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Effect of partial and complete variable loop deletions of the human immunodeficiency virus type 1 envelope glycoprotein on the breadth of gp160-specific immune responses

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Abstract

Induction of cross-reactive cellular and humoral responses to the HIV-1 envelope (env) glycoprotein was examined after DNA immunization of BALB/c mice with $gp140_{89.6}$-derived constructs exhibiting partial or complete deletions of the V1, V2, and V3 domains. It was demonstrated that specific modification of the V3 loop (mV3) in combination with the V2-modified (mV2) or V1/V2-deleted (ΔV1/V2) region elicited increased levels of cross-reactive $CD8^+$ T cell responses. Mice immunized with the mV2/mV3 or ΔV1/V2/mV3 $gp140_{89.6}$ plasmid DNA were greater than 50-fold more resistant to challenge with recombinant vaccinia virus (rVV) expressing heterologous env gene products than animals immunized with the wild-type (WT) counterpart. Sera from mV2/mV3- and ΔV1/V2/mV3-immunized mice exhibited the highest cross-neutralizing activity and displayed intermediate antibody avidity values which were further enhanced by challenge with rVV expressing the homologous gp160 glycoprotein. In contrast, complete deletion of the variable regions had little or no effect on the cross-reactive antibody responses. The results of these experiments indicate that the breadth of antibody responses to the HIV-1 env glycoprotein may not be increased by removal of the variable domains. Instead, partial deletions within these regions may redirect specific responses toward conserved epitopes and facilitate approaches for boosting cross-reactive cellular and antibody responses to the env glycoprotein.
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Keywords: HIV-1; DNA vaccine; Envelope glycoprotein; Cellular responses; Neutralizing antibodies

Introduction

The current challenge for the design of effective HIV-1 vaccines is to develop immunization strategies to elicit both stronger and broader immunity against diverse viral species (Chakrabarti et al., 2002; Schulke et al., 2002). The envelope (env) glycoprotein is an important component of an efficacious vaccine for AIDS because gp120 is the major viral surface glycoprotein and a key mediator of the entry process (Binley et al., 2000; Kwong et al., 2000; Moore and Binley, 1998; Moore and Sodroski, 1996; Parren et al., 1999; Reitter et al., 1998; Wyatt et al., 1998). However, to persist as a chronic infection, HIV-1 has evolved ways to escape from cytotoxic T lymphocyte (CTL) recognition and to limit the generation of neutralizing antibodies. This includes mutations arising within regions of the viral genome encoding immunodominant epitopes that contribute to viral spread and the inability of anti-HIV-1 immunity to prevent the onset of AIDS (Goulder et al., 1999). Additionally, there is extensive shielding of conserved regions by carbohydrates (Reitter et al., 1998) and hiding of the coreceptor-binding site by variable loops of gp120 until the CD4 interaction occurs, thereby minimizing the time and space available for the antibody to intervene against this stage of the fusion process (Kwong et al., 1998; Moore and Binley, 1998). Because the overall structure of gp120 must be at least partially conserved for the protein to function in

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receptor binding and cellular entry, therefore the receptor-binding sites may provide a target for structural modifications to induce broadly neutralizing antibodies.

Although mechanisms responsible for induction of cross-protective antibody responses to the HIV-1 env glycoprotein remain to be elucidated, several lines of evidence indicate that exposure of certain neutralizing epitopes participating in the env-CD4 and -coreceptor binding can be increased by structural modification of the variable domains of gp120 (Barnett et al., 2001; Cao et al., 1997; Malenbaum et al., 2000; Stamatatos and Cheng-Mayer, 1998). Changes in the gp120 V2 loop or V1/V2 stem region that are responsible for both CD4-independent entry into cells and gp120 binding to CCR5 in the absence of CD4 (Kolchinsky et al., 1999, 2001) can alter immunogenicity of the single or double mutant. For example, deletion of the V1 and V2 loops from the env glycoprotein of the HIV-1_{HXBc2} was shown to increase susceptibility of the virus to neutralization by anti-V3 and certain CD4-induced monoclonal antibodies (Cao et al., 1997). Deletion of the central region of the V2 loop in gp120 of the HIV-$1_{SF162\Delta V2}$ isolate enhanced induction of cross-neutralizing antibodies by altering the immunogenicity of the V3 and V1 loops and rendering the C5 region immunogenic (Barnett et al., 2001; Srivastava et al., 2003). Deletion of the V1/V2 variable loop also leads to a significant reduction of the carbohydrate content (Reitter et al., 1998) that modulates the conformation of the V1/V2 stem-loop and affects the exposure of conserved, discontinuous structures on the HIV-1 gp120 glycoprotein (Wyatt et al., 1995). Studies in rhesus monkeys revealed that simian immunodeficiency virus mac239 variants lacking specific N-linked carbohydrate attachment sites within and around the V1 and V2 regions of gp120 were more sensitive to antibody-mediated neutralization and better elicitors of neutralizing antibody responses (Reitter et al., 1998).

The third hypervariable loop is another domain of gp120 that modulates immunogenicity of the env glycoprotein (Trkola et al., 1996). A number of studies based on the construction of chimeric env glycoproteins of X4 and R5 strains have demonstrated that the V3 loop is the primary determinant of tropism and coreceptor usage (Hung et al., 1999; Hwang et al., 1991), although other domains of gp120 are also involved (Basmaciogullari et al., 2002; Ross and Cullen, 1998). It has been reported that V3 loop-derived peptides can inhibit viral entry into target cells or syncytium formation between cells expressing the env glycoproteins of HIV-1 and cells that co-express CD4 and CCR5 or CXCR4 in a coreceptor-specific manner (Verrier et al., 1999). Recent studies have demonstrated that human monoclonal antibodies (mAbs) which recognize epitopes at the crown of the V3 loop derived from the HIV-$1_{JR\text{-}CSF}$ isolate showed cross-clade binding to native, intact virions of clades A, B, C, D, and F (Gorny et al., 2002). Additionally, the N-terminal V3 loop glycan was found to block access to the binding site for CD4 and modulated the chemokine receptor binding site of phenotypically diverse clade A and clade B isolates (Malenbaum et al., 2000). Because the majority of primary HIV-1 isolates reveals good exposure of the V3 region on the surface of intact virions (Nyambi et al., 2000a, 2000b), it has been suggested that antibodies which react with conformation-sensitive epitopes of the V3 loop may have potent cross-neutralizing activities (Park et al., 2000).

While "sterilizing" immunity mediated by broadly neutralizing antibody responses remains the ultimate goal for prophylactic HIV-1 vaccines, env-specific CTL responses may represent an important component of therapeutic vaccines. For example, the clearance of HIV-1 from plasma during the primary infection occurs before the appearance of neutralizing antibodies in newly infected individuals (Moore et al., 1994). In many studies, vaccinated macaques were able to efficiently control a virus challenge in the absence of detectable neutralizing antibodies, particularly those animals that were immunized with live, attenuated virus vaccines (Bogers et al., 1995; Cho et al., 2001; Dunn et al., 1997). Additional studies in pigtailed macaques demonstrated that animals immunized with a mixture of HIV-1 env glycoproteins exhibited lower levels of plasma virus than monkeys in the control group despite the absence of any detectable neutralizing antibody, suggesting induction of superior cellular responses by the polyvalent vaccine (Cho et al., 2001). Our previous studies have demonstrated that immunization of the HLA-A2/K^b transgenic mice with the ΔV3 env vaccine enhanced $CD8^+$ T-cell responses to conserved epitopes of gp160 and broaden cross-reactive CTL responses (Kiszka et al., 2002). However, the ability of the ΔV3 $gp140_{89.6}$ mutant to induce cross-reactive neutralizing antibody responses has not been examined. Therefore, it was of interest to analyze neutralization activities in ΔV3-immunized mice and compare them with responses induced by $gp140_{89.6}$-derived constructs exhibiting partial or complete deletions of the V1, V2, and V3 domains. This head-to-head analysis of cellular and humoral responses demonstrated that partial deletion of the V3 domain in combination with V2 or V1/V2 region modifications increased the breadth and potency of cellular, and to a lesser degree, protective antibody responses.

Results

Variable domain-modified env vaccines

To analyze the effect of specific modifications within the variable region of the env glycoprotein on the repertoire of cellular and humoral responses, we have generated DNA vaccines with partial or complete deletions of the V1, V2, and V3 domains on the background of gp140 of the HIV-$1_{89.6}$ isolate. Several reasons underlay this choice: (i) the HIV-$1_{89.6}$ virus is a primary, dual tropic virus (Doranz et al., 1996); (ii) it is a molecularly cloned virus with a well-

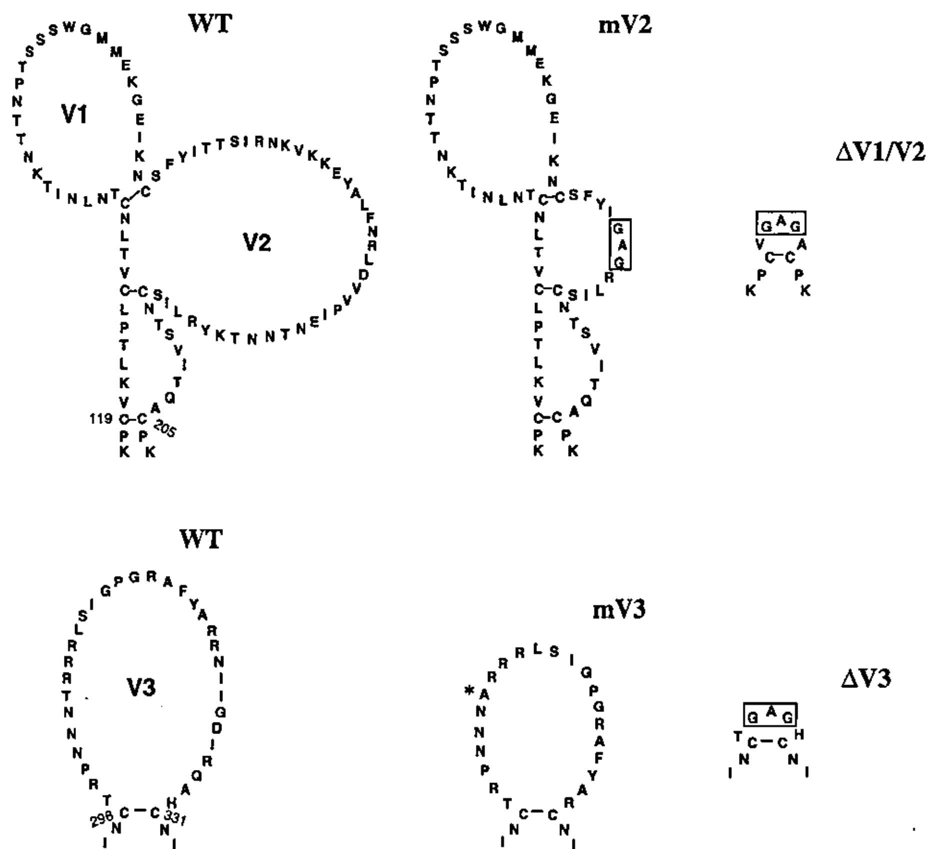

Fig. 1. Schematic representation of the variable domain-modified HIV-1$_{89.6}$ gp140 mutants. The amino acid sequences of V1 and V2 domains of the WT env glycoprotein and the mV2 and ΔV1/V2 mutants are shown in the upper panel. The lower panel depicts the amino acid sequence of the V3 loop of the WT env glycoprotein and the mV3 and ΔV3 mutants. In all mutants, the GAG sequence replaced the deleted residues. The 303T/A substitution in the mV3 domain is indicated by asterisk. The numbering of amino acids is based on the HxBc2 sequence available from the HIV Sequence Database.

characterized env gene (Kim et al., 1995); and (iii) we have previously expressed the gp140 protein from HIV-1$_{89.6}$ and analyzed its immunogenicity in mice (Kiszka et al., 2002).

To generate the variable region-modified gp140$_{89.6}$ mutants with deletion of the V1 and V2 domains (ΔV1/V2), residues 121 to 203 were replaced with a 3-aa sequence

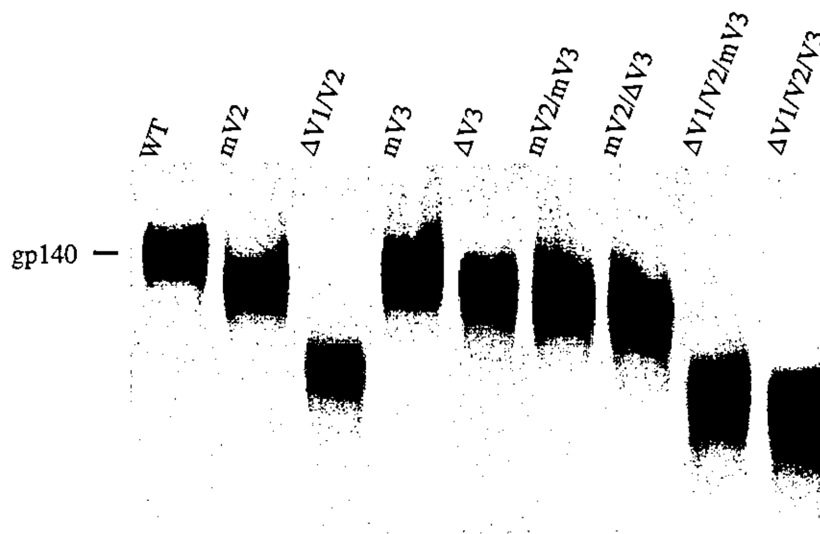

Fig. 2. Immunoprecipitation of WT and domain-modified HIV-1$_{89.6}$ gp140 glycoproteins. The 293T cells transiently transfected with plasmid DNA encoding the WT or the variable domain-modified env glycoproteins were radiolabeled with [^{35}S]methionine/cysteine and lysed in Nonident P-40 buffer. The env proteins from cell lysates were immunoprecipitated with a mixture of sera from HIV-1-infected individuals followed by polyclonal rabbit anti-human Ig and protein A-Sepharose CL-4B.

