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Neutralizing Antibodies in Sera from Macaques Infected with Chimeric Simian-Human Immunodeficiency Virus Containing the Envelope Glycoproteins of either a Laboratory-Adapted Variant or a Primary Isolate of Human Immunodeficiency Virus Type 1

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The magnitude and breadth of neutralizing antibodies raised in response to infection with chimeric simian-human immunodeficiency virus (SHIV) in rhesus macaques were evaluated. Infection with either SHIV-HXB2, SHIV-89.6, or SHIV-89.6PD raised high-titer neutralizing antibodies to the homologous SHIV (SHIV-89.6P in the case of SHIV-89.6PD-infected animals) and significant titers of neutralizing antibodies to human immunodeficiency virus type 1 (HIV-1) strains MN and SF-2. With few exceptions, however, titers of neutralizing antibodies to heterologous SHIV were low or undetectable. The antibodies occasionally neutralized heterologous primary isolates of HIV-1; these antibodies required >40 weeks of infection to reach detectable levels. Notable was the potent neutralization of the HIV-1 89.6 primary isolate by serum samples from SHIV-89.6-infected macaques. These results demonstrate that SHIV-HXB2, SHIV-89.6, and SHIV-89.6P possess highly divergent, strain-specific neutralization epitopes. The results also provide insights into the requirements for raising neutralizing antibodies to primary isolates of HIV-1.

An important goal in the development of a successful vaccine for human immunodeficiency virus type 1 (HIV-1) is to generate an effective neutralizing antibody response. The surface gp120 and, to a lesser extent, transmembrane gp41 envelope glycoproteins of the virus are major targets for neutralizing antibodies (for a review, see reference 3) and have been the basis for several candidate vaccines. To date, most HIV-1 envelope vaccines that have advanced to clinical trials in humans are derived from T-cell line-adapted (TCLA) strains of virus and are administered as molecularly cloned monomeric subunits (43). Some of these vaccines have induced significant levels of neutralizing antibodies against the vaccine strain of virus and, to a lesser extent, against heterologous TCLA strains (1, 12, 13, 23, 32, 38, 45). However, with the exception of a rare clade B variant that is highly sensitive to neutralization (isolate BZ167) (46), sera from vaccinated volunteers have failed to neutralize low-passaged field strains (i.e., primary isolates) of the virus (14, 23, 24). Neutralizing antibodies raised by these vaccines have been shown to recognize primarily linear epitopes (41), including those in the V3 loop of gp120 (25).

It seems prudent to develop an HIV-1 vaccine that will induce antibodies capable of neutralizing a broad spectrum of primary isolates. The fact that primary isolates are only occasionally neutralized by sera from infected individuals (4, 17, 26, 29, 33, 42) is an indication that this will not be an easy task. One approach in preclinical development has been to use oligomeric forms of the viral envelope glycoproteins as immunogens (2, 9, 36). This approach gains support from the observation that native gp120 and gp41 exist as oligomeric complexes on virus particles (8, 21, 44) and that virus neutralization is associated with antibodies that bind these complexes efficiently (10, 30, 37, 40). Although the neutralization epitopes on primary isolates are ill defined, the broad and potent neutralization of TCLA variants and primary isolates by human monoclonal antibodies b12, 2G12, and 2F5 has revealed the presence of several neutralization epitopes that are highly conserved (for a review, see reference 3). These epitopes must be poorly immunogenic, however, since primary isolates are not broadly neutralized by sera from most HIV-1-infected individuals. The fact that primary isolates are neutralized sporadically by these sera (17, 26, 29, 33) suggests the presence of additional neutralization epitopes that are strain specific and are both antigenic and immunogenic. Oligomeric gp120 may differ antigenically from the monomer (2, 9, 36), but the effect this has on the immunogenicity of primary isolate neutralization epitopes remains uncertain.

The exploration of new vaccine approaches for raising neutralizing antibodies to HIV-1 would benefit greatly from an appropriate animal model. The recent development of chimeric simian-human immunodeficiency virus (SHIV), in which the *env*, *tat*, and *rev* of molecularly cloned SIVmac239 are replaced with the corresponding regions of HIV-1 (15, 16, 19, 20, 22, 34, 35, 39), affords novel opportunities to dissect the molecular determinants of HIV-1 pathogenesis and to assess HIV-1 envelope glycoprotein vaccines in a highly relevant animal model. The magnitude and strain specificity of neutraliz-

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ing antibody responses in SHIV-infected macaques were evaluated in this study to gain a better understanding of how the SHIV model may be best utilized to assess HIV-1 vaccine efficacy and associated immunologic correlates and to provide insights into the design of a vaccine that will raise antibodies capable of neutralizing primary isolates of HIV-1.

