP and Rh418, infected with SHIV DH12, showed extensive neutralization of SHIV DH12 yet had no effect against the heterologous SHIV KB9 (Fig. 1A and B, respectively, and Table 1). Conversely, plasma from monkeys 477-99 and 481-99, infected with SHIV KB9, strongly neutralized SHIV KB9, but not SHIV DH12 (Fig. 1C and D, respectively, and Table 2). Most or all of the neutralizing activity was accounted for by purified immunoglobulin fractions, indicating that the neutralization was antibody mediated (data not shown).

Further studies were directed at determining whether the variable loops in gp120 were principally responsible for the strain specificity of the neutralizing antibody response and, if so, which variable loop or loops were primarily involved.

Construction of recombinant variable-loop chimeric SHIVs. SHIV DH12 and SHIV KB9 were originally constructed using SIVmac239 as the backbone and differ genetically in the *env*, *tat*, *rev*, *vpu*, and *vpr* genes, which are derived from multiple different HIV-1 species (47, 79, 86). Gp120 envelope sequences outside of the variable loops share an 82% amino acid identity between these two SHIV clones. Within the variable loops, however, homology is dramatically reduced, maintaining only 58% amino acid identity (Fig. 2A). To investigate the extent to which the variable loops of gp120 contribute to the strain restriction of the neutralizing antibody response, each variable-loop sequence was exchanged alone or in combination with other loop sequences between SHIV DH12 and SHIV KB9 (Fig. 2B). The nomenclature of each SHIV-derived variable-loop chimera reflects this construction. For each variable-loop chimeric virus, the backbone SHIV envelope sequence is identified by the first abbreviated letter (D for SHIV DH12 and K for SHIV KB9), the SHIV clone from which the exchanged variable loop was derived is indicated by the second letter, and the exchanged variable loop is identified by the numerical designation of the loop sequence. For example, SHIV DKV12 was constructed in the SHIV DH12 background to contain the V1/V2 loop derived from SHIV KB9. Virus stocks were generated by HEK 293T cell transfection, and equivalent p27 quantities were used for all comparative experiments.

TABLE 2. Neutralization of SHIV-KB9 chimeras with positive plasmas

Animal	Infecting SHIV	Time point of sera (wk)	DH12 wt[a,b]	Neutralization activity for SHIV-KB9 and KB9-derived chimeric virus[b]					
				KB9 wt	KDV12	KDV3	KDV4	KDV124	KDV1234
RHTPP	DH12	10	9,000	–	ND	–	–	ND	3,000
		12	7,000	–	ND	–	–	ND	5,000
		24	2,000	–	ND	–	–	ND	2,000
Rh418	DH12	12	20,000	–	ND	–	–	ND	10,500
		24	10,000	–	ND	–	–	ND	5,300
477-99	KB9	12	–	2,500	ND	5,000	5,000	ND	80
		24	–	5,000	ND	5,000	10,000	ND	100
481-99	KB9	12	–	1,000	ND	1,500	1,280	ND	–
		24	–	1,100	ND	1,280	1,280	ND	–

[a] wt, wild type.
[b] The numbers indicate the reciprocals of the dilutions of plasma required to reduce the infectivity of the indicated viruses by 50%. –, 50% neutralization was not achieved, even at the lowest dilution (highest concentration) of plasma tested. ND, not determined due to inadequate infectivity of designated viruses.