(GAG) as indicated in Fig. 1 (upper panel). Similarly, the GAG sequence replaced the deleted residues 298 to 329 in the ΔV3 mutant (Fig. 1, lower panel). We have also prepared an additional set of V2 and V3 domain-modified $gp140_{89.6}$ constructs wherein the variable loops were only partially deleted (Fig. 1). In the V2 loop-modified mutant (mV2), the residues 162 to 191 were replaced with GAG, leaving the RLISC sequence in V2 as it represents a part of the CTL epitope RLISCNTSV with the A2-binding motif (Korber et al., 1999). The V3 loop-modified construct (mV3) retained the N-terminal 23-aa fragment from cysteine 296 to arginine 320. This segment contained the GPGR sequence recognized by the broadly neutralizing 447-52D human mAb (Conley et al., 1994) and the RRLSIGP sequence, in the N-terminal side of the V3 loop, that shares antigenic feature recognized by human mAbs with intra- and interclade cross-reactivity (Gorny et al., 1997). The mV3 loop also contained several CTL epitopes with multiple HLA-binding motifs (Korber et al., 1999), and had the N-linked glycan at residue 303 eliminated by the NNT to NNA amino acid replacement. This N-linked glycan was shown to block access to the binding site for CD4 and modulated the chemokine receptor binding site of phenotypically diverse clade A and clade B isolates (Malenbaum et al., 2000).

The variable region deletions were introduced in the HIV-1 $gp140_{89.6}$ construct together or separately, and cloned in frame with the human tissue plasminogen activator (tPA) signal sequence for expression in the pNGVL-7 plasmid as DNA vaccines. The expressions of the WT and domain-modified gp140 proteins were analyzed by immunoprecipitation of transiently transfected 293 cells. The env glycoproteins were immunoprecipitated from cellular lysates of $[^{35}S]$methionine/cysteine-labeled transfectants with a mixture of sera from HIV-1-infected individuals and resolved by SDS-PAGE. Because the $HIV-1_{89.6}$ gp140 was originally

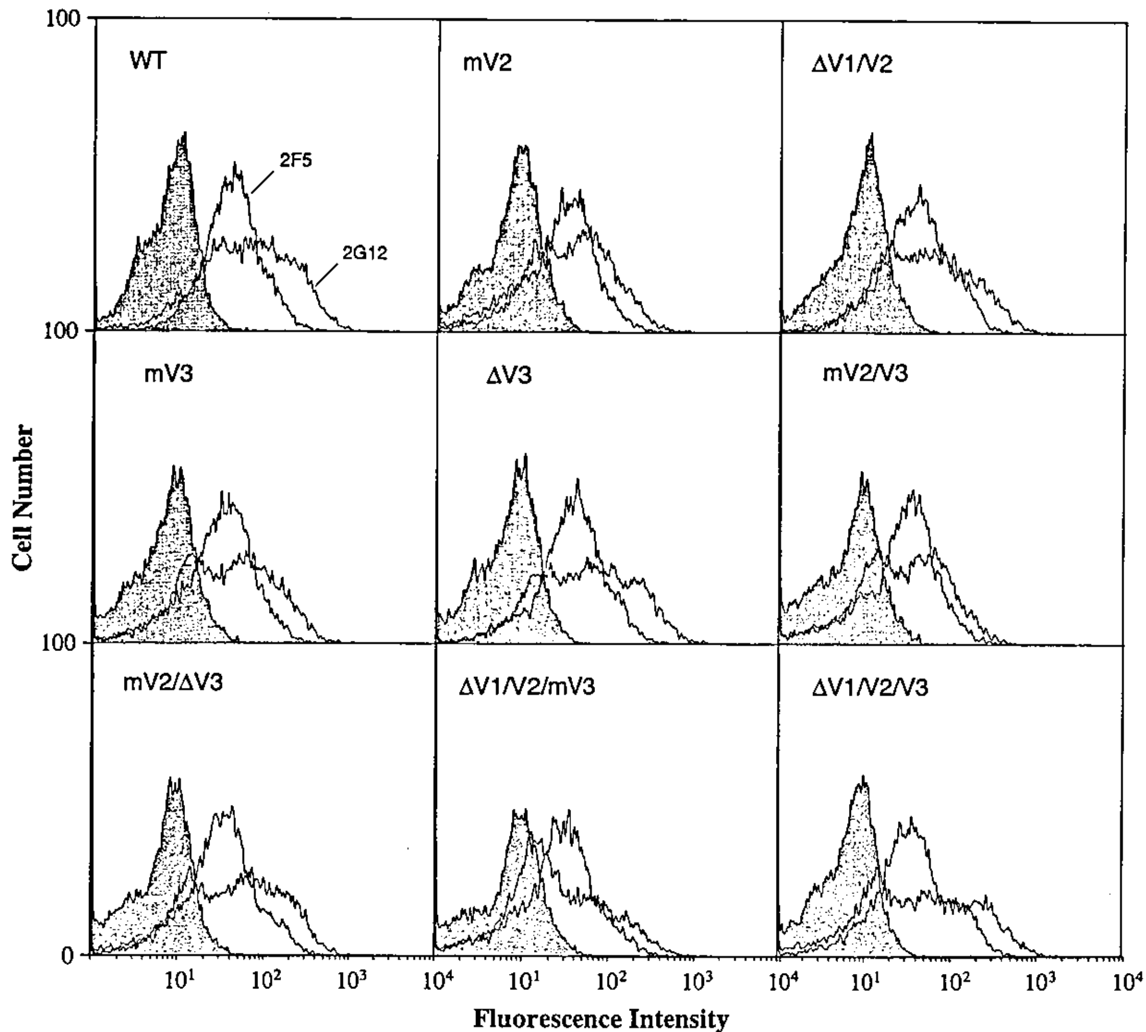

Fig. 3. Immunofluoresce staining and flow cytometry analysis of the full-length and variable domain-modified $HIV-1_{89.6}$ gp140 proteins expressed in 293T transfectants. The expression of the env glycoproteins was analyzed 48 h after transfection by intracellular staining with 2F5 and 2G12 human mAbs followed by the FITC-conjugated $F(ab')_2$ portion of goat anti-human Ig. Cells were analyzed by flow cytometry on FACScan. The light gray areas denote transfectants stained only with the secondary antibody. Continuous and dotted lines indicate transfectants stained with 2F5 and 2G12 mAbs, respectively.

generated as a gp120–gp41 cleavage site mutant wherein the furin cleavage motif was replaced with a hexameric Leu–Arg motif (Binley et al., 2000), the full-length and domain-modified env glycoproteins were immunoprecipitated as single bands (Fig. 2). The mutants could be distinguished based on differences in the molecular weight due to complete or partial deletions of the variable regions. Variations in the expression levels between the WT and the env mutants were less than 30%, suggesting that the expression of the gp140 mutants was not largely altered by the variable loop modifications. Furthermore, immunostaining of intracellularly expressed env mutants in transiently transfected 293 cells with broadly neutralizing antibodies revealed that the gp140 mutants retained native antigenic determinants similar to the unmodified env protein (Fig. 3). The broadly neutralizing mAbs 2G12 and 2F5 used for staining recognize a carbohydrate-dependent epitope located on the gp120 outer domain and the NEQELLSLWN epitope near the C-terminal end of the gp41 ectodomain, respectively (Parker et al., 2001; Trkola et al., 1996).

Breadth of cellular responses induced by immunization with the WT and domain-modified mutants of HIV-1$_{89.6}$ gp140 protein

In the first set of experiments, we compared the ability of domain-modified env vaccines to induce cross-reactive $CD8^+$ T cell responses. Groups of BALB/c mice (n = 5) were immunized intramuscularly (i.m.) with DNA vaccines expressing the complete or domain-modified gp140$_{89.6}$ glycoproteins. Three weeks after the last immunization, frequencies of IFN-γ-secreting splenocytes were analyzed by ELISPOT assay against P815 (H-2^d) target cells infected with recombinant vaccinia virus (rVV) expressing homologous (vBD3) or heterologous (vPE16 or vV1) env gene products. Cells infected with vSC8 virus expressing β-galactosidase served as negative control. This analysis permitted detection of env-specific $CD8^+$ T cells because the P815 mastocytoma cell line expresses only MHC class I molecules (Gherardi and Esteban, 1999).

As shown in Fig. 4, immunization with WT and domain-modified env mutants induced comparable frequencies of env-specific IFN-γ-secreting $CD8^+$ splenocytes against the homologous gp160 gene products. However, the profile of $CD8^+$ T cell responses directed to heterologous gp160 varied among mice immunized with different env vaccines. For example, $CD8^+$ T cells derived from mice immunized with the WT gp140$_{89.6}$ construct exhibited a rather restricted pattern of reactivity, in which the heterologous responses were at approximately 35% (vPE16) and 15% (vV1) of those directed to the homologous env gene products. Immunization with the mV2 or ΔV1/V2 mutant had no effect on the profile of cross-reactive IFN-γ responses in cultures stimulated with vPE16 or vV1-infected target cells. On the other hand, the ΔV3 and mV3 env-induced cellular immunity showed a broader pattern of reactivity responding to vPE16- and vV1-infected target cells with approximately 40% higher efficiencies than responses elicited by the WT env vaccine. Among the double deletants, the highest levels of cross-reactive responses were induced with the mV2/mV3 and ΔV1/V2/mV3 gp140$_{89.6}$ vaccines. In mice immunized with the mV2/mV3 gp140$_{89.6}$ mutant, the responses to vPE16- and vV1-infected cells represented approximately 75% and 60% of those directed to target cells expressing the homologous env gene products, respectively. They were also significantly higher than the heterologous responses in animals immunized with the WT gp140$_{89.6}$ vaccine (P = 0.003 and P = 0.0002, respectively). A similar profile of

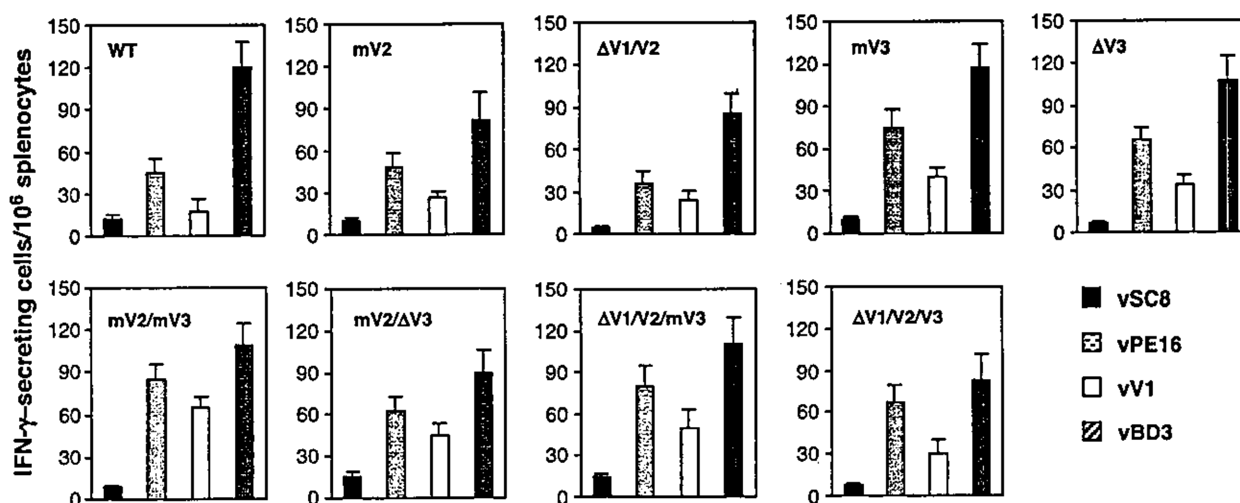

Fig. 4. Induction of cross-reactive cellular responses by DNA immunization with WT and variable domain-modified HIV-1$_{89.6}$ gp140 mutants in BALB/c mice. The numbers of IFN-γ-secreting $CD8^+$ T cells in splenocyte cultures established from mice immunized with the env vaccines were determined by ELISPOT assay with P815 cells infected with vPE16, vV1, and vBD3. Control cells were infected with vSC8 expressing β-galactosidase. Results are presented as the means ± SD of at least three independent experiments. The frequencies of IFN-γ-secreting cells were determined by regression analysis from a curve generated by plotting the number of spots versus the number of effector cells.

reactivity was detected in ΔV1/V2/mV3-immunized mice whose cross-reactive $CD8^+$ T cell activities directed to vPE16- and vV1-infected targets were 2- and 3-fold higher compared to those elicited by the unmodified vaccine ($P = 0.02$ and $P = 0.009$, respectively). The latter responses represented 70% and 45% of IFN-γ-secreting $CD8^+$ splenocytes directed to vBD3-infected cells, respectively.

Protection against challenge with rVV expressing homologous and heterologous env glycoproteins

The significance of WT and domain-modified env vaccine-induced cellular responses was further examined in protective studies against challenge with vPE16, vV1, and vBD3 as surrogates for HIV-1 (Belyakov et al., 1998). BALB/c mice immunized with the full-length or variable loop-modified mutants of the $gp140_{89.6}$-derived DNA constructs were challenged on day 28 by intraperitoneal (i.p.) injection with 5×10^6 PFU of vPE16, vV1, or vBD3 as described (Kiszka et al., 2002). To determine whether any protection induced by the vaccines was env-specific, additional groups of immunized and control mice were challenged with vSC8 virus expressing β-galactosidase. Because the vaccinia virus replicates most efficiently in ovaries (Belyakov et al., 1998), ovaries were removed 5 days after the challenge and tested for vaccinia titer on a monolayer of $HuTK^-$ 143B cell line.