Serum samples were obtained from rhesus macaques (*Macaca mulatta*) at various time points after intravenous inoculation with one of five SHIV variants. The first variant, SHIV-HXB2, contained the envelope glycoproteins of HIV-1 strain IIIB (19, 20). A second variant, SHIV-89.6, contained the envelope glycoproteins of a primary isolate that is dualtropic for T cells and macrophages and can utilize both CXCR4 and CCR5 as coreceptors for virus entry (5–7, 34). The third and fourth variants were generated by serial passage of SHIV-89.6 in rhesus macaques to generate SHIV-89.6′ (second passage) and SHIV-89.6PD (fourth passage) (16, 34). The latter virus was obtained from plasma and was isolated in CEMx174 cells. SHIV-89.6P, which was used only in neutralization assays, was obtained directly from blood and lymphoid tissue mononuclear cells from the same monkey after the fourth passage of SHIV-89.6 and, therefore, is closely related to SHIV-89.6PD (16, 34). The fifth SHIV used for infection of macaques is a molecular clone of SHIV-89.6P, designated SHIV-KB9 (16).

SHIV-HXB2 and SHIV-89.6 produce transient viremia but have caused no immune suppression or disease in animals observed for up to 2 to 4 years of infection (19, 20, 35; unpublished observations). In contrast, rapid immune suppression and death from AIDS are induced by infection with either SHIV-89.6PD (unpublished observations) or SHIV-89.6P (34). Four of four macaques infected with SHIV-89.6P progressed very rapidly and failed to seroconvert as shown by Western blot and virus neutralization assays, and, therefore, their sera were not included in the study. Animals were housed at either the New England Regional Primate Research Center, the GTC Mason primate facility, or the National Institutes of Health (NIH)-National Center for Research Resources primate housing facilities and were maintained in accordance with guidelines set forth in *Guide for the Care and Use of Laboratory Animals* (31).

Sera from SHIV-infected macaques were assessed for their ability to neutralize multiple SHIV variants and heterologous TCLA strains of HIV-1 (MN and SF-2) in either MT-2 or CEMx174 cells as described previously (27, 33). Seed stocks of MN and SF-2 were obtained from the NIH AIDS Research and Reference Reagent Program; the derivation of these viruses has been reported elsewhere (11, 18). Neutralizing antibodies were further assessed with a panel of six heterologous primary isolates consisting of three isolates with a syncytium-inducing phenotype (V89872, V67970, and W179273) and three others having a non-syncytium-inducing phenotype (P59423, W25798, and W79290) (26). These primary isolates are occasionally neutralized by sera from HIV-1-infected individuals (26, 33), and none of them would be considered unusually sensitive or resistant to neutralization compared with the majority of primary isolates evaluated by others. The fact that these isolates are neutralized sporadically by sera from HIV-1-infected individuals (26, 33) is evidence that they possess a variety of antigenically distinct neutralization determinants that are useful when assessing the breadth of neutralizing antibodies in serum samples. Some assessments of neutralizing antibodies were also made with the uncloned HIV-1 89.6 primary isolate and its molecularly cloned counterpart, HIV-1 89.6mc (6). All primary isolates were obtained by peripheral blood mononuclear cell (PBMC) coculture and were of low passage number in PBMCs exclusively (one or two passages of the original coculture supernatant). Antibody-mediated neutralization of primary isolates was assessed by a reduction in p24 synthesis in human PBMCs as described previously (26, 33).

As shown in Table 1, infection with SHIV-HXB2 raised high-titer neutralizing antibodies to SHIV-HXB2 and HIV-1 strains MN and SF-2 but little or no detectable neutralizing antibodies to SHIV-89.6. These results are in agreement with those obtained in a previous study (20). Animals infected with either SHIV-89.6 or SHIV-89.6PD developed high-titer neutralizing antibodies to the homologous SHIV and significant titers of neutralizing antibodies to MN and SF-2, although the titers to SHIV-HXB2 were low or undetectable. Similar strain-specific neutralization of SHIV-HXB2 and SHIV-89.6 has been observed for sera from macaques immunized with IIIB and 89.6 envelope glycoprotein subunit vaccines (unpublished observations). Finally, infection with the intermediate animal-passaged SHIV-89.6′ raised neutralizing antibodies to SHIV-89.6, SHIV-89.6P, MN, and SF-2 but no detectable neutralizing antibodies to SHIV-HXB2.