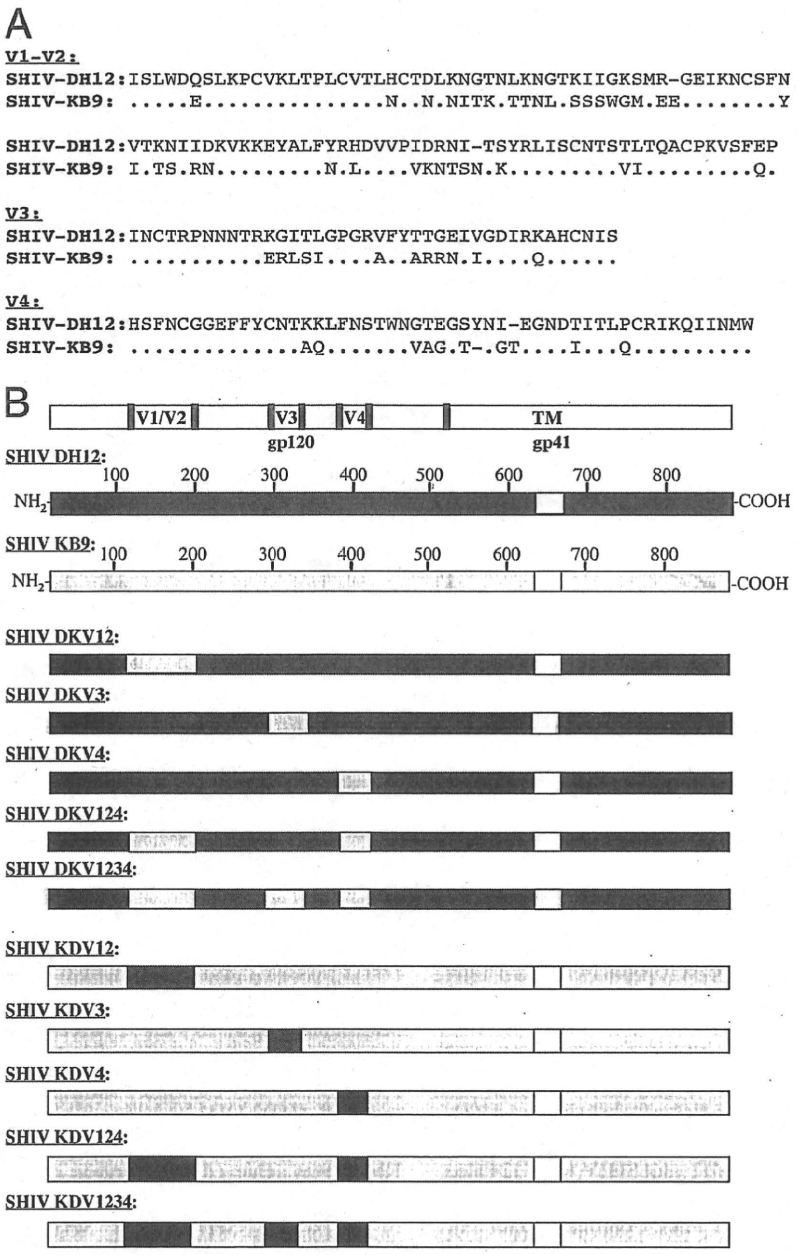

FIG. 2. Variable-loop exchanges made between SHIV DH12 and SHIV KB9. (A) Comparative amino acid alignment of variable-loop sequences between SHIV DH12 and SHIV KB9. (B) Schematic representation of a variable-loop exchange(s) within the SHIV DH12 and SHIV KB9 gp120 envelope glycoproteins.

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)