As shown in Table 1, the vaccinia titers in ovaries of mice immunized with the full-length env or the domain-modified mutants and challenged with vBD3 were approximately 3 log_{10} lower than in vSC8-challenged animals. The protection levels against the homologous challenge with vBD3 were comparable in animals immunized with the unmodified or domain-modified vaccines. Consistent with the profile of $CD8^+$ T cell responses, immunization with the WT env gene products elicited approximately 10- and 100-fold lower protection levels against heterologous challenge with vPE16 and vV1, respectively. A similar level of protection was detected in mice immunized with the mV2 or ΔV1/V2 deletant. Animals immunized with the ΔV3 or mV3 mutant and challenged with the heterologous vPE16 and vV1 viruses had approximately 10-fold lower viral titers in ovaries than WT env-vaccinated mice. The highest level of protection against the heterologous challenge was detected in mV2/mV3- and ΔV1/V2/mV3-immunized mice. In these animals, resistance to heterologous challenge with vPE16 and vV1 was similar and approximately 50-fold higher compared with mice immunized with WT $gp140_{89.6}$ ($P < 0.01$). There was no significant difference between vSC8 titers in control mice and those immunized with the WT env or any of the domain-modified mutants, indicating that the protection elicited by the mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ mutants could not be mediated by nonspecific inflammatory responses.

Development of env-specific antibodies in mice immunized with the domain-modified $gp140_{89.6}$ vaccines

The full-length and domain-modified HIV-$1_{89.6}$ gp140-derived immunogens induced antibodies capable of binding to the homologous env glycoprotein expressed on the surface of vBD3-infected cells (Table 2). Although variations in the antibody titers were measured throughout the immunization schedule, no statistically significant differences were recorded among groups of mice vaccinated with different env constructs. The endpoint binding antibody titers in animals immunized with the unmodified or the modified env vaccines were in the range of 2×10^3 at the end of the vaccination schedule. These results suggest that, based on the assay used here to determine antibody titers, the domain-modified gp140 vaccines were as effective as the unmodified env in eliciting antibody responses even if

Table 1
Env vaccine-induced protection against challenge with rVV expressing homologous and heterologous gp160 of primary HIV-1 isolates

Envelope vaccine	Challenge[a]						
	vSC8	vBD3		vPE16		vV1	
	Virus titer[b] (log_{10}/ovaries)	Virus titer[b] (log_{10}/ovaries)	Protection[c] (Δlog_{10})	Virus titer[b] (log_{10}/ovaries)	Protection[c] (Δlog_{10})	Virus titer[b] (log_{10}/ovaries)	Protection[c] (Δlog_{10})
WT	9.1 ± 0.8	6.0 ± 0.4	3.1	7.4 ± 0.5	1.7	8.0 ± 0.6	1.1
mV2	9.2 ± 0.6	6.3 ± 0.6	2.9	7.2 ± 0.7	2.0	7.6 ± 0.5	1.6
ΔV1/V2	9.0 ± 0.8	6.2 ± 0.5	2.8	7.1 ± 0.7	1.9	7.7 ± 0.6	1.3
mV3	8.7 ± 0.8	5.6 ± 0.4	3.1	6.6 ± 0.4	2.1	6.8 ± 0.5	1.9
ΔV3	9.1 ± 0.5	6.1 ± 0.4	3.0	6.8 ± 0.2	2.3	7.0 ± 0.4	2.1
mV2/mV3	8.8 ± 0.8	5.3 ± 0.2	3.5	5.7 ± 0.3	3.1	5.9 ± 0.2	2.9
mV2/ΔV3	9.2 ± 0.6	5.6 ± 0.2	3.6	6.2 ± 0.3	3.0	6.4 ± 0.3	2.8
ΔV1/V2/mV3	8.8 ± 0.7	5.4 ± 0.3	3.4	5.9 ± 0.2	2.9	6.1 ± 0.4	2.7
ΔV1/V2/V3	8.6 ± 0.7	5.8 ± 0.2	2.8	6.3 ± 0.2	2.3	6.5 ± 0.4	2.1

[a] On day 28 after immunization with the WT or domain-modified $gp140_{89.6}$ DNA vaccines, BALB/c mice were challenged i.p. with 5×10^6 PFU of vPE16, vV1, vBD3, or vSC8. Five days later, mice were sacrificed, ovaries were removed, homogenized, sonicated, and assayed for vaccinia virus titers by plating serial 10-fold dilutions on human $HuTK^-$ 143B cells, staining with crystal violet, and counting plaques at each dilution.
[b] The rVV titers are presented as the mean log_{10} ± SD of PFU per ovaries of four mice per group.
[c] Protection is reduction of vBD3, vPE16, or vV1 titers (Δlog_{10} PFU) in ovaries compared with the value for the control group challenged with vSC8 virus.

Table 2
Env-specific antibody responses and neutralization activities induced by the domain-modified HIV-$1_{89.6}$ gp140 vaccines

Domain-modified HIV-$1_{89.6}$ gp140 vaccines	Antibody titer to the homologous envelope glycoprotein (endpoint titer ± SD)[a]	Specific neutralization (% inhibition ± SD)[b]	
		HIV-$1_{89.6}$	HIV-1_{SF162}
WT	2100 ± 461	36 ± 16	17 ± 8
mV2	2933 ± 562	70 ± 19	49 ± 15
ΔV1/V2	1600 ± 800	48 ± 44	28 ± 26
mV3	2000 ± 565	28 ± 11	ND
ΔV3	1867 ± 661	24 ± 9	26 ± 22
mV2/mV3	2400 ± 800	91 ± 19	86 ± 9
mV2/ΔV3	2240 ± 661	81 ± 10	67 ± 11
ΔV1/V2/mV3	1800 ± 546	95 ± 13	88 ± 14
ΔV1/V2/V3	3133 ± 1194	67 ± 34	60 ± 16

[a] The env-specific antibody responses to native viral env glycoprotein expressed in vBD3-infected 293 cells were analyzed in sera of mice immunized with the WT or the variable domain-modified gp$140_{89.6}$ vaccine using the ELISA assay. Sample dilutions were considered positive if the optical density recorded for that dilution was at least 2-fold higher than the optical density recorded for a naive sample at the same dilution (Staats et al., 1996). Results are from three independent experiments.

[b] The neutralizing antibody responses induced by the WT and variable domain-modified env vaccines were examined using the homologous HIV-$1_{89.6}$ isolate and CEMx174 cells, or the heterologous HIV-1_{SF162} virus and PHA-stimulated PBMC as target cells. Neutralizing activity was evaluated at 1:20 dilution, taking into consideration the nonspecific neutralization recorded with sera collected from animals vaccinated with the DNA vector alone. Results represent means ± SD from three independent experiments.

the former immunogens lack up to 86 amino acids from the V1/V2 and 31 amino acids from the V3 region.

Next, we investigated the ability of unmodified and domain-modified HIV-$1_{89.6}$ gp140 vaccines to generate neutralizing antibodies to the functional env glycoprotein on the HIV-1 virus. In the initial set of experiments, the neutralizing assay was performed using the homologous HIV-$1_{89.6}$ isolate and the susceptible CEMx174 cells as a target. Analysis of p24-antigen levels in culture supernatants of CEMx174 cells infected with HIV-$1_{89.6}$ in the presence or absence of the immune sera revealed that immunization with the unmodified gp$140_{89.6}$ vaccine was not very effective in inducing neutralizing antibodies (Table 2). The neutralizing activity of the WT gp$140_{89.6}$-induced antibody responses measured at a serum dilution of 1:20 revealed 36 ± 16% inhibition of infection. A similar profile of neutralizing activity was observed with sera from animals immunized with the single mV3 or ΔV3 deletant. A trend toward higher neutralization levels was recorded in sera collected from animals vaccinated with the mV2 mutant, and the neutralizing activities of the serum antibodies were further augmented in mV2/mV3- and ΔV1/V2mV3-immunized mice ($P < 0.02$).

To examine whether domain-modified gp140 vaccines were effective in eliciting cross-reactive neutralizing antibody responses, we tested the HIV-1_{SF162} isolate whose susceptibility to neutralization by sera collected from animals immunized with the ΔV2 gp140_{SF162} mutant had previously been examined (Barnett et al., 2001; Srivastava et al., 2003). At a 1:20 serum dilution, the sensitivity of the HIV-1_{SF162} virus to neutralization by sera obtained from mice immunized with the unmodified gp$140_{89.6}$ vaccine was at least 2-fold lower than that detected using the homologous HIV-$1_{89.6}$ virus (17 ± 8% inhibition of infection, Table 2). At this dilution, a mixture of sera from HIV-1-infected individuals, used as a positive control in the assay, inhibited the infection by >95%. Removal of the entire V1/V2 region or the V3 loop did not augment the neutralizing responses. The antibody-neutralizing potency was 2–3-fold enhanced in sera derived from animals immunized with the mV2, mV2/ΔV3, or ΔV1/V2/V3 mutant ($P < 0.03$). The highest, approximately 5-fold, increases in susceptibility of the HIV-1_{SF162} virus to neutralization were elicited by mV2/mV3 and ΔV1/V2/mV3 gp$140_{89.6}$ vaccines ($P = 0.0006$ and $P = 0.002$, respectively), indicating that specific modifications of the variable loops might be capable of redirecting antibody responses toward conserved epitopes on the env glycoprotein.

Env-specific antibody avidity

Previous studies with attenuated SIV vaccines in rhesus macaques demonstrated that the establishment of long-term protective immunity was associated with maturation of antibody responses (Cole et al., 1997; Montelaro et al., 1998). These results prompted us to evaluate the nature of anamnestic responses to rVV challenge in mice immunized with domain-modified mutants that elicited the highest cross-reactive neutralizing antibody responses after DNA priming. Because variations in priming specificity by the variant env immunogens could be reflected in differences in the protective immune responses to the viral challenge, we measured the association between env-specific antibody avidity and neutralizing activities in sera of mice immunized with the WT, mV2/mV3, or ΔV1/V2/mV3 vaccine after challenge with vBD3 virus expressing homologous env gene products. Mice immunized with the env-specific DNA vaccines and challenged with vSC8 virus expressing β-galactoside served as controls. The antibody avidity was determined by measuring the relative stability of the native env antigen–antibody complexes to an 8 M urea wash in concanavalin A (ConA) ELISA assay as described (Cole et al., 1997).

Table 3
Env-specific antibody responses after rVV challenge in mice immunized with the WT, mV2/mV3, or ΔV1/V2/mV3 $gp140_{89.6}$ vaccine

Envelope vaccine	Challenge[a]	Avidity index[b] (%)	Specific neutralization[c]			
			HIV-$1_{89.6}$		HIV-1_{SF162}	
			50%	90%	50%	90%
WT	ND	25	–	–	–	–
WT	vBD3	37	+	–	–	–
mV2/mV3	vSC8	43	+	+	+	–
mV2/mV3	vBD3	56	+	+	+	+
ΔV1/V2/mV3	vSC8	34	+	+	+	–
ΔV1/V2/mV3	vBD3	49	+	+	+	–

[a] BALB/c mice were immunized with plasmid DNA expressing the WT, mV2/mV3, or ΔV1/V2/mV3 mutant of $gp140_{89.6}$ and challenged i.p. with 5×10^6 PFU of vBD3 or vSC8 3 weeks after DNA vaccine. The env-specific antibody responses to the native env glycoprotein were analyzed in the ConA ELISA with or without an 8 M urea wash. ND, not determined.
[b] The avidity index was determined by measuring the relative stability of the native viral env antigen–antibody complexes to an 8-M urea wash in ConA ELISA, and values were calculated by using the equation (urea-washed wells/PBS-washed wells) × 100. The avidity index values $x \geq 50\%$, $50\% > x > 30\%$, and $x \leq 30\%$ have been defined high, intermediate, and low avidity, respectively. Results represent average values from three independent experiments.
[c] Neutralizing activity was calculated at 1:20 dilution, taking into consideration the nonspecific neutralization recorded with sera collected from animals vaccinated with the DNA vector alone. –, 50% specific neutralization was not recorded; +, 50% or 90% specific neutralization was recorded. Results are from three independent neutralization experiments.

The results summarized in Table 3 demonstrated that antibody avidity values in mice immunized with the env-specific DNA constructs showed approximately 30% increases after challenge with vBD3 vaccinia virus compared to responses in vSC8-challenged mice. In the latter group of mice, the antibody avidity index varied between 25% and 43% with the lowest and highest values detected in animals immunized with the WT and mV2/mV3 vaccines, respectively. The antibody responses progressed from low to intermediate or to high avidity in vBD3-challenged mice at 11 days postinfection. The increases in antibody avidity after vBD3 challenge were also associated with small enhancement of neutralization activities against the HIV-$1_{89.6}$ and HIV-1_{SF162} isolates in animals immunized with the WT and mV2/mV3 vaccines, respectively (Table 3). However, this association was not observed in ΔV1/V2/mV3-vaccinated mice whose sera displayed similar neutralizing activities after challenge with vBD3 or vSC8 virus.

Discussion

Potent neutralizing antibody responses that prevent primary infection or reduce the viral load at the site of HIV-1 entry remain the ultimate goal for prophylactic AIDS vaccines. However, the same vaccines should also induce cross-protective cellular responses capable of increasing the level of immune control and inhibit spreading infection in HIV-1-positive individuals. Thus, efforts to improve the cross-reactivity of env-specific vaccine might require the creation of a polyvalent vaccine composed of env glycoproteins from multiple primary isolates or a vaccine in which the structure of the env is modified to elicit cellular and humoral responses to conserved epitopes that are otherwise poorly immunogenic. Previously, we have demonstrated that immunization of the HLA-A2/K^b transgenic mice with the ΔV3 env mutant induced qualitative changes at the level of env peptide-specific $CD8^+$ T cell responses that were associated with an increased recognition of target cells expressing heterologous env gene products (Kiszka et al., 2002). In these studies, we showed that the ΔV3 env vaccine was not effective in eliciting neutralizing antibody responses. These findings are consistent with previous studies which demonstrated that immunization strategies using variable loop-deleted env constructs failed to generate cross-reactive neutralizing antibodies (Kim et al., 2003; Lu et al., 1998). The experiments presented here also indicated that partial deletions within the V3 loop in combination with the V2-modified or V1/V2-deleted region were more effective in eliciting broader levels of cross-reactive $CD8^+$ T cell and neutralizing antibody responses than the single V3 loop-deleted mutant or the WT $gp140_{89.6}$ vaccine.