The above results indicate that the neutralization determinants of SHIV-HXB2 differ from those of SHIV-89.6 and SHIV-89.6P. Poor neutralization of SHIV-89.6P by sera from SHIV-89.6-infected animals indicates that these two viruses differ in their neutralization determinants also. Failure to detect neutralizing antibodies to SHIV-89.6 in serum samples obtained after 11 weeks of infection with SHIV-89.6PD (Table 1) supports this conclusion. A notable exception was the detection of neutralizing antibodies to SHIV-89.6 after 38 weeks of infection with SHIV-89.6PD, making it possible that the animal-passaged stock of SHIV-89.6PD contained a mixture of genetic quasispecies, including a minor population of SHIV-89.6 that raised these antibodies. To minimize the possible effects of genetic quasispecies, we evaluated sera from two animals infected for up to 41 weeks with molecularly cloned SHIV-KB9. High titers of neutralizing antibodies to SHIV-KB9 and SHIV-89.6P were detected by 8 and 17 weeks of infection, respectively (Table 2). No neutralizing antibodies to SHIV-89.6 were detected for up to 29 weeks of infection, although low-titer neutralizing antibodies to this virus were detected after 41 weeks of infection (Table 2). Thus, the cross-reactive neutralizing antibodies to SHIV-89.6 generated by SHIV-89.6PD infection could have been due to long-term exposure to antigen rather than to a mixture of genetic quasispecies in the viral stock. Nonetheless, multiple animal passages clearly resulted in a loss of strain-specific neutralization epitopes on SHIV-89.6 and the acquisition of new and different neutralization epitopes on SHIV-89.6P. Associated with these changes are 12 amino acid substitutions found throughout the gp120 and gp41 of the KB9 molecular clone of SHIV-89.6P, including the loss of two potential N-glycosylation sites (16). Similar changes might occur when other SHIVs are passaged multiple times in animals.

The demonstration of highly divergent, strain-specific neutralization epitopes on SHIV-HXB2, SHIV-89.6, and SHIV-89.6P (including molecularly cloned SHIV-KB9) has important implications for the choice of challenge virus when assessing HIV-1 envelope glycoprotein vaccines in monkeys. For example, it might be essential to match the challenge virus to the vaccine strain in order to achieve maximum benefit from neutralizing antibodies raised by experimental HXB2, 89.6, and 89.6P vaccines. These results also provide a foundation for studies in which animals may be challenged with a SHIV that is heterologous to the vaccine strain as far as in vitro neutralization determinants are concerned. This will be particularly useful when assessing correlates of immunity and breadth of efficacy in the SHIV model. Finally, these SHIV variants may be used in addition to other strains of HIV-1

TABLE 1. Breadth of neutralizing antibody responses in SHIV-infected macaques

Macaque	Wk of infection	NAb titer against[a]:					No. of primary isolates neutralized[b]
		SHIV-89.6	SHIV-89.6P	SHIV-HXB2	HIV-1 strain		
					MN	SF-2	
SHIV-HXB2 infected							
471-92	21	<20	NT	245	<20	NT	0
L28	21	NT	NT	1,536	NT	NT	0
149-93	32	<20	NT	172	24	NT	0
L23	40	87	NT	>3,200	5,796	751	0
H123	76	98	NT	>3,200	1,565	1,610	1
8A2	76	NT	NT	>3,200	291	459	2
T06	100	<10	NT	845	212	347	1
R96	100	16	NT	783	88	326	0
L3	102	NT	NT	168	62	246	0
8A2	114	21	NT	>3,200	874	711	3
L28	124	18	NT	2,427	195	1,076	3
SHIV-89.6 infected							
123-93	32	1,962	156	<20	387	NT	0
123-93	124	2,484	30	40	659	388	0
504-92	124	3,539	13	<10	124	48	0
SHIV-89.6′ infected							
351-80	96	514	77	<10	1,518	866	0
145-84	96	95	42	<10	196	41	0
SHIV-89.6PD infected							
RQ1060	11	<20	1,503	<20	139	<20	NT
RQ1060	38	106	333	<20	1,753	129	0
RQ1128	11	<20	140	<20	37	<20	NT
RQ1128	38	1,628	4,755	33	411	117	0

[a] HIV-89.6 and SHIV-89.6P were grown in human PBMCs, whereas SHIV-HXB2, HIV-1 MN, and HIV-1 SF-2 were grown in CEMx174 cells. Cell-free viruses were incubated with multiple dilutions of serum samples in triplicate for 1 h at 37°C in 96-well plates. MT-2 cells (SHIV-89.6, SHIV-89.6P, SHIV-HXB2, and HIV-1 MN) or CEMx174 cells (HIV-1 SF2) were added, and the incubation continued until virus-induced cell killing was observed in virus control wells (no test sample), which usually took 3 to 6 days. Neutralizing antibody (NAb) titers are given as the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing. NT, not tested.