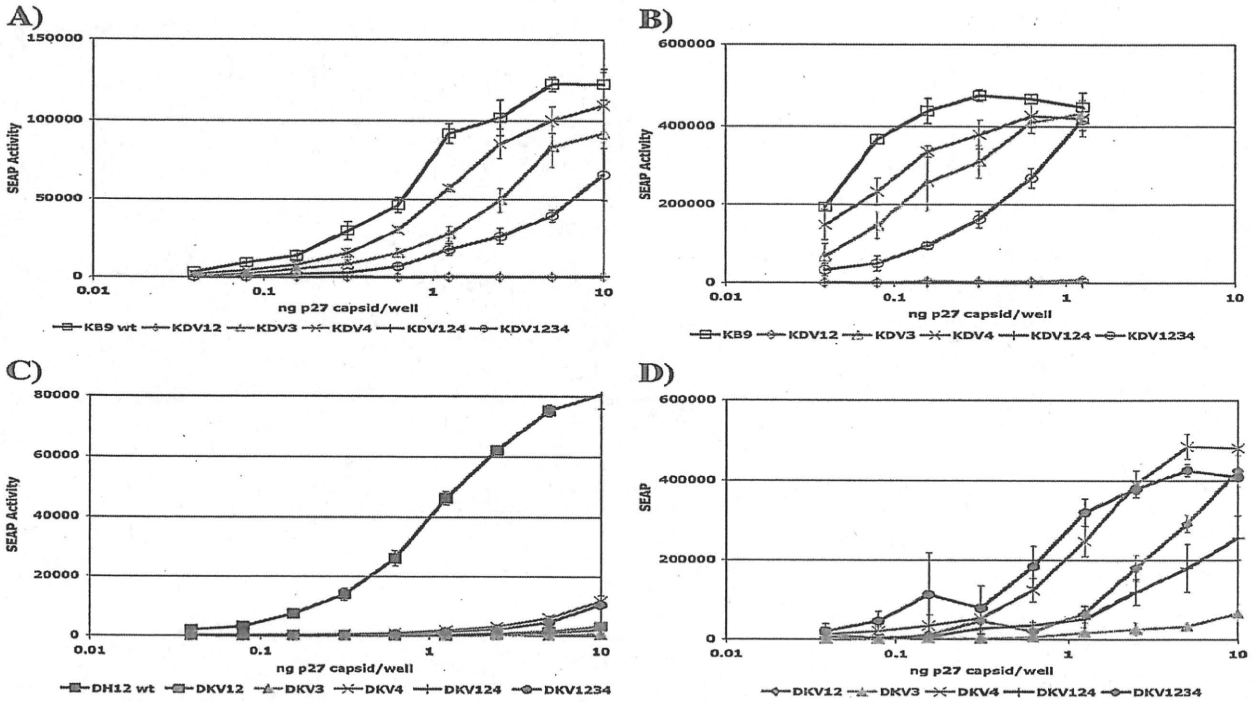

FIG. 3. Comparative infectivities of parental and SHIV-derived variable-loop chimeric viruses. Virus stocks were obtained from transfection of HEK 293T cells, normalized by the amount of p27, and used to infect C8166-45 LTR-SEAP cells. (A) SEAP activity was measured at 3 days postinfection with SHIV 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