Using the challenge experiments with rVV-expressing homologous or heterologous env gene products, we demonstrated that modifications within the V3 domain that increased the level of cross-reactive cellular responses in vitro also elicited higher protection levels against challenge with rVV expressing heterologous gp160 in the immunized mice. Although immunization with the mV2 or the ΔV1/V2 mutant affected neither the level of cross-reactive $CD8^+$ T cell responses nor the protection against the heterologous rVV challenge, adding mV3 into these constructs significantly increased their immunogenic efficacies. Our findings in the murine model together with the data available from the HIV Molecular Database, which demonstrated the presence of only three CTL epitopes with the HLA-A2- and Cw8-binding motifs in the V1–V2 region (Korber et al., 1999), indicate that this region might not be targeted by $CD8^+$ T cell responses. However, specific deletions within the V1–V2 region could result in structural changes that indirectly affected the immunogenicity of the mV2/mV3 and ΔV1/V2/mV3 mutants at the level of antigen processing or presentation. Because the quantity of the epitope–MHC complex on the cell surface may regulate CTL responses (Yewdell and Bennink, 1992), it is possible that increases in cross-reactive cellular responses induced by mV2/mV3 and ΔV1/V2/mV3 vaccines could be associated with a higher pool of conserved epitopes available for presentation in cells expressing the double mutants than the WT env gene products. This can be due to the appearance of new CTL epitopes that are more frequently present on heterologous envelopes than are those presented by unmodified $gp140_{89.6}$ vaccine. Alternatively, the modified gp140 immunogen may increase the relative concentration of conserved epitopes by reducing a competition with less desirable epitopes during

antigen processing and presentation. Although further studies are required to determine the mechanism that contributes to the increased immunogenicity of the conserved epitopes in mV2/mV3 and ΔV1/V2/mV3 vaccines, results presented here suggest that one possible approach to broaden the repertoire of env-specific cellular responses is by modifications of the variable sequences within the env glycoprotein.

Consistent with the notion that both cellular and humoral responses will be required for an effective AIDS vaccine, understanding of the immunogenicity of env glycoproteins from several primary HIV-1 isolates will be necessary for the development of broadly protective immune responses. Our results with the gp140 mutants of the HIV-$1_{89.6}$ isolate demonstrated that despite different constrains for the induction of cross-reactive cellular and humoral responses, structural modifications of the env glycoprotein can be introduced to redirect both types of immune responses toward conserved epitopes that can be recognized on more than one strain of HIV-1. It is also noteworthy that the variable loop modifications had a more pronounced effect on induction of cross-reactive cellular than neutralizing antibody responses. This could be due to the inherent difficulties of generating cross-neutralizing antibodies related to the highly variable structure and glycosylation of gp160 as well as the poor exposure and immunogenicity of the receptor-biding sites on the env molecules (Kim et al., 2003; Kwong et al., 1998; Wyatt et al., 1998). In addition, the single immunization approach based on a DNA vaccine that elicited low- to intermediate-avidity antibodies might have reduced efficacy of the domain-modified vaccines. The use of mouse sera in our assay and that of Kim et al. (2003), which suffers from low neutralizing activities against primary isolates, could also contribute to the weak cross-neutralizing responses induced by the variable domain-deleted mutants. However, regardless of the differences in the experimental models and HIV-1 isolates, in both systems complete removal of the variable regions was not effective in inducing cross-reactive cellular and antibody responses, suggesting that partial deletions within these regions may facilitate more effective approaches for boosting the breadth of protective immunity.

In summary, we demonstrated that specific modifications of the variable domains of the env glycoprotein could be introduced with favorable antigenic properties. Additional studies that would examine in detail the existence and epitope-specificity of the cross-protective cellular and antibody responses elicited by mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ mutants may help to establish to what extent the specific deletions differentially alter the structure of certain env regions that are already immunogenic or render certain regions immunogenic. Similarly, further studies with a panel of heterologous primary isolates are also required to determine susceptibility of these HIV-1 isolates to neutralization by antibodies elicited by mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ vaccines. These results will help to establish whether the cross-reactive epitopes present on those $gp140_{89.6}$ mutants are absent from the heterologous isolates that are resistant to neutralization or whether they are more efficiently masked on these particular isolates than on the viruses susceptible to neutralization. As such, this model system will provide a comprehensive means for selecting the most effective envelope vaccines for future vaccine trials.

Materials and methods

Vector construction

The HIV-$1_{89.6}$ gp140 plasmid (accession number U39362) was used for the construction of env mutants with complete or partially deleted V1/V2, V2, and V3 domains using a two-step PCR protocol using as described (Kiszka et al., 2002). For each mutant, two fragments of the env gene were synthesized with an overlap. One fragment, with a specific deletion within the variable domains, was synthesized with primer A containing the *KpnI* cleavage site (5′-AAT*GGTACC* TGTGTGGAGAGAAGCAACCAC-3′) together with anti-sense primer B that contains overlapping sequences with primer C that was used for synthesis of the second fragment (Table 4). The second fragment was synthesized with sense primer C in conjunction with the anti-sense primer D (5′-CCT*CCTGAGG* ATTGATTAAAGGCTATTGTT-3′) containing the *Bsu36I* restriction site. The nucleotide sequences for the restriction enzyme cleavage sites are underlined. These two fragments were then used together in a second reaction along with primers A and D to generate the indicated domain-modified env fragments. The final product was digested with *KpnI* and *Bsu36I* and exchanged with the corresponding segment of WT $gp140_{89.6}$ fused in frame with the tPA signal sequence under the control of human cytomegalovirus immediate early promoter in the pNGVL-7 plasmid (University of Michigan, Ann Arbor, MI) as described (Kiszka et al., 2002). Rev was not included in the gp140-expressing plasmids as expression of env with the tPA signal sequence in pNGVL-7 allows env expression without Rev (Lu et al., 1998). The integrity of the plasmid was verified by restriction enzyme cleavage and DNA sequence analysis. After DNA sequence verification, the expression of the env gene products was confirmed by immunoprecipitation from radiolabeled 293T cells transfected with the respective env-pNGVL-7 plasmid.

Transfection, labeling, and immunoprecipitation

Transient transfection of 293T cells was performed by calcium phosphate precipitation with 50 μg of plasmid DNA encoding the respective env protein. For radioimmunoprecipitation analysis, [^{35}S]methionine/cysteine (400 μCi per plate; DuPont-New England Nuclear, Boston, MA)

Table 4
Nucleotide sequence of partially overlapping primers B and C used for generation of the variable domain-modified HIV-$1_{89.6}$ gp140 vaccines

V1/V2 and V3-modified HIV-$1_{89.6}$ gp140[a]	Modified V1 and V2 domains		Modified V3 domain	
	B	C	B	C
WT	N/A	N/A	N/A	N/A
mV2	5′-TAACCTACCGGCACC GATATAGAAAGAGCA-3′	5′-GGTGCCGTTAGGTTA ATAAGTTGTAACACC-3′	N/A	N/A
ΔV1/V2	5′-ACAGGCACCGGCACC TACACATGGCTTTAG-3′	5′-GGTGCCGGTGCCT GTCCAAAGGTATCC-3′	N/A	N/A
mV3	N/A	N/A	5′-TCTCCCTGGTCCTATA GATAACCTTCTTCTTGC ATTGTT-3′	5′-ATAGGACCAGGGAGA GCATTTTATGCAAGATGT AACATTAGTAGAGCA-3′
ΔV3	N/A	N/A	5′-ACAATGGCCGGCACC TGTACAATTAATTAC-3′	5′-GGTGCCGGCCATTGT AACATTAGTAGAGCA-3′
mV2/mV3	5′-TAACCTACCGGCACC GATATAGAAAGAGCA-3′	5′-GGTGCCGGTAGGTTA ATAAGTTGTAACACC-3′	5′-TCTCCCTGGTCCTATA GATAACCTTCTTCTTGC ATTGTT-3′	5′-ATAGGACCAGGGAGA GCATTTTATGCAAGATGT AACATTAGTAGAGCA-3′
mV2/ΔV3	5′-TAACCTACCGGCACC GATATAGAAAGAGCA-3′	5′-GGTGCCGTTAGGTTA ATAAGTTGTAACACC-3′	5′-ACAATGGCCGGCACC TGTACAATTAATTAC-3′	5′-GGTGCCGGCCATTGT AACATTAGTAGAGCA-3′
ΔV1/V2/mV3	5′-ACAGGCACCGGCACC TACACATGGCTTTAG-3′	5′-GGTGCCGGTGCCT GTCCAAAGGTATCC-3′	5′-TCTCCCTGGTCCTATA GATAACCTTCTTCTTGC ATTGTT-3′	5′-ATAGGACCAGGGAGA GCATTTTATGCAAGATGT AACATTAGTAGAGCA-3′
ΔV1/V2/V3	5′-ACAGGCACCGGCACC TACACATGGCTTTAG-3′	5′-GGTGCCGGTGCCT GTCCAAAGGTATCC-3′	5′-ACAATGGCCGGCACC TGTACAATTAATTAC-3′	5′-GGTGCCGGCCATTGT AACATTAGTAGAGCA-3′

[a] The HIV-$1_{89.6}$ gp140 vaccines with complete or partially deleted V1, V2, and V3 regions were constructed using a two-step PCR method and partially overlapping primers B and C as described in Materials and methods section.

was added on the second day of transfection for an additional 12-h period. At the end of the incubation period, the cells were pelleted and lysed using Nonidet P-40 buffer (0.5% Nonidet P-40, 0.5 M NaCl, and 10 mM Tris–HCl, [pH 7.5]). Equal amounts of radiolabeled cell lysates, based on the protein concentration, were immunoprecipitated with a mixture of sera from HIV-1-infected individuals followed by polyclonal rabbit anti-human Ig (ICN Biomedicals, Inc. Costa Mesa, CA) and protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ). Immunoprecipitates were separated by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE). The gels were fluorographed to visualize [^{35}S]methionine/cysteine-labeled proteins, and the intensity of individual bands was quantified with a LabImage Program (Copyright 1999–2001, Kapelan GmbH).

Flow cytometry analysis of the env glycoprotein expression

For intracellular staining of transfectants expressing the WT and domain-modified env glycoproteins, 293T cells were harvested 48 h after transfection, washed twice with PBS containing 2% FCS and 0.1% sodium azide, and stained with env-specific human mAbs 2F5 and 2G12 (Buchacher et al., 1994) (NIH AIDS Research and Reference Reagent Program, Rockville, MD). After washing, the cells were incubated with a 1:40 dilution of FITC-conjugated F(ab′)$_2$ of goat anti-human Ig (ICN Biomedicals, Inc.) and analyzed by flow cytometry (FACScan). The intracellular staining was carried out using BD Cytofix/Cytoperm kit (Pharmingen, San Diego, CA) according to the manufacturer's protocol.

Recombinant vaccinia viruses

The rVV's expressing the full-length gp160 of HIV-1_{IIIB} (vPE16) (Earl et al., 1991) or β-galactosidase (vSC8) (Earl et al., 1991) were provided by Dr. B. Moss (Laboratories of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The rVV's expressing the env glycoprotein of the primary VI-06 (vV1) and 89.6 (vBD3) HIV-1 isolates were provided by Drs. K. Luzuriaga (University of Massachusetts Medical Center, Worcester, MA) and R. Collman (University of Pennsylvania, Philadelphia, PA), respectively. VI-06 is a "transmitted" primary isolate derived from an infant with perinatal HIV-1 infection (Pikora et al., 1997) that exhibits 8–10% sequence variation compared with the env glycoprotein of the HIV-1_{IIIB} isolate. The second env glycoprotein derives from a dual-tropic HIV-$1_{89.6}$ primary isolate which uses CXCR4 and CCR5 as well as many other chemokine co-receptors for entry (Doranz et al., 1996). The HIV-$1_{89.6}$ isolate exhibits 82% identity with HIV-1_{IIIB} gp160 sequence with the highest (92%) and the lowest (64%) sequence homology in the C1 region and the V3 loop, respectively.

Mice and immunization

Six-week-old BALB/c (H-2^d) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free microisolator environment. Mice were injected i.m. in the quadriceps with 100 μg of plasmid encoding WT or domain-modified gp140 mutants on days 0, 14, 28, and 42. Control mice were immunized with a sham plasmid. To augment the level of envelope-specific immune

responses, plasmid DNA encoding IL-2/Ig (10 μg) was administered i.m. 2 days after immunization with the env-specific vaccine as described (Barouch et al., 1998; Wierzbicki et al., 2002).

ELISPOT assay

The number of IFN-γ-secreting T cells specific for the env gene products in splenocytes of the immunized mice was determined by the ELISPOT method 3 weeks after the last immunization as described (Gherardi and Esteban, 1999). Briefly, 96-well nitrocellulose plates (Millipore multiscreen-MAIP; Millipore, Bedford, MA) were coated with 15 μg/ml of rat anti-mouse mAb directed to IFN-γ (MAB785, R and D Systems Inc., Minneapolis, MN) in 0.05 M carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4 °C, the wells were washed with PBS containing 0.05% Tween 20 and blocked for 1 h with RPMI 1640 medium containing 10% FCS. For the analysis of frequencies of IFN-γ-secreting cells specific for the env gene products, splenocytes were combined with target cells infected with vPE16, vBD3, vV1, or vSC8 at a ratio of 3:1 and placed in 2-fold dilutions into the antibody-coated wells. For each dilution duplicate samples were used. P815 cells were used as antigen-presenting cells (Gherardi and Esteban, 1999). After 24 h of incubation at 37 °C, the plates were washed six times with PBS containing 0.05% Tween 20 and incubated for 2 h with 50 μl of 1 μg/ml of biotinylated mAb directed to mouse IFN-γ (MAB485, R&D Systems Inc.). The plates were washed and incubated for 1 h with 50 μl of 1:1000 diluted streptavidin-conjugated alkaline phosphatase (SA-5100, Vector Laboratories, Burlingame, CA). After a final wash with PBS, spots were developed with an alkaline phosphatase BCIP/NBT (5-bromo-4-chloro-3-indolyl-1-phosphate/nitroblue tetrazolium) substrate (SK-5400, Vector Laboratories) and counted under a stereomicroscope. The frequencies of IFN-γ-secreting cells were determined by regression analysis from a curve generated by plotting the number of spots versus the number of effector cells.