[b] Cell-free viruses were incubated with a 1:4 final dilution of serum samples in triplicate for 1 h at 37°C in 96-well plates. PBMCs in interleukin 2 (IL-2) growth medium were added, and the incubation continued for another 3 h. The cells were then washed three times, resuspended in IL-2 growth medium, and incubated at 37°C in fresh 96-well plates. Immediately after resuspension, 25 μl was removed and mixed with 225 μl of 0.5% Triton X-100 spiked with a known amount of HIV-1 p24 to test for interference by residual anti-p24 antibody in the antigen enzyme-linked immunosorbent assay. An additional 25 μl of culture fluids was removed every 2 days for measurement of p24 production; the 25 μl removed was replaced each time with fresh IL-2 growth medium. Quantities of p24 in virus control wells (no test sample) were determined for each collection day. Neutralization was assessed in the remaining wells at a time when p24 in virus control wells exceeded 10 ng/ml but had not yet reached peak values (usually 4 to 6 days). Neutralization was considered to have occurred when p24 synthesis was reduced by >80% relative to the virus control. Results are given as the number of primary isolates neutralized per total number of isolates tested (n = 6). All positive results are considered true antibody-mediated neutralization, since we found no evidence of interference by anti-p24 antibodies. NT, not tested.

to assess the breadth of neutralizing antibodies raised by experimental vaccines.

Neutralizing antibody responses in SHIV-infected macaques have additional relevance to HIV-1 vaccine development that was worth investigating. For example, infection with highly attenuated SHIV-HXB2 and SHIV-89.6 is analogous to immunizing with a mixture of different forms of molecularly cloned viral envelope glycoproteins from a single strain of virus, with the understanding that the glycoproteins may undergo sequence variation during infection. The different forms of envelope glycoproteins would include native oligomers on intact virus particles in addition to monomeric gp120 and gp41 and fragments thereof released into circulation by infected cells or when infected cells are lysed by cytotoxic T lymphocytes. It was therefore of interest to determine whether SHIV infection raised antibodies that are capable of neutralizing primary isolates. This became more of an interest once it was determined that some of these antibodies had potent neutralizing activity against heterologous TCLA variants of HIV-1. Similar assessments in humans have shown that antibodies raised in response to HIV-1 infection neutralize primary isolates occasionally but not with nearly the frequency or titer by which they neutralize TCLA variants (3, 4, 17, 26, 29, 33, 42). Assessments in the SHIV macaque model are of particular interest because the infecting strain of virus is known and, in some cases, is molecularly cloned and highly attenuated.

Antibodies raised in response to chronic SHIV-HXB2 infection occasionally neutralized one or more primary isolates, but only after the animals had been infected for more than 40 weeks (Table 1). No neutralization of heterologous primary isolates was seen for serum samples from macaques infected with either SHIV-89.6, SHIV-89.6′, or SHIV-89.6PD (Table 1), although sera from SHIV-89.6-infected macaques were able to neutralize the parental, uncloned HIV-1 89.6 primary isolate (Table 3). The last of these serum samples had similar neutralization titers against HIV-1 89.6, molecularly cloned HIV-1 89.6mc, and SHIV-89.6 (Table 3), indicating that the molecular and biological manipulations used in the construction of HIV-1 89.6mc and SHIV-89.6 produced little if any changes in the neutralization determinants of the parental virus. Due to the small number of SHIV-89.6-infected animals evaluated and to possible immunosuppression in animals in-

TABLE 2. Neutralizing antibodies in sera from macaques infected with SHIV-KB9

Macaque	Wk of infection	NAb titer against[a]:		
		SHIV-89.6	SHIV-89.6P	SHIV-KB9
15434	Pre[b]	<20	<20	<20
	8	<20	<20	<20
	17	<20	351	209
	29	<20	514	227
	41	28	42	117
18429	Pre	<20	<20	<20
	8	<20	3,415	2,226
	17	<20	4,374	3,064
	29	<20	1,719	369
	41	162	124	180

[a] All viruses were grown in human PBMCs and were assayed in an MT-2 cell-killing assay. Neutralizing antibody (NAb) titers are given as the reciprocal of the last serum dilution that was positive for neutralization.
[b] Pre, preinfection.

fected with SHIV-89.6′ and SHIV-89.6PD, our results do not necessarily imply that infection with SHIV-HXB2 is more likely to raise neutralizing antibodies to primary isolates than is infection with other SHIV variants.