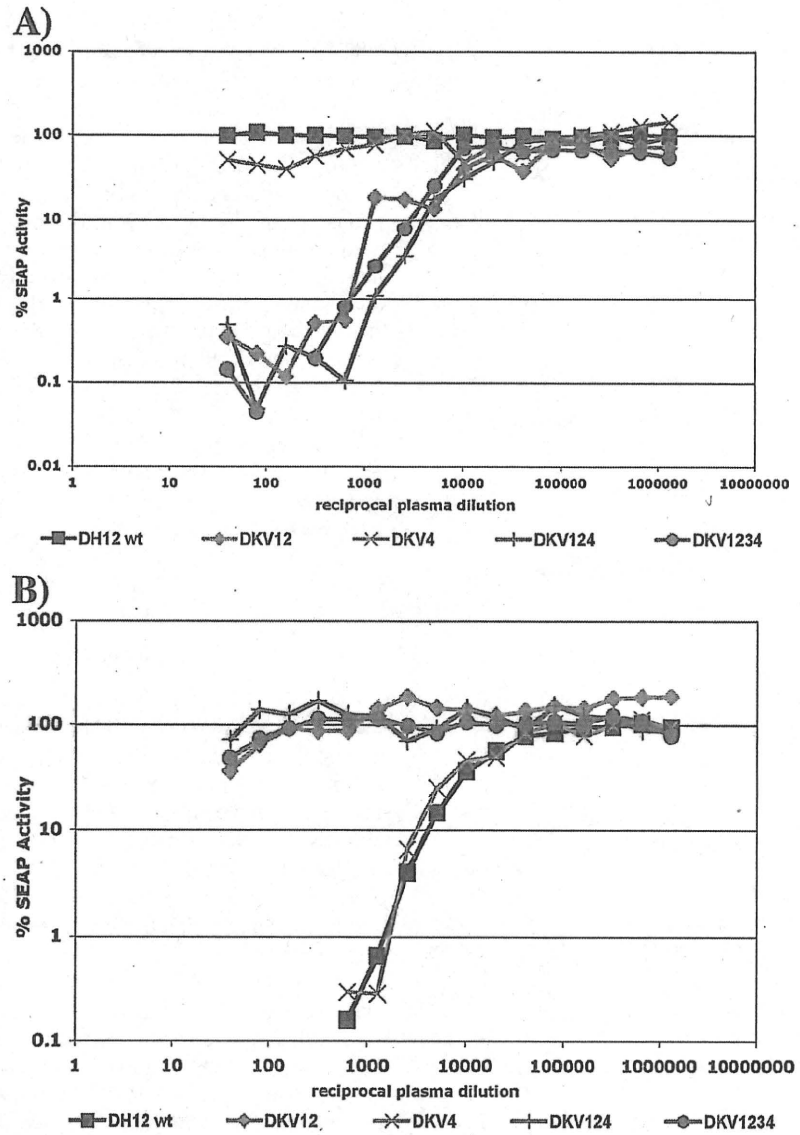

FIG. 4. Representative neutralization curves of SHIV DH12 and SHIV DH12-derived loop chimeras with plasma from SHIV-infected monkeys. (A) Neutralization of SHIV DH12 and SHIV DH12-derived loop chimeras by plasma from a SHIV DH12-infected monkey, Rh418, collected 13 weeks postinfection. (B) Neutralization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

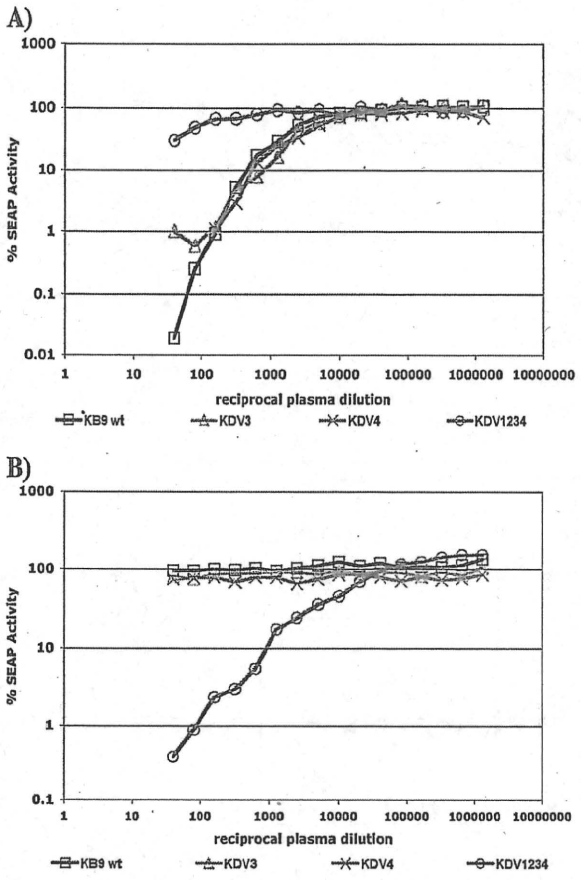

FIG. 5. Representative neutralization curves of SHIV KB9 and SHIV KB9-derived loop chimeras with plasma from SHIV-infected monkeys. (A) Neutralization of SHIV KB9 and SHIV KB9-derived loop chimeras by plasma from a SHIV DH12-infected monkey, Rh418, collected 13 weeks postinfection. (B) Neutralization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

ACKNOWLEDGMENTS

This work was supported by PHS grants AI 025328 (R.C.D.) and RR00168 (NEPRC) and by funding from the International AIDS Vaccine Initiative. Additionally, this work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (M.A.M.).

We thank the NEPRC DNA-sequencing core for technical support and the NEPRC Division of Primate Resources for experimental monkey infection and blood sampling. We also thank Thomas Postler, Elizabeth MacKenzie, and Jacqueline Bixby for technical and editing assistance.

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JOURNAL OF VIROLOGY, Feb. 2009, p. 2055
0022-538X/09/$08.00+0 doi:10.1128/JVI.02472-08

Vol. 83, No. 4

ERRATUM

Importance of the V1/V2 Loop Region of Simian-Human Immunodeficiency Virus Envelope Glycoprotein gp120 in Determining the Strain Specificity of the Neutralizing Antibody Response

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Volume 82, no. 22, p. 11054–11065, 2008. Page 11060, legend to Figure 4. Line 2: "plasma from a SHIV DH12-infected monkey, Rh418," should read "plasma from a SHIV KB9-infected monkey, 477-99," and lines 3 and 4: "plasma from a SHIV KB9-infected monkey, 477-99," should read "plasma from a SHIV DH12-infected monkey, Rh418."

Page 11061, legend to Figure 5. Line 4: "plasma from a SHIV DH12-infected monkey, Rh418," should read "plasma from a SHIV KB9-infected monkey, 477-99," and lines 6 and 7: "plasma from a SHIV KB9-infected monkey, 477-99," should read "plasma from a SHIV DH12-infected monkey, Rh418."