Protection against challenge with rVV expressing gp160 proteins of HIV-1 isolates

On day 28 after the last immunization with the env-specific vaccines, mice were challenged i.p. with 5 × 10^6 PFU of vPE16, vBD3, vV1, or vSC8. Five days later, mice were sacrificed, ovaries removed, homogenized, sonicated, and assayed for vaccinia virus titer by plating serial 10-fold dilutions on human $HuTK^-$ 143B indicator cells, staining with crystal violet and counting plaques at each dilution as described (Kiszka et al., 2002).

Measurement of HIV-1 env-specific antibody endpoint titers

Serum samples from control or env-vaccine immunized mice were analyzed for their reactivity to native viral env glycoprotein on vBD3-infected 293T cells in the ELISA assay. Briefly, 293T cells (4 × 10^4 cells/well) cultured on 96-well plates were infected with vBD3 at a multiplicity of infection of 10. After 12 h of infection, cells were fixed by 10-min treatment with 3.7% paraformaldehyde in PBS. Following a wash with PBS containing 0.05% Tween 20 (PBS/Tween 20), the wells were blocked for 2 h with a solution containing 2% BSA (Sigma) and 0.05% Tween 20 in PBS. Sera were prepared from murine blood samples, serially diluted in PBS/Tween 20, and added to the wells. For each dilution, duplicate wells were used. After 1-h incubation at room temperature, the plates were washed three times and incubated with a 1:500 dilution of a peroxidase-conjugated goat anti-mouse Ig (IgG, IgM, and IgA; Sigma) in PBS/Tween 20. After washing, the reaction was developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma), stopped with 0.4 N sulfuric acid, and analyzed at 450 nm with an ELISA plate reader (Dynatech MRX, Chantilly, VA). Sample dilutions were considered positive if the optical density recorded for that dilution was at least twofold higher than the optical density recorded for a naive sample at the same dilution (Staats et al., 1996).

Neutralizing antibody responses induced by the unmodified and variable domain-modified env vaccines

The ability of env vaccines to generate neutralizing antibodies was tested to determine which of these env mutants could elicit a response to the functional env glycoprotein on the homologous and heterologous HIV-1 viruses. The assay was performed using the homologous HIV-$1_{89.6}$ isolate and CEMx174 cells obtained from Dr. R. Collman (University of Pennsylvania) or the heterologous HIV-1_{SF162} virus and PHA-activated human peripheral blood mononuclear cells (PBMC) as targets (Wierzbicki et al., 2002). The HIV-$1_{89.6}$ isolate exhibits 82% identity with HIV-1_{SF162} gp160 sequence with the highest (90%) and the lowest (62%) sequence homology in the C1 region and the V1 loop, respectively.

The HIV-$1_{89.6}$ and HIV-1_{SF162} isolates were grown and titrated in CEMx174 cells and in PHA-activated human PBMC, respectively. The stocks were aliquoted and kept at −80 °C until further use. For antibody-mediated neutralization, the virus stock diluted to one hundred 50% tissue culture infectious doses ($TCID_{50}$) in 50 μl of complete RPMI medium was preincubated with an equal volume of heat-inactivated (35 min at 56 °C) sera for 1 h at 37 °C in 96-well tissue culture plates. For each sample, triplicate wells were used. To account for the relatively high background and variability frequently observed with mouse antisera, samples were collected from control animals vaccinated with the sham vector and served as controls for nonspecific neutralization. To each well, 0.1 ml of medium containing 10^5 target cells was added. Following 4-h incubation at 37 °C, half of the

volume of each well was replaced with fresh, complete RPMI medium. Following centrifugation of the plate (5 min at 1500 rpm), half of the volume of each well was again replaced with fresh medium. This procedure was repeated twice. The p24 antigen concentration in each well was evaluated 6 days after infection by a p24-antigen-capture assay (NEN Life Science Products, Inc., Boston, MA) according to the manufacturer's procedures. Each assay plate contained uninfected and infected cell controls and a serum from an individual with AIDS as a positive control (Kmieciak et al., 1998). Because the infection was reduced in the presence of control sera, we calculated the difference between the percent of inhibition recorded with the immune sera minus that recorded with control sera. The 1:20 serum dilution indicated in the text refers to that present during the entire period of virus-cell incubation.

Measurement of HIV-1 env-specific antibody avidity

The avidity index values of serum antibodies to the native viral env were determined by measuring the resistance of antibody–env glycoprotein complexes to an 8 M urea wash in ConA ELISA (Cole et al., 1997). For avidity assays, the ConA-anchored native viral env glycoprotein substrate was purified from cell culture supernatants of vBD3-infected 293T cells by *Galanthus nivalis* lectin affinity column (Vector Laboratories) as described (Trkola et al., 1996). For the ConA ELISA assay, all test sera were diluted in 5% nonfat dry milk in PBS (BLOTTO) and plated in two sets of duplicate wells. After the serum incubation, one set of wells was treated in parallel for 5 min with either PBS or a solution of 8 M urea in PBS. Following this treatment, the wells were thoroughly washed with PBS and incubated with a 1:500 dilution of a peroxidase-conjugated goat anti-mouse Ig in PBS. After washing, the reaction was developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma), stopped with 0.4 N sulfuric acid, and analyzed at 450 nm with an ELISA plate reader. The avidity index was then calculated from the ratio of the absorbance value obtained with urea treatment to that observed with PBS treatment multiplied by 100. Antibodies with avidity index values of $\leq$30% were designated low avidity, those with index values between 30% and 50% were designated intermediate avidity, and those with values $\geq$50% were designated high avidity. While measurements of antibody avidity were performed at the dilution producing an OD at 450 nm of about 1.0 in the endpoint titer ConA ELISA procedure, experiments using several different dilutions within this linear range were performed to ensure that the variation in actual values was within 10%.

Data analysis

Amino acid sequences of gp160 proteins of primary HIV-1 isolates were obtained through accession numbers available from the HIV Sequence Database (http://hiv-web.lanl.gov). The percentage of amino acid sequence identity within immunogenic regions of gp160 proteins of primary HIV-1 isolates was determined by the BLAST 2 program (http://www.ncbi.nlm.nih.gov/BLAST). The numbering of amino acids was based on the HxBc2 sequence available from the HIV Sequence Database (http://hiv-web.lanl.gov) using the HIV/SIV Sequence Locator Tool.

The significance of differences in the number of IFN-γ-secreting $CD8^+$ cells specific for the env gene products and the percentage of inhibition of HIV-1_{SF162} infection induced by the full-length and domain-modified gp140 vaccines was determined by the unpaired Student's *t* test using JMP software (SAS Institute Inc., Cary, NC). Mixed model analysis of variance (Winer, 1971) was used to compare mean values of the rVV titers between control mice and those immunized with the unmodified and domain-modified vaccines after challenge with vPE16, vBD3, vV1, or vSC8.

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References

Barnett, S.W., Lu, S., Srivastava, I., Cherpelis, S., Gettie, A., Blanchard, J., Wang, S., Mboudjeka, I., Leung, L., Lian, Y., Fong, A., Buckner, C., Ly, A., Hilt, S., Ulmer, J., Wild, C.T., Mascola, J.R., Stamatatos, L., 2001. The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. J. Virol. 75 (12), 5526–5540.

Barouch, D.H., Santra, S., Steenbeke, T.D., Zheng, X.X., Perry, H.C., Davies, M.E., Freed, D.C., Craiu, A., Strom, T.B., Shiver, J.W., Letvin, N.L., 1998. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. J. Immunol. 161 (4), 1875–1882.

Basmaciogullari, S., Babcock, G.J., Van Ryk, D., Wojtowicz, W., Sodroski, J., 2002. Identification of conserved and variable structures in the human immunodeficiency virus gp120 glycoprotein of importance for CXCR4 binding. J. Virol. 76 (21), 10791–10800.

Belyakov, I.M., Derby, M.A., Ahlers, J.D., Kelsall, B.L., Earl, P., Moss, B., Strober, W., Berzofsky, J.A., 1998. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. Proc. Natl. Acad. Sci. U.S.A. 95 (4), 1709–1714.

Binley, J.M., Sanders, R.W., Clas, B., Schuelke, N., Master, A., Guo, Y., Kajumo, F., Anselma, D.J., Maddon, P.J., Olson, W.C., Moore, J.P., 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. J. Virol. 74 (2), 627–643.

Bogers, W.M., Niphuis, H., ten Haaft, P., Laman, J.D., Koornstra, W., Heeney, J.L., 1995. Protection from HIV-1 envelope-bearing chimeric simian immunodeficiency virus (SHIV) in rhesus macaques infected with attenuated SIV: consequences of challenge. AIDS 9 (12), F13–F18.

Buchacher, A., Predl, R., Strutzenberger, K., Steinfellner, W., Trkola, A., Purtscher, M., Gruber, G., Tauer, C., Steindl, F., Jungbauer, A., et al., 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein–Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res. Hum. Retroviruses 10 (4), 359–369.

Cao, J., Sullivan, N., Desjardin, E., Parolin, C., Robinson, J., Wyatt, R., Sodroski, J., 1997. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. J. Virol. 71 (12), 9808–9812.

Chakrabarti, B.K., Kong, W.P., Wu, B.Y., Yang, Z.Y., Friborg, J., Ling, X., King, S.R., Montefiori, D.C., Nabel, G.J., 2002. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. J. Virol. 76 (11), 5357–5368.

Cho, M.W., Kim, Y.B., Lee, M.K., Gupta, K.C., Ross, W., Plishka, R., Buckler-White, A., Igarashi, T., Theodore, T., Byrum, R., Kemp, C., Montefiori, D.C., Martin, M.A., 2001. Polyvalent envelope glycoprotein vaccine elicits a broader neutralizing antibody response but is unable to provide sterilizing protection against heterologous Simian/human immunodeficiency virus infection in pigtailed macaques. J. Virol. 75 (5), 2224–2234.

Cole, K.S., Rowles, J.L., Jagerski, B.A., Murphey-Corb, M., Unangst, T., Clements, J.E., Robinson, J., Wyand, M.S., Desrosiers, R.C., Montelaro, R.C., 1997. Evolution of envelope-specific antibody responses in monkeys experimentally infected or immunized with simian immunodeficiency virus and its association with the development of protective immunity. J. Virol. 71 (7), 5069–5079.

Conley, A.J., Gorny, M.K., Kessler II, J.A., Boots, L.J., Ossorio-Castro, M., Koenig, S., Lineberger, D.W., Emini, E.A., Williams, C., Zolla-Pazner, S., 1994. Neutralization of primary human immunodeficiency virus type 1 isolates by the broadly reactive anti-V3 monoclonal antibody, 447-52D. J. Virol. 68 (11), 6994–7000.

Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G., Doms, R.W., 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 85 (7), 1149–1158.

Dunn, C.S., Hurtrel, B., Beyer, C., Gloeckler, L., Ledger, T.N., Moog, C., Kieny, M.P., Mehtali, M., Schmitt, D., Gut, J.P., Kirn, A., Aubertin, A.M., 1997. Protection of SIVmac-infected macaque monkeys against superinfection by a simian immunodeficiency virus expressing envelope glycoproteins of HIV type 1. AIDS Res. Hum. Retroviruses 13 (11), 913–922.

Earl, P.L., Koenig, S., Moss, B., 1991. Biological and immunological properties of human immunodeficiency virus type 1 envelope glycoprotein: analysis of proteins with truncations and deletions expressed by recombinant vaccinia viruses. J. Virol. 65 (1), 31–41.

Gherardi, M.M., Esteban, M., 1999. Mucosal and systemic immune responses induced after oral delivery of vaccinia virus recombinants. Vaccine 17 (9–10), 1074–1083.

Gorny, M.K., VanCott, T.C., Hioe, C., Israel, Z.R., Michael, N.L., Conley, A.J., Williams, C., Kessler II, J.A., Chigurupati, P., Burda, S., Zolla-Pazner, S., 1997. Human monoclonal antibodies to the V3 loop of HIV-1 with intra- and interclade cross-reactivity. J. Immunol. 159 (10), 5114–5122.

Gorny, M.K., Williams, C., Volsky, B., Revesz, K., Cohen, S., Polonis, V.R., Honnen, W.J., Kayman, S.C., Krachmarov, C., Pinter, A., Zolla-Pazner, S., 2002. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. J. Virol. 76 (18), 9035–9045.

Goulder, P.J., Rowland-Jones, S.L., McMichael, A.J., Walker, B.D., 1999. Anti-HIV cellular immunity: recent advances towards vaccine design. AIDS 13 (Suppl. A), S121–S136.

Hung, C.S., Vander Heyden, N., Ratner, L., 1999. Analysis of the critical domain in the V3 loop of human immunodeficiency virus type 1 gp120 involved in CCR5 utilization. J. Virol. 73 (10), 8216–8226.

Hwang, S.S., Boyle, T.J., Lyerly, H.K., Cullen, B.R., 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science 253 (5015), 71–74.

Kim, F.M., Kolson, D.L., Balliet, J.W., Srinivasan, A., Collman, R.G., 1995. V3-independent determinants of macrophage tropism in a primary human immunodeficiency virus type 1 isolate. J. Virol. 69 (3), 1755–1761.

Kim, Y.B., Han, D.P., Cao, C., Cho, M.W., 2003. Immunogenicity and ability of variable loop-deleted human immunodeficiency virus type 1 envelope glycoproteins to elicit neutralizing antibodies. Virology 305 (1), 124–137.

Kiszka, I., Kmieciak, D., Gzyl, J., Naito, T., Bolesta, E., Sieron, A., Singh, S.P., Srinivasan, A., Trinchieri, G., Kaneko, Y., Kozbor, D., 2002. Effect of the V3 loop deletion of envelope glycoprotein on cellular responses and protection against challenge with recombinant vaccinia virus expressing gp160 of primary human immunodeficiency virus type 1 isolates. J. Virol. 76 (9), 4222–4232.

Kmieciak, D., Wasik, T.J., Teppler, H., Pientka, J., Hsu, S.H., Takahashi, H., Okumura, K., Kaneko, Y., Kozbor, D., 1998. The effect of deletion of the V3 loop of gp120 on cytotoxic T cell responses and HIV gp120-mediated pathogenesis. J. Immunol. 160 (11), 5676–5683.