Differences in either the amount of antigen or the ratio of oligomeric to monomeric envelope glycoproteins produced during infection might explain why only SHIV-HXB2 raised antibodies that occasionally neutralized primary isolates. SHIV-89.6 has been shown to replicate to higher levels than SHIV-HXB2 in rhesus macaques (35), which means there should have been no relative shortage of antigen in SHIV-89.6-infected animals. Measurements of oligomeric and monomeric envelope glycoprotein in infected animals are technically difficult and were not attempted here. It is also possible that sequence variation in the SHIV-HXB2 *env* gave rise to a wide range of neutralization epitopes during infection. In a previous study, 5 to 14 amino acid changes were shown to be present in molecularly cloned *env* from two monkeys infected for 55 to 66 weeks with SHIV-HXB2, and one of these clones was less sensitive to neutralization than the parental HXB2 clone (20). Neutralizing antibodies to primary isolates raised by SHIV-HXB2 in our studies were detected after 76 to 124 weeks of infection but not after 21 to 40 weeks of infection (Table 1). This requirement for long-term infection is consistent with sequence variation being a critical component of the ability to raise antibodies that neutralize primary isolates. Less variation in *env* might have occurred during infection with the SHIV-89.6 series of variants, possibly owing to differences in their coreceptor usage and cellular tropism compared to that of HXB2 (5–7, 28) or to the overall host response to infection. Finally, we cannot exclude the possibility that the antibody response in SHIV-infected macaques requires a long time to mature (e.g., antibody affinity), independently of the source and sequence variation of *env*, before the antibodies are capable of neutralizing primary isolates.

TABLE 3. Neutralization of HIV-1 89.6, HIV-1 89.6mc, and SHIV-89.6 in human PBMCs by sera from macaques infected with SHIV-89.6

Macaque	Wk of infection	NAb titer against[a]:		
		HIV-1 strain		SHIV-89.6
		89.6	89.6mc	
123-93	124	25	25	25
504-92	124	125	125	125
504-92	32	125	NT	NT

[a] Serum samples from SHIV-89.6-infected macaques were evaluated for neutralizing antibodies (NAb) to viruses grown and assayed in human PBMCs as described in footnote *b* to Table 1, except that 10 μl of undiluted virus was incubated with 40 μl of diluted serum samples in triplicate such that the final serum dilutions were 1:5, 1:25, 1:125, and 1:625 after adding virus. Titers are given as the reciprocal of the last serum dilution that was positive for neutralization. Virus neutralization was considered positive when the production of p24 (HIV-1 89.6 and 89.6mc) or p27 (SHIV-89.6) was reduced by >80% relative to the virus control (no test sample). Culture fluids harvested after the last wash showed no interference by residual anti-p24 or anti-p27 antibodies in the antigen enzyme-linked immunosorbent assay. HIV-1 89.6mc is a molecular clone of HIV-1 89.6 (see text). NT, not tested.

The limited ability for SHIV infection to raise antibodies that neutralize heterologous primary isolates suggests that the use of oligomeric rather than monomeric envelope glycoproteins as immunogens is unlikely to solve the problem of raising broadly cross-reactive neutralizing antibodies to primary isolates when the immunogens are derived from a single strain of virus. In this regard, the ability of sera from SHIV-89.6-infected macaques to neutralize HIV-1 89.6 indicates that vaccines derived from molecularly cloned envelope glycoproteins will raise antibodies that are at least capable of neutralizing the homologous vaccine strain of virus when it is a primary isolate, as has been the case for TCLA strains. Whether this will require the structure of the immunogen to be monomeric or oligomeric remains to be determined. Efforts to improve the breadth of neutralization might require either a polyvalent vaccine composed of envelope glycoproteins from multiple primary isolates or a vaccine in which the structure of the envelope glycoproteins is modified so as to be capable of raising antibodies to conserved neutralization epitopes that are otherwise poorly immunogenic. These approaches will require greater knowledge of the immunogenicity of primary isolate envelope glycoproteins and of the structures on these proteins that are targets for neutralizing antibodies.

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