Biochemical and Biophysical Research Communications 377 (2008) 889–893

Contents lists available at ScienceDirect

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

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ARTICLE INFO

Article history:
Received 11 October 2008
Available online 23 October 2008

Keywords:
Rhesus macaque
CD1
Mycobacteria
Glucose monomycolate

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.

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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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doi:10.1016/j.bbrc.2008.10.075

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

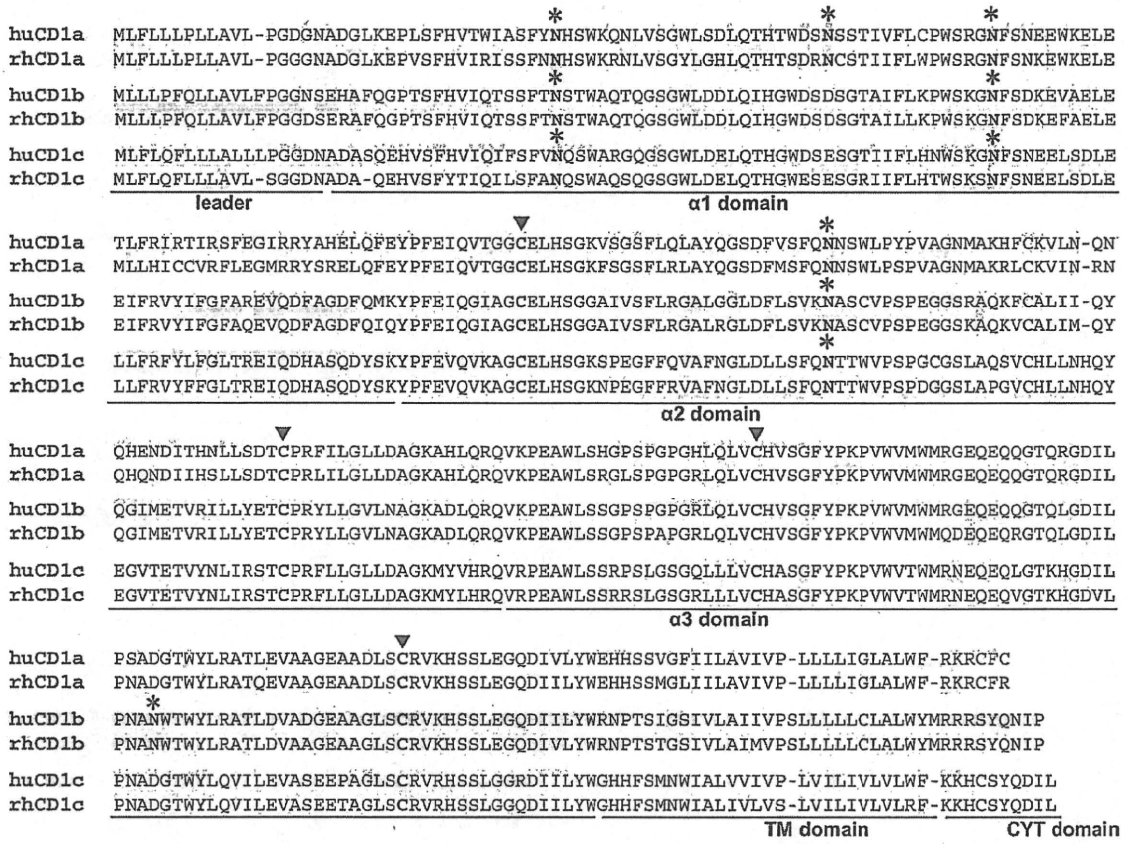

Fig. 1. Alignment of deduced amino acid sequences of human (hu) and rhesus macaque (rh) group 1 CD1 proteins. Residues conserved between the two species are shaded in light gray. Solid triangles denote cysteines conserved in all the group 1 CD1 proteins of both species that are presumed to be involved 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).