Kolchinsky, P., Mirzabekov, T., Farzan, M., Kiprilov, E., Cayabyab, M., Mooney, L.J., Choe, H., Sodroski, J., 1999. Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication. J. Virol. 73 (10), 8120–8126.

Kolchinsky, P., Kiprilov, E., Bartley, P., Rubinstein, R., Sodroski, J., 2001. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. J. Virol. 75 (7), 3435–3443.

Korber, B.T.M., Moore, J.P., Brander, C., Walker, B.D., Haynes, B.F., Koup, R.E., 1999. HIV Molecular Immunology Database. Los Alamos National Library: Theoretical Biology and Biophysics, Los Alamos, NM.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393 (6686), 648–659.

Kwong, P.D., Wyatt, R., Sattentau, Q.J., Sodroski, J., Hendrickson, W.A., 2000. Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. J. Virol. 74 (4), 1961–1972.

Lu, S., Wyatt, R., Richmond, J.F., Mustafa, F., Wang, S., Weng, J., Montefiori, D.C., Sodroski, J., Robinson, H.L., 1998. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. AIDS Res. Hum. Retroviruses 14 (2), 151–155.

Malenbaum, S.E., Yang, D., Cavacini, L., Posner, M., Robinson, J., Cheng-Mayer, C., 2000. The N-terminal V3 loop glycan modulates the interaction of clade A and B human immunodeficiency virus type 1 envelopes with CD4 and chemokine receptors. J. Virol. 74 (23), 11008–11016.

Montelaro, R.C., Cole, K.S., Hammond, S.A., 1998. Maturation of immune responses to lentivirus infection: implications for AIDS vaccine development. AIDS Res. Hum. Retroviruses 14 (Suppl. 3), S255–S259.

Moore, J.P., Binley, J., 1998. HIV. Envelope's letters boxed into shape. Nature 393 (6686), 630–631.

Moore, J.P., Sodroski, J., 1996. Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. J. Virol. 70 (3), 1863–1872.

Moore, J.P., Cao, Y., Ho, D.D., Koup, R.A., 1994. Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1. J. Virol. 68 (8), 5142–5155.

Nyambi, P.N., Mbah, H.A., Burda, S., Williams, C., Gorny, M.K., Nadas, A., Zolla-Pazner, S., 2000a. Conserved and exposed epitopes on intact, native, primary human immunodeficiency virus type 1 virions of group M. J. Virol. 74 (15), 7096–7107.

Nyambi, P.N., Nadas, A., Mbah, H.A., Burda, S., Williams, C., Gorny, M.K., Zolla-Pazner, S., 2000b. Immunoreactivity of intact virions of human immunodeficiency virus type 1 (HIV-1) reveals the existence of fewer HIV-1 immunotypes than genotypes. J. Virol. 74 (22), 10670–10680.

Park, E.J., Gorny, M.K., Zolla-Pazner, S., Quinnan Jr., G.V., 2000. A global neutralization resistance phenotype of human immunodeficiency virus type 1 is determined by distinct mechanisms mediating enhanced infectivity and conformational change of the envelope complex. J. Virol. 74 (9), 4183–4191.

Parker, C.E., Deterding, L.J., Hager-Braun, C., Binley, J.M., Schulke, N., Katinger, H., Moore, J.P., Tomer, K.B., 2001. Fine definition of the epitope on the gp41 glycoprotein of human immunodeficiency virus type 1 for the neutralizing monoclonal antibody 2F5. J. Virol. 75 (22), 10906–10911.

Parren, P.W., Moore, J.P., Burton, D.R., Sattentau, Q.J., 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. AIDS 13 (Suppl. A), S137–S162.

Pikora, C.A., Sullivan, J.L., Panicali, D., Luzuriaga, K., 1997. Early HIV-1 envelope-specific cytotoxic T lymphocyte responses in vertically infected infants. J. Exp. Med. 185 (7), 1153–1161.

Reitter, J.N., Means, R.E., Desrosiers, R.C., 1998. A role for carbohydrates in immune evasion in AIDS. Nat. Med. 4 (6), 679–684.

Ross, T.M., Cullen, B.R., 1998. The ability of HIV type 1 to use CCR-3 as a coreceptor is controlled by envelope V1/V2 sequences acting in conjunction with a CCR-5 tropic V3 loop. Proc. Natl. Acad. Sci. U.S.A. 95 (13), 7682–7686.

Schulke, N., Vesanen, M.S., Sanders, R.W., Zhu, P., Lu, M., Anselma, D.J., Villa, A.R., Parren, P.W., Binley, J.M., Roux, K.H., Maddon, P.J., Moore, J.P., Olson, W.C., 2002. Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. J. Virol. 76 (15), 7760–7776.

Srivastava, I.K., VanDorsten, K., Vojtech, L., Barnett, S.W., Stamatatos, L., 2003. Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. J. Virol. 77 (4), 2310–2320.

Staats, H.F., Nichols, W.G., Palker, T.J., 1996. Mucosal immunity to HIV-1: systemic and vaginal antibody responses after intranasal immunization with the HIV-1 C4/V3 peptide T1SP10 MN(A). J. Immunol. 157 (1), 462–472.

Stamatatos, L., Cheng-Mayer, C., 1998. An envelope modification that renders a primary, neutralization-resistant clade B human immunodeficiency virus type 1 isolate highly susceptible to neutralization by sera from other clades. J. Virol. 72 (10), 7840–7845.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J.P., Katinger, H., 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. 70 (2), 1100–1108.

Verrier, F., Borman, A.M., Brand, D., Girard, M., 1999. Role of the HIV type 1 glycoprotein 120 V3 loop in determining coreceptor usage. AIDS Res. Hum. Retroviruses 15 (8), 731–743.

Wierzbicki, A., Kiszka, I., Kaneko, H., Kmieciak, D., Wasik, T.J., Gzyl, J., Kaneko, Y., Kozbor, D., 2002. Immunization strategies to augment oral vaccination with DNA and viral vectors expressing HIV envelope glycoprotein. Vaccine 20 (9–10), 1295–1307.

Winer, B.J., 1971. Design and analysis of factorial experiments. Statistical Principles in Experimental Design, 2nd ed. McGraw-Hill, New York, pp. 224–251.

Wyatt, R., Moore, J., Accola, M., Desjardin, E., Robinson, J., Sodroski, J., 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. J. Virol. 69 (9), 5723–5733.

Wyatt, R., Kwong, P.D., Desjardins, E., Sweet, R.W., Robinson, J., Hendrickson, W.A., Sodroski, J.G., 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature 393 (6686), 705–711.

Yewdell, J.W., Bennink, J.R., 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. Adv. Immunol. 52, 1–123.

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Update on HIV/AIDS in Japan, 2003

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INTRODUCTION

In Japan, the first case of HIV-1 infection was discovered in 1983 (Miyoshi *et al.*, 1983) and its incidence continued to increase among Japanese hemophiliacs through 1985 by repeated inoculations of coagulation factor products VIII and IX imported from the United States into Japan. Approximately 2000 hemophiliacs were estimated to be infected with HIV, demonstrating a 40% infectivity rate of HIV among a total of 5000 hemophiliacs treated with the contaminated blood products. Furthermore, 12 non-hemophiliacs were retrospectively defined to be infected with HIV-1 (hereafter referred to simply as HIV) by their treatment with the contaminated products.

Surveillance of HIV/AIDS in Japan began in 1984 on a voluntary basis and become mandatory by the AIDS Prevention Law in 1989. According to this law, all HIV/AIDS individuals except those infected through the use of coagulation factor products are to be reported to the National AIDS Surveillance Committee, Ministry of Health and Welfare, as the repository of National HIV/AIDS Surveillance in Japan. Six AIDS patients were initially reported in 1985 and 55 HIV-positive individuals were detected in 1987. The number of HIV/AIDS cases has since increased every year. As of October 28, 2003, 5573 HIV infections had been reported, along with 2776 cases of AIDS, for a total of 8349 reported cases. This number corresponds to 4.05 HIV cases and 2.014 AIDS patients per 100,000 population in Japan (Infectious Disease Surveillance Center, 2003). Since sexual behavior among young people has been changing, there is concern over the prediction that the number of HIV-positive individuals may reach 100,000 by the end of the year 2010 (Kihara, 2003).

INITIAL DETECTION OF HIV IN JAPAN

The first report of HIV infection was detected in a non-hemophiliac co-infected with adult T cell leukemia virus in 1983 (Miyoshi *et al.*, 1983), followed by the identification of two additional AIDS cases and one HIV infection in Japan (Aoki *et al.*, 1984; Kobayashi

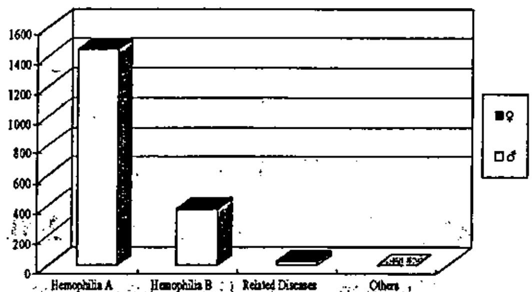

FIGURE 1. Total numbers of HIV-positive hemophiliacs treated with coagulant factor products prior to May 1996 in Japan. HIV-infected individuals treated with coagulant factors were categorized into 4 groups: hemophilia A, hemophilia B, related diseases, and others.

et al., 1984), suggesting that there had already existed an unidentified HIV-1 reservoir among non-hemophiliacs prior to 1983.

Since hemophiliacs seropositive for HIV had been reported in the United States from 1982, Abe *et al.* (1985) screened 50 samples of sera from Japanese hemophiliacs through collaboration with Dr. Gallo of the US National Cancer Institute and the National Institutes of Health in 1985. Surprisingly, 46% (23 hemophiliacs) were seropositive for HIV, including two hemophiliacs who had developed AIDS (Abe *et al.*, 1985). These hemophiliacs had been injected with imported and domestic coagulant factor VIII or IX for over six years. Based on these results, researchers recommended preparation of virus-free blood products for the prevention of HIV infection.

To establish a profile of HIV infection among hemophiliacs in Japan, the National Survey for HIV-positive Hemophiliacs began in 1987 .()(Yamada, 1996). Through May of 1996, 1862 hemophiliacs had been defined as HIV-positive, 1448 cases were among hemophilia A patients, and 382 cases were among hemophilia B patients (Figure 1). In 1996, 630 cases (33.7%) progressed to AIDS—33.4% of the hemophilia A patients and 35.1% of the hemophilia B patients; approximately 70% of the cases from both groups died in the same year (Figure 2).

The hemophiliac population provided an opportunity to study and identify the risk factors for HIV infection and its disease progression. Interestingly, the rate of long-term non-progressor status (more than 10-years maintenance of $CD4^+$ T-cells above 500 cells/mm^3) was as high as 14% among HIV-infected Japanese hemophiliacs (Yamada, 1996).

SURVEILLANCE OF HIV IN NON-HEMOPHILIACS IN JAPAN

Since HIV was not detected in hemophiliacs treated with coagulant factor products after 1986, due to the increased safety of the blood supply, the National AIDS Surveillance Committee examined analyses of reported cases of HIV infection and released its updated HIV/AIDS surveillance information twice a year.

In the most recent report in 2003 (Infectious Disease Surveillance Center, 2003), newly reported HIV infections and AIDS cases numbered 614 and 308, respectively (Figure 3A) and Japanese males dominated

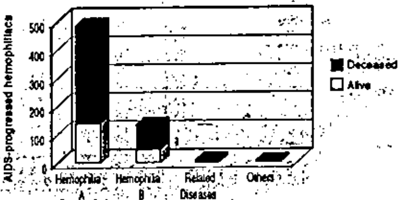

FIGURE 2. Total numbers of AIDS-progressed hemophiliacs treated with coagulant factor products prior to May 1996 in Japan. Deaths are shown in black.

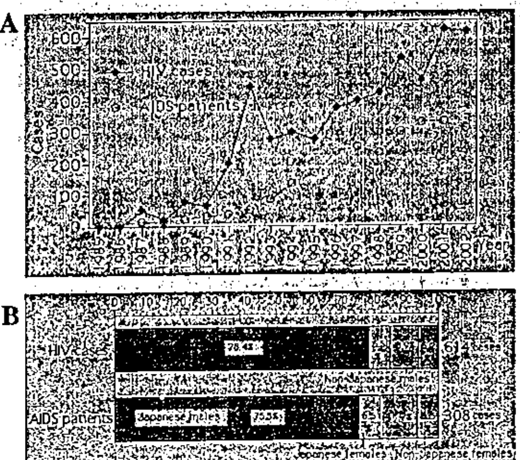

FIGURE 3. Increase of HIV/AIDS incidence in Japan. Increase of annual incidence of HIV/AIDS from 1985 to 2002 (A). HIV and AIDS cases in 2002 by gender and Japanese nationality (B). Japanese males predominated among both HIV-positive individuals and people with AIDS in 2002. Non-Japanese represented 15.1% of HIV-positive non-hemophiliac individuals and 18.7% of people with AIDS in 2002 (MHLW, 2002; NIID, 2003).

both of these groups, 78% and 75%, respectively (Figure 3B).

HIV infections among Japanese males have been continuously increasing; cases among Japanese females are also increasing but the total number has been less than 50 per year (Figure 4). It is important to note that HIV infections among Japanese men who have sex with men (MSM) are rapidly increasing (Figures 3 and 4). Cases among non-Japanese males are gradually increasing, and cases among non-Japanese females peaked

FIGURE 4. Study of the increase of HIV incidence by nationality, gender, and mode of infection from 1985 to 2002 in Japan (MHLW, 2002; NIID, 2003).

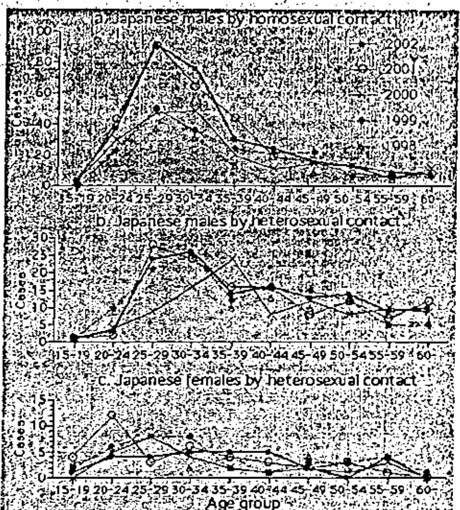

FIGURE 5. Age distribution analysis of HIV incidence by gender and mode of infection from 1998 to 2002 in Japan (MHLW, 2002; NIID, 2003).

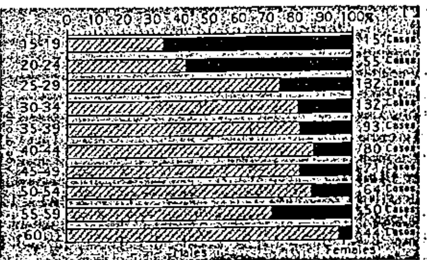

FIGURE 6. Distribution of heterosexual HIV infections by gender and age group, 1998–2002. Japanese females predominated among young HIV-positive Japanese contracting HIV by heterosexual contact from 1998 to 2002. Ten age categories were created to cover ages 15 to over 60 (MHLW, 2002; NIID, 2003).

in 1992, but the percentage of non-Japanese in the total of HIV cases is more than 18%, higher than that of other countries.

HIV infections are considered to be mainly due to sexual transmission among Japanese males in Japan (Figures 5 and 6). Although the prevalence is low as described above, heterosexually acquired HIV has been rapidly increasing since the early 1990s in Japan (Umeda, 1999).

Interestingly, the first HIV-antibody-positive case among blood donors was

FIGURE 7. Rate of HIV-antibody positives per 100,000 blood donors, 1987–2002. HIV infections among blood donors were rapidly increasing from 1987 to 2002, at a rate much higher than that of European countries. Numbers of HIV-positive blood donors detected in 2000, 2001 and 2002 were 67, 79, and 82, respectively, and only 6 of 228 positives were detected by the nucleic acid amplification test (MHLW, 2002; NIID, 2003).

detected in 1987, and the positive rate per 100,000 donations increased to 1.368 (2.259 in males and 0.043 in females) in 2001, a rate approximately 10–100 times higher than that of European and American countries (Figure 7). Contaminated blood was found in 67, 79, and 82 donors in 2000, 2001, and 2002, respectively; six infections were detected by nucleic acid test with PCR detection. These data suggest the need for careful evaluation of whether HIV-positive blood donors may be more likely to donate blood frequently for the purpose of HIV testing, as speculated, or whether HIV may truly be spreading vigorously in Japan.

PHENOTYPIC CHARACTERIZATION OF HIV IN JAPAN

The molecular epidemiology of HIV in Southeast Asian countries has been well characterized by studying this unique geographical hot spot, where extensive recombination events appear to be ongoing. These events suggests the presence of highly exposed individuals and social networks of HIV transmission (Motomura *et al.*, 2003; Takebe *et al.*, 2003; Weniger *et al.*, 1994; Yang *et al.*,

2003). In Japan, hemophiliacs who received frequent transfusions of HIV-contaminated blood products comprised the largest population of HIV-positive cases until 1998 (Kamakura *et al.*, 1998). The HIV-1s detected in the hemophiliacs were all HIV-1 clade B; the major HIV clade circulating in Japan has also been identified as clade B by genotypic analysis of HIV-1 *env* C2-V3 region (Hattori *et al.*, 1991; Yamanaka *et al.* 1997). However, the National Survey of HIV/AIDS in 1999 found sexual transmission to be a major risk factor in the population of HIV-positive individuals in Japan. During the rapid increase in sexually transmitted HIV infections in Japan, which took place from 1996 to 1998, 40% of the HIV-positive individuals infected via MSM contact were grouped into subtype E. Others were subtype B, including Japanese males infected by heterosexual contact with multiple partners both abroad and in Japan (Kitsutani *et al.*, 1998). Furthermore, a replication-competent molecular clone of HIV-1 was isolated from an HIV-1 NH1 isolate in Japan as an CRF01_AE strain, and the infectious molecular clone was designated p93JP-NH1 (Kusagawa *et al.*, 2002). Further epidemiological research of HIV in Japan has detected a gradual shift toward an increase with predominantly heterosexual and MSM contact. (Infectious Disease Surveillance Center, 2003). Apart from subtypes B and E, other HIV-1 subtypes A, C, D, and G, and HIV-2 have also been sporadically found in Japan (Hara *et al.*, 2001; Kusagawa *et al.*, 2003; Nakasone *et al.*, 1998). Efforts to isolate infectious clones of various HIV subtypes are continuing to define the HIV phenotypes spreading in the field (Mochizuki *et al.*, 1999).

ANTI-HIV CHEMOTHERAPY IN JAPAN

Between 1988 and 1999, HIV was isolated from 614 HIV-positive individuals out of a total 2785 cases. During those 11 years, annual HIV isolation rates were found to decrease, reflecting the success of anti-HIV chemotherapy with single, double, and triple combinations of antiretroviral drugs (Figure 8) (Nakamura *et al.*, 1987).

In 1997, three protease inhibitors, Saquinavir, Ritonavir, and Indinavir became available for clinical use in Japan, and highly active antiretroviral therapy (HAART) began being used for treatment of people with HIV/AIDS (PLWHA) in Japan. Among 446 cases, 87 (60.0%) out of 145 naïve cases and 150 (49.8%) out of 301 drug-treatment-experienced cases showed a reduction of plasma viral load to undetectable levels. Successful therapy with HAART was maintained for more than twelve months among 50% of the medicated patients (Oishi *et al.*, 1999). The prevalence of emerging drug resistance-related mutations has been systematically monitored to evaluate the efficacy of HAART in Japan (Sugiura, 2001; Sugiura *et al.*, 1999).

VERTICAL TRANSMISSION OF HIV IN JAPAN

A national collaborative group for studying vertical transmission of HIV in pregnant women and their infants was established by Dr. Ryozo Totani in Japan in 1989. Through their research, 42 infants, including 13 HIV-positive, 25 uninfected, four of undetermined status, and 15 control children born to HIV-positive mothers were diagnosed and followed from birth to 1.5 years (Yoshino *et al.*, 1998). All strains from HIV-positive infants were either clade E (eight infants, 61.5%) or B (five infants, 38.5%) according to DNA sequencing specific for the HIV-1 C2-V3 region. The 42 HIV-positive mothers were women with sexual risk behaviors from all regions, but were concentrated in the Kanto District. In this group of HIV-positive children, there was no significant difference between the transmissibility of their mother's clade E and B viruses. Eight (61.5%) of the 13 HIV-positive babies were Japanese and five (62.5%) of the

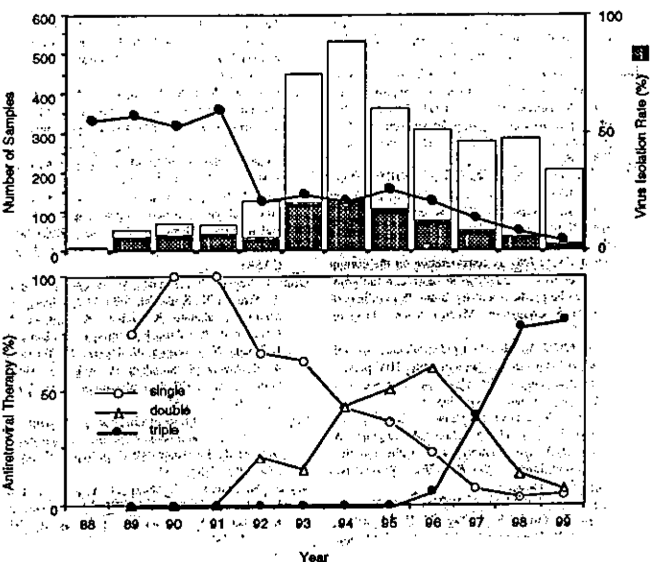

FIGURE 8. Viral isolation rate and antiretroviral therapy coverage, 1988–1999. Decline in the HIV-1 isolation rate in Japan may correspond to anti-HIV chemotherapy. Upper panel: light and dark dotted bars indicate the total number of samples, and sample numbers positive for HIV-1 isolation, respectively. Bold line indicates the kinetics of the rate of HIV-1 isolation. Lower panel: open circle, open triangle and closed circle lines indicate percentage of patients treated with single, double, and triple anti-HIV chemotherapies, respectively.

eight were positive for HIV-1 clade E. The V3 loop region of the clade E virus of the babies was conserved, but approximately 60% of the sequences showed a substitution of aspartic acid by asparagine at position 29. These results suggest that HIV clade E may be predominant in cases of vertical transmission and are phenotypically different from HIV in persons with various other risk behaviors in Japan. Prevention efforts against vertical transmission have been quite successfully undertaken through a combination of Caesarian-section deliveries and chemotherapy during the delivery stage.

REFERENCES

Abe, T., Kinoshita, T., Matsuda, J., Ryu, T., Ting, R.C., Aoki, T., Robert-Guroff, M., Miyakoshi, H., Gallo, R.C. (1985). Detection of antibodies to HTLV-I and -III in sera from Japanese hemophiliacs. *Cancer Res.* 45:4621s–4623s.

Aoki, T., Miyakoshi, H., Usuda, Y., Chermann, J.C., Barre-Sinoussi, F., Ting, R.C., Gallo, R.C. (1984). Antibodies to HTLV I and III in sera from two Japanese patients, one with possible pre-AIDS. *Lancet* 2:936–937.

Hara, T., Yoshino, N., Takayama, N., Minamitani, M., Naganawa, S., Ohkubo, H., Takizawa, M., Izumi, Y., Kantake, M., Suzuki, S., Takano, M., Kita, T., Totani, R., Nagai, Y., Honda, M., Nakasone, T.

(2001). Presence of multiple HIV type 1 subtypes among mothers and children in Japan. *AIDS Res. Hum. Retroviruses* 17:569–575.

Hattori, T., Shiozaki, K., Eda, Y., Tokiyoshi, S., Matsushita, S., Inaba, H., Fujimaki, M., Meguro, T., Yamada, K., Honda, M., *et al.* (1991). Characteristics of the principal neutralizing determinant of HIV-1 prevalent in Japan. *AIDS Res. Hum. Retroviruses* 7:825–830.

National Institute of Infectious Diseases (NIID). (2003). HIV/AIDS in Japan, 2002. Infectious Agent Surveillance Report 24:203–204. Infectious Disease Surveillance Center, Tokyo.

Kamakura, M., Soda, K., Hashimoto, S., Morio, S., Kitamura, T. (1998). A consideration on the current epidemic of HIV infection in Japan and the problems in the prediction of its future. Proceedings of Japan-U.S. Cooperative Medical Science Program 9:21–22.

Kihara, M. (2003, October 1). Epidemiological surveillance in Japan and enlightenment for HIV prevention. Proceedings of UNAIDS; Dr. Peter Piot welcoming symposium. Tokyo, Japan.

Kitsutani, P.T., Naganawa, S., Shiino, T., Matsuda, M., Honda, M., Yamada, K., Taki, M., Sugiura, W. (1998). HIV type 1 subtypes of nonhemophiliac patients in Japan. *AIDS Res. Hum. Retroviruses* 14:1099–1103.

Kobayashi, M., Yoshimoto, S., Fujishita, M., Yano, S., Niiya, K., Kubonishi, I., Taguchi, H., Miyoshi, I. (1984). HTLV-positive T-cell lymphoma/leukaemia in an AIDS patient. *Lancet* 1:1361–1362.

Kusagawa, S., Imamura, A., Yasuoka, A., Hoshino, H., Oka, S., Takebe, Y. (2003). Identification of HIV type 2 subtype B transmission in East Asia. *AIDS Res. Hum. Retroviruses* 19:1045–1049.

Kusagawa, S., Sato, H., Tomita, Y., Tatsumi, M., Kato, K., Motomura, K., Yang, R., Takebe, Y. (2002). Isolation and characterization of replication-competent molecular DNA clones of HIV type 1 CRF01_AE with different coreceptor usages. *AIDS Res. Hum. Retroviruses* 18:115–122.

Ministry of Health, Labor and Welfare (MHLW) Japan. (2002). Annual Report on HIV/AIDS Surveillance in Japan, the National Surveillance Committee.

Miyoshi, I., Kobayashi, M., Yoshimoto, S., Fujishita, M., Taguchi, H., Kubonishi, I., Ohtsuki, Y. (1983). ATLV in Japanese patient with AIDS. *Lancet* 2:275.

Mochizuki, N., Otsuka, N., Matsuo, K., Shiino, T., Kojima, A., Kurata, T., Sakai, K., Yamamoto, N., Isomura, S., Dhole, T.N., Takebe, Y., Matsuda, M., Tatsumi, M. (1999). An infectious DNA clone of HIV type 1 subtype C. *AIDS Res. Hum. Retroviruses* 15:1321–1324.

Motomura, K., Kusagawa, S., Lwin, H.H., Thwe, M., Kato, K., Oishi, K., Yamamoto, N., Zaw, M., Nagatake, T., Takebe, Y. (2003). Different subtype distributions in two cities in Myanmar: Evidence for independent clusters of HIV-1 transmission. *AIDS* 17:633–636.

Nakamura, K., Yoshida, Y., Ando, T., Yamazaki, K., Ohnuki, N., Ito, T., Yano, K., Sekine, S., Hayashi, Y., Sinkai, T., *et al.* (1987). A serological survey for human immunodeficiency virus types 1 and 2 of individuals who visited health centers in Tokyo. *Jpn. J. Med. Sci. Biol.* 40:159–164.

Nakasone, T., Totani, R., Yamazki, S., Honda, M. (1998). HIV-1 subtype A in Japan. *AIDS* 12:950–952.

National Institute of Infectious Diseases (NIID) Japan (year). Infectious Agents Surveillance Report (IASR).

Oishi, T., Sugiura, W., Matsuda, M., Abumi, H., Okano, A., Yamada, K., Koike, M., Taki, M., Ishikawa, M., Miura, T., Fukutake, K., Gouchi, K., Ajisawa, A., Iwamoto, A., Hanabusa, H., Mimaya, J., Takamatsu, J., Takata, N., Kakishita, E., Higasa, S., Yoshioka, A., Kashiwagi, S., Shirahata, A., Nagai, Y. (1999). Status of anti-HIV-1 chemotherapy in Japan. *Jpn. J. Infect. Dis.* 52:51–52.