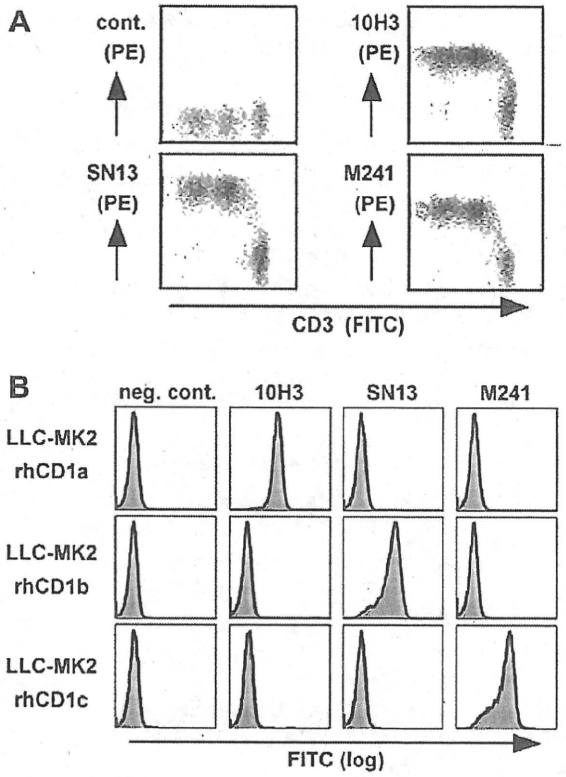

Fig. 2. Cross-reactivity of anti-human CD1 mAbs to rhesus macaque group 1 CD1 proteins. (A) Rhesus macaque thymocytes were double-labeled with the SP34 anti-CD3 mAb and either the 10H3 anti-human CD1a mAb, the SN13 anti-human CD1b mAb, the M241 anti-human CD1c mAb, or negative control Abs, followed by 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.

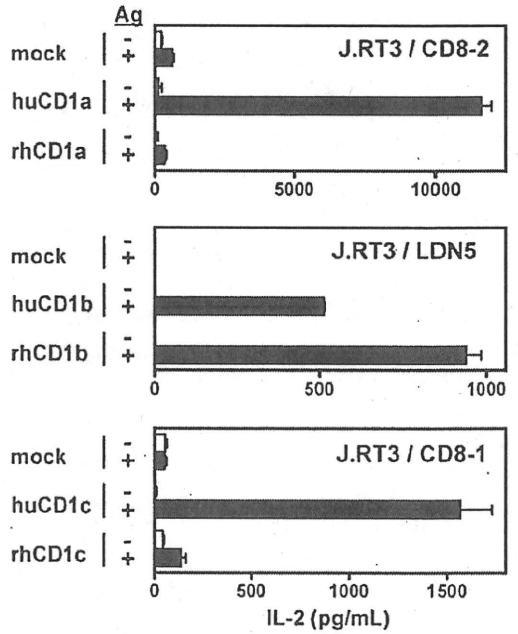

Fig. 3. Ag presentation function of rhesus macaque CD1b molecules. The J.RT3/CD8-2 cells were cultured in the presence or absence of the organic extract of *M. tuberculosis* (50 mg/ml) with T2 cells expressing either human CD1a (huCD1a) or rhesus macaque CD1a (rhCD1a) or those that were mock-transfected (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.

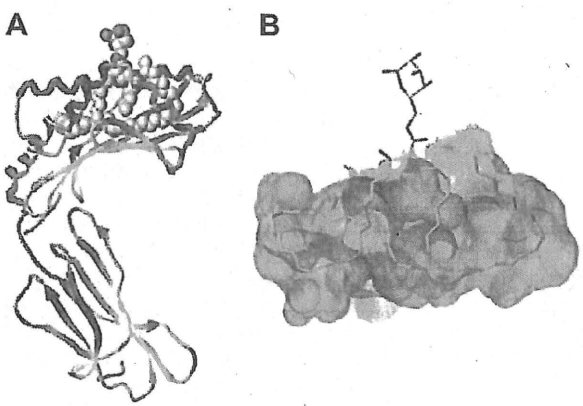

Fig. 4. A molecular model of rhesus macaque CD1b proteins. The rhesus macaque CD1b structure was constructed, based on the crystal structure of the human CD1b-GMM complex. (A) The overall structure of the rhesus macaque CD1b-GMM complex is shown, in which the CD1b heavy chain is depicted in ribbon diagram 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).

Acknowledgments

We thank Drs. M. Brenner, D. Olive, and C. Mawas for their gifts of reagents. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Grant-in-Aid from Scientific Research on Priority Areas), from the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research

(B)), and from the Ministry of Health, Labour, and Welfare (Research on Emerging and Re-emerging infectious Diseases) (to M.S.).

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Biochemical and Biophysical Research Communications 380 (2009) 838–843

Contents lists available at ScienceDirect

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Inhibition of human immunodeficiency virus type 1 (HIV-1) nuclear import via Vpr–Importin α interactions as a novel HIV-1 therapy

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ARTICLE INFO

Article history:
Received 18 January 2009
Available online 4 February 2009

Keywords:
HIV-1 inhibitor
Vpr
Nuclear import inhibition
Importin α
Small molecule
Macrophage

ABSTRACT

The development of multidrug-resistant viruses compromises the efficacy of anti-human immunodeficiency virus (HIV) therapy and limits treatment options. Therefore, new targets that can be used to develop novel antiviral agents need to be identified. One such target is the interaction between Vpr, one of the accessory gene products of HIV-1 and Importin α, which is crucial, not only for the nuclear import of Vpr, but also for HIV-1 replication in macrophages. We have identified a potential parent compound, hematoxylin, which suppresses Vpr–Importin α interaction, thereby inhibiting HIV-1 replication in a Vpr-dependent manner. Analysis by real-time PCR demonstrated that hematoxylin specifically inhibited nuclear import step of pre-integration complex. Thus, hematoxylin is a new anti-HIV-1 inhibitor that targets the nuclear import of HIV-1 via the Vpr–Importin α interaction, suggesting that a specific inhibitor of the interaction between viral protein and the cellular factor may provide a new strategy for HIV-1 therapy.

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Macrophages are a major target of human immunodeficiency virus type 1 (HIV-1) and serve as a viral reservoir for the release of small amounts of viral particles in symptomatic carriers [1]. HIV-1 in latently infected macrophages in some lymphoreticular tissues cannot be eradicated by highly active anti-retroviral therapy (HAART), and these cells may produce viral particles that can spread throughout the body [2]. A striking feature of HIV-1 is its ability to replicate in non-dividing cells, in particular, macrophages. Replication in non-dividing cells depends on the active nuclear import of the viral pre-integration complex (PIC) [3]. The HIV-1 PIC exhibits biophysical properties typical of a large nucleo–protein complex and contains the viral proteins reverse transcriptase, integrase (IN), nucleocapsid, Vpr, and small amounts of matrix (MA), in addition to the viral nucleic acids [4–7]. Three PIC-associated proteins, MA, IN, and Vpr, have been proposed as karyophilic agents that act probably via their interactions with Importin (Imp) α, Imp β, and/or Imp 7 [8]. In addition, a recent study indicates that transportin-SR2, which shuttles the essential splicing factor, mediates PIC nuclear import, thereby facilitating HIV infection [9]. Moreover, a novel partner, tRNA, has been shown to facilitate PIC nuclear import [10]. However, the molecular mechanisms of PIC nuclear import and its role in viral replication in macrophages are still not completely understood.

Several studies have shown that Vpr is essential for the nuclear import of PIC in macrophages [11,12], while others do not support such observations [13]. However, our studies have clearly shown that Vpr is targeted to the nuclear envelope and then transported into the nucleus by Imp α alone, in an Imp β-independent manner [12,14]. Furthermore, the interaction between Imp α and the N-terminal α-helical domain (αH1) of Vpr, amino acid residues 17–34, is indispensable, not only for nuclear import of Vpr, but also for HIV-1 replication in macrophages [12]. Thus, it appears that Vpr–Imp α binding precedes a novel nuclear import process, which is a potential target for therapeutic intervention in macrophages, which is crucial for subsequent viral spread to lymphoid organs and T-helper lymphocytes [15]. In addition to nuclear transport, Vpr also has other important functions, including the induction of cell cycle arrest at the G_2 phase [16], the regulation of apoptosis [16,17] and splicing [18,19], carried out through interactions with a variety of cellular partners. These observations suggest that drug targeting of Vpr may lead to pleiotropic effects on the HIV life cycle.

As a promising target for blocking HIV-1 replication in macrophages, by screening a large collection of chemical compounds,

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doi:10.1016/j.bbrc.2009.01.180