Sugiura, W. (2001). Effect of introduction of highly active antiretroviral treatment and the changes in patterns of drug-resistant HIV-1 in Japan. *J. Infect. Chemother.* 7:127–132.

Sugiura, W., Matsuda, M., Abumi, H., Yamada, K., Taki, M., Ishikawa, M., Miura, T., Fukutake, K., Gouchi, K., Ajisawa, A., Iwamoto, A., Hanabusa, H., Mimaya, J., Takamatsu, J., Takada, N., Kakishita, E., Yoshioka, A., Kashiwagi, S., Shirahata, A., Nagai, Y. (1999). Prevalence of drug resistance-related mutations among HIV-1s in Japan. *Jpn. J. Infect. Dis.* 52:21–22.

Takebe, Y., Motomura, K., Tatsumi, M., Lwin, H.H., Zaw, M., Kusagawa, S. (2003). High prevalence of diverse forms of HIV-1 intersubtype recombinants in Central Myanmar: Geographical hot spot of extensive recombination. *AIDS* 17:2077–2087.

Takeda, A., Igarashi, H., Nakamura, H., Kano, M., Iida, A., Hirata, T., Hasegawa, M., Nagai, Y., Matano, T. (2003). Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. *J. Virol.* 77:9710–9715.

Umeda, T. (1999). Increase in heterosexually acquired AIDS among Japanese, 1986 to 1996. *Jpn. J. Infect. Dis.* 52:167–168.

Weniger, B.G., Takebe, Y., Ou, C.Y., Yamazaki, S. (1994). The molecular epidemiology of HIV in Asia. *AIDS* 8 Suppl 2:S13–S28.

Yamada, K. (1996). Annual report on AIDS prevention and clinical control. *Proceedings of HIV/AIDS Prevention and Therapy* 10:1–360.

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Japan's Collaboration with Thailand in the Development of an HIV/AIDS Vaccine

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INTRODUCTION

On March 26, 1998, the Japan Science and Technology Corporation, Japan's Ministry of Education, Science and Culture, Ministry of Health, Labor and Welfare, and Thailand's Department of Medical Science and Ministry of Public Health jointly launched a bilateral collaborative HIV vaccine project with equal partnership. This international collaboration primarily focused on in-country capacity building for HIV vaccine research and development, and preclinical development of the Thailand-Japan AIDS vaccine candidates that targeted the prevalent HIV-1 subtype in Thailand—HIV CRF01_AE—with potential for work with other types of vaccines as well in Thailand.

The Thailand-Japan Cooperative AIDS Vaccine Research Project (T-J CAVRP) initially targeted development of a recombinant Bacillus Calmette-Guérin (rBCG)-based HIV CRF01_AE *env* vaccine (Chujoh *et al.*, 2002). However, a more realistic goal of a preventive HIV vaccine may be to limit HIV infection by producing a cellular immune response to control viral load and prevent disease progression (Nabel, 2001; Schultz and Bradac, 2001). Therefore, the vaccine strategy was changed to apply the experimental results of a prime-boost regimen consisting of rBCG-SIV priming followed by recombinant

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collected and virus was isolated from HIV-1-infected individuals throughout Thailand by various local hospitals and institutions, including the Siriraj Hospital, Bamurasnaradura Hospital, Rajavithi Hospital, the Queen Sirikit National Institute of Child Health, and others. Sequence analysis of HIV-1 genes *env*, *env*-V3 (the variable loop 3 of the *env* gene), *pol*, *gag*, *tat*, and *nef* were performed. These studies demonstrated that HIV CRF01_AE is spreading all over Thailand. Most of these viruses had the amino acid sequence GPGQ at the tip of V3 of their *env* genes (Naganawa *et al.*, 1997; Sutthent *et al.*, 2001; Balachandra *et al.*, 2002). It was also shown that the HIV CRF01_AE isolates from genital fluid samples appeared to have less genetic divergence than those from the blood samples of the same individuals (Sutthent *et al.*, 2001). Moreover, the sero-reactivity of an HIV-infected individual appeared to decrease when the GPGQ sequence at the tip of V3 was substituted, and its reactivity in AIDS patients was inversely correlated with their tip sequences. The sequence substitutions at the V3 also seemed to correlate with the appearance of HIV-1 mutants that become unrecognizable by the neutralizing antibody in the hosts (Balachandra *et al.*, 2002). HIV CRF01_AE in cerebrospinal fluid was defined to be non-syncytium-inducing even when the patients progressed to the disease state, suggesting that central nervous tissue cells may be one of the reservoirs of CCR5-tropic virus in individuals with advanced disease (Albright *et al.*, 1999). The Gag sequence of HIV CRF01_AE also had a characteristic deletion of six amino acids in the p15 peptide, which was approximately 90% homologous to that of HIV-1 subtype B primary isolates. Another recent study of HIV-1 CRF01_AE showed that the consensus sequence of the *gag* and *env*-V3 region was very close to the consensus sequence of sero-converters in the early epidemic period in Thailand. These data suggest that CRF01_AE might have evolved randomly, and the consensus sequence strategy should be applicable to selecting appropriate vaccine antigens (Hamano *et al.*, 2004).

Various HIV-1 subtypes spread predominately in different human populations in the world. Our studies, together with studies from many other investigators, showed that the predominant HIV-1 subtype in Thailand, CRF01_AE, has significant genetic differences from the predominant HIV-1 subtype B in Japan (Weniger *et al.*, 1994; Kitsutani *et al.*, 1998; Kusagawa *et al.*, 2002). In order to make the candidate Thailand-Japan HIV-1 vaccines beneficial for the people in Thailand where future vaccine efficacy trials will be conducted, we devoted our efforts to match the origins of HIV-1 antigens in our vaccine designs with the HIV CRF01_AE that is prevalent in Thailand. In our opinion, in the absence of convincing evidence showing that the subtype of HIV-1 antigens in a vaccine design has nothing to do with potential vaccine efficacy, developing the Thailand-Japan HIV-1 vaccines that include CRF01_AE was the only ethically correct way to proceed.

HUMAN GENETICS, CELLULAR IMMUNE RESPONSES, AND HIV VACCINE DESIGN

Numerous factors and cofactors are believed to affect the infectivity of HIV and the disease progression of AIDS, some of which warrant special consideration in the HIV/AIDS vaccine design for Thailand. The design was focused on specifically mapping the HIV viral epitopes that are responsible for eliciting HLA restricted anti-HIV cytotoxic T lymphocytes (CTL) responses in HIV-infected people in Thailand. Using the classical chromium release CTL assay, intracellular cytokine staining, and ELISPOT assays, new CTL epitopes were identified in HIV-infected people in Thailand that differed from the dominant CTL epitopes in HIV-infected people in the United States (Ruxrungtham and Phanuphak, 2001; Ariyoshi *et al.*, 2002; Ananworanich *et al.*, 2003). Significant cross-clade CTL responses were also found in Thai HIV-infected people, characterized by reactions to peptide epitopes

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derived from different subtypes, including Gag, Env, Pol and Tat antigens (Ruxrungtham *et al.*, 2003). HLA typing in Thai people revealed that the frequencies of HLA-A2, -A11, -B46, -B57 and DQB1*0303 were significantly increased as compared to controls from other populations ($p < 0.05$) (Ward *et al.*, 1995; Vejbaesya *et al.*, 1998; Fukada *et al.*, 2002; Pimtanothai *et al.*, 2003).

These studies provided additional support to the designing principle of the T-J CAVRP that the proposed HIV vaccine candidates for Thailand should be based on CRF01_AE rather than the HIV-1 subtype B that is prevalent in the United States and Japan. By characterizing the immune responses of Thai infected people to CRF01_AE, we also obtained useful information about potential CTL responses in Thailand where the proposed HIV vaccine would be evaluated. Moreover, by collaboratively conducting these state-of-art immunology and molecular virology studies in Thailand with necessary laboratory and clinical capacity and infrastructure building, new vaccine research capability was introduced into Thailand, to benefit future vaccine studies in the country.

HIV VACCINE DEVELOPMENT STRATEGY SPECIFICALLY DESIGNED FOR THAILAND

Increasing evidence suggests that cell-mediated immune responses play critical roles in controlling HIV-1 replication and disease progression. It is widely believed that an effective HIV vaccine should elicit both humoral and cell-mediated immunity in order to provide protection against HIV. Our approach to develop an HIV vaccine for Thailand is mainly focused on the induction of protective cellular immune responses to HIV. In the past five years, T-J CAVRP focused primarily on two types of recombinant vectors: *Mycobacterium bovis* BCG, which has been used in Thailand and Japan for many years as the vaccine against tuberculosis (TB), and vaccinia virus DIs (Tagaya *et al.*, 1961; Kitamura *et al.*, 1967), which had been tested in Japan as the vaccine against smallpox. These two vaccines not only have had excellent safety records for human use, they also have a number of features that are highly desirable for practical use in Thailand. BCG is known to induce long-lasting T helper type 1 responses, which are preferable for induction and maintenance of antigen-specific CTL responses. In addition, BCG can be produced at low cost and can be given by oral, nasal, and rectal administration for targeting mucosal delivery of recombinant antigens to induce effective mucosal immunity (Lagranderie *et al.*, 1997).

Based on the reports that previously used vaccinia strains posed potential safety hazards in immune-compromised individuals, associated with replication of the viruses, we sought to select and evaluate highly attenuated vaccinia virus (DIs) as another vaccine vector for T-J CAVRP. One desirable feature of this particular virus vector is its lack of replication capacity in mammalian cells.

The strategy of using recombinant BCG-Tokyo 172 and attenuated vaccinia virus DIs as the vectors for the proposed Thailand-Japan HIV vaccine also resulted from considerations beyond the proposed clinical testing. If these vaccine candidates turn out to be effective in future large-scale vaccine efficacy trials in Thailand, they should be made available immediately for the Thai government to use. Based on this principle, the transfers of these two recombinant vaccine technologies from Japan to Thailand, including molecular construction, pilot production, and quality control and assurances, has been a major goal of the T-J CAVRP. Moreover, the organizing committee of the T-J CAVRP has also initiated discussions about the intellectual property issues that will affect the use of these vaccines in Thailand. The intellectual property covering any new vaccine of the prime-boost regimen targeting HIV CRF01 AE Gag, achieved by the Thailand-Japan Collaborative Project, will be equally shared between both countries.

The experimental part of T-J CAVRP has produced encouraging results. First, it was established that whole HIV-1 *gag* genes from subtype B, CRF01_AE, and SIV could be productively expressed by the recombinant BCG-vector. After optimizing the codon usage for viral antigen expression in mycobacteria, we demonstrated that the expression of the HIV-1 p24 antigen by the recombinant BCG increased more than 20-fold (Kanekiyo *et al.*, 2003). We obtained similar results using recombinant non-replicative vaccinia vector DIs, which productively expressed the Gag proteins from HIV subtype B, CRF01_AE, and SIV (Ishii *et al.*, 2002; Izumi *et al.*, 2003). In small animal experiments, these potential HIV vaccine candidates were shown to effectively induce strong anti-HIV specific CTL responses in mice (Ishii *et al.*, 2002).

The same prime-boost regimen, in which the recombinant BCG bacteria were used first and followed by a boost immunization with the recombinant vaccinia DIs that expressed the same HIV antigens, also showed a strong ability to induce CTL in immunized macaque monkeys (Matsuo *et al.*, 2002). This result was critically important to advance the T-J CAVRP into the preclinical testing stage of the HIV vaccine candidates.

PRECLINICAL EVALUATION OF THE THAILAND-JAPAN AIDS VACCINE CANDIDATES

As part of the research capacity building efforts of the T-J CAVRP, facilities and expertise were collaboratively established in the Sasakawa Memorial Animal Facility Building, Nonthaburi, Thailand, where the HIV vaccines would be evaluated in a hu-PBL-NOD-SCID mouse model. Such a complicated but useful animal model enabled us to demonstrate the capability of our prime-boost regimen to induce the desirable neutralizing antibodies that would be required in order to protect human vaccinees from HIV/AIDS. In one particular experiment, the IgG fraction collected from HIV-1-positive individuals, which has neutralizing activity *in vitro* checked by the MAGIC5 cell line, inhibited virus growth in hu-PBL-NOD-SCID mice more than 100 times compared to negative controls (Ogura *et al.*, 1996; Okamoto *et al.*, 1997; Okamoto *et al.*, 1998).

Next, we established the macaque monkey HIV/AIDS models using chimeric human-simian immunodeficiency virus (SHIV) that was specifically developed for this project. A panel of challenging SHIV stocks was prepared for the proposed vaccine efficacy studies, including the nonpathogenic SHIV-MN, SHIV-HXB, the pathogenic SHIV-C2/1, and its molecular clone, SHIV-C2/1 KS661 (Shinohara *et al.*, 1999; Sakai *et al.*, 2001). In order to demonstrate the potential vaccine efficacy for candidates with the SHIV model, we constructed new recombinant BCG and non-replicative vaccinia DIs that productively express the SIV *gag* gene to match the SIV *gag* gene in the challenging SHIV viruses. Results showed that the recombinant BCG-vectored and recombinant non-replicative vaccinia virus DIs-vectored SIV Gag vaccines induced strong CTL responses in immunized monkeys, respectively. However, the best immune responses in monkeys were obtained when the two vaccines were used in combination as a prime-boost regimen. When the immunized monkeys were challenged with the pathogenic SHIV-C2/1, the vaccination provided full protection for the monkey hosts from developing simian AIDS (Matsuo *et al.*, 2002). The CD4 T-cell decline in the vaccinated monkeys after the lethal challenge of SHIV, which is the hallmark of simian AIDS as well as AIDS in humans, was prevented. The plasma virus load in the vaccinated monkeys after the SHIV challenge also showed approximately 100- to 1000-fold reductions in comparison with unvaccinated monkeys (Matsuo *et al.*, 2002). Moreover, it was demonstrated that oral administration of the recombinant

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