Human Rhinovirus Type 14:Human Immunodeficiency Virus Type 1 (HIV-1) V3 Loop Chimeras from a Combinatorial Library Induce Potent Neutralizing Antibody Responses against HIV-1

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In an effort to develop a useful AIDS vaccine or vaccine component, we have generated a combinatorial library of chimeric viruses in which the sequence IGPGRFYTKN from the V3 loop of the MN strain of human immunodeficiency virus type 1 (HIV-1) is displayed in many conformations on the surface of human rhinovirus 14 (HRV14). The V3 loop sequence was inserted into a naturally immunogenic site of the cold-causing HRV14, bridged by linkers consisting of zero to three randomized amino acids on each side. The library of chimeric viruses obtained was subjected to a variety of immunoselection schemes to isolate viruses that provided the most useful presentations of the V3 loop sequence for potential use in a vaccine against HIV. The utility of the presentations was assessed by measures of antigenicity and immunogenicity. Most of the immunoselected chimeras examined were potently neutralized by each of the four different monoclonal anti-V3 loop antibodies tested. Seven of eight chimeric viruses were able to elicit neutralizing antibody responses in guinea pigs against the MN and ALA-1 strains of HIV-1. Three of the chimeras elicited HIV neutralization titers that exceeded those of all but a small number of previously described HIV immunogens. These results indicate that HRV14:HIV-1 chimeras may serve as useful immunogens for stimulating immunity against HIV-1. This method can be used to flexibly reconstruct varied immunogens on the surface of a safe and immunogenic vaccine vehicle.

The development of a suitable vaccine for the prevention of AIDS remains a formidable challenge after more than 15 years of worldwide AIDS research. The immunological correlates of protection against infection by the human immunodeficiency virus (HIV) are currently unclear. It has been shown that passive immunization can provide protection against HIV (19, 20, 25, 50, 56) and the related lentiviruses, simian immunodeficiency virus (SIV) (11) and feline immunodeficiency virus (FIV) (34). Furthermore, correlations between serum neutralizing antibody levels and protective immune responses have been reported in some vaccination-and-challenge studies involving HIV-1 in chimpanzees (7, 8, 13, 18, 28), SIV in macaques (3, 36, 41, 43, 58, 69), and FIV in cats (35, 70, 71). Thus, it is likely to be advantageous for an HIV vaccine to elicit a long-lasting neutralizing antibody response. Such a response should be elicited both systemically and mucosally since HIV can be transmitted both directly into blood and across mucosal surfaces. It may also be critical in the case of HIV-1 to stimulate an effective cell-mediated immune response.

Traditional vaccine approaches, such as those involving live-attenuated or whole-inactivated HIV, are associated with safety concerns that need to be addressed before their wide-

spread use can be considered. To develop a suitable vaccine for the prevention of AIDS, we have been investigating the vaccine potential of recombinant human rhinoviruses that display HIV-1 epitopes on their surfaces. The goal of this research is to identify one epitope, or more likely a combination of epitopes, that can act in concert to provide safe and protective immunity.

Chimeric human rhinoviruses have the potential to serve as safe and effective vaccine vectors. Rhinoviruses cause common colds and are capable of stimulating robust immune responses including significant systemic and mucosal responses (reviewed in references 14 and 17). Furthermore, since nasal administration of antigens appears to be one of the most effective means for inducing both systemic and mucosal immune responses (16, 22, 23, 61), it is especially favorable that the natural site of infection for human rhinoviruses is the nasal epithelium and associated lymphoid tissues (reviewed in references 14 and 33).

To achieve the goal of creating an effective rhinovirus-based vaccine for HIV, we have been generating libraries of live recombinant human rhinoviruses that display HIV epitopes. To find the members of such libraries that best present the foreign sequences in conformations capable of inducing strong neutralizing responses, we have used immunoselection techniques. Human rhinovirus type 14:HIV-1 (HRV14:HIV-1) chimeras containing V3 loop sequences recognized and neutralized by multiple neutralizing anti-HIV-1 V3 loop antibodies should have an increased likelihood of inducing potent neutralizing immune responses against HIV.

This paper describes the production of an HRV14:HIV-1 library encoding a V3 loop sequence from the MN strain of HIV-1. The V3 loop was chosen because it is one of the regions of HIV-1 that elicits a significant neutralizing immunogenic response in the majority of HIV-infected individuals (65). The

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sequence IGPGRFYTTKN was chosen for transplantation for several reasons. First, it is representative of sequences found in clade B, the most prevalent clade found in North America and western Europe (38, 46). Second, this segment has been shown to bind to and elicit the production of neutralizing antibodies (30, 49, 54). Third, this region of the V3 loop has also been demonstrated to contain or be part of both human and murine cytotoxic T-lymphocyte and T-helper epitopes (55, 62, 63). In addition, there are well characterized anti-HIV-1_{MN} antibodies available for immunoselecting and characterizing chimeric viruses from the library. The V3 loop sequence was flanked by randomized linkers of variable sequence and length, resulting in the presentation of the V3 loop sequence in many conformations. An immunoselection scheme using up to four monoclonal antibodies (MAbs) consecutively to identify HIV-like presentations of the V3 loop sequences was employed.

The value of this approach was demonstrated by the observation that most of the chimeras selected were potentially neutralized by anti-HIV antibodies and were capable of eliciting the production of antibodies that could neutralize HIV-1 in cell culture with significant titers. The ability of three of eight HRV14:HIV-1 chimeras to elicit serum antibodies capable of inhibiting 90% of HIV infectivity in cell culture with reciprocal neutralizing titers of approximately 400 or higher is indeed noteworthy. The number of HIV immunogens reported to block the infectivity of HIV with this level of stringency is very limited (some mixtures of gp160 with peptides or proteins [6, 27, 28], a gp120 construct [7], a few V3 loop peptides [1, 2, 64, 66], an influenza:V3 loop chimera [40], a Ty:V3 loop chimera [31], and several HRV14:HIV-1 chimeras [this work and reference 51]). Thus, HRV14:HIV-1 chimeras are among the most potent immunogens for HIV neutralization that have been reported.

MATERIALS AND METHODS

Cells, viruses, and media. H1-HeLa cells (39) were used for the propagation of HRV14 and HRV14:HIV-1 chimeras. Medium M and PA medium have been described previously (51). *Escherichia coli* JS4 cells (Bio-Rad Laboratories) were used for electroporation of plasmid DNA. H9/FDA cells (42) were used for propagation of HIV-1 (67). HIV-1 neutralization experiments were performed with CEM-SS cells (47) (from S. Zolla-Pazner) and three strains of HIV-1: MN (24, 59) (National Institutes of Health AIDS Research and Reference Reagent Program; from R. Gallo), IIB (24) (from S. Zolla-Pazner), and ALA-1 (21) (from S. Zolla-Pazner).

Antibodies. Four monoclonal neutralizing anti-HIV antibodies were used to select for and characterize chimeric viruses that would ideally have HIV-like antigenic and immunogenic properties. These MAbs are as follows: (i) human MAb (HuMAb) 694/98-D (mapped to V3 loop sequence GRAF [29]; from M. Gorny and S. Zolla-Pazner), (ii) HuMAb 447-52-D (mapped to GPXR, where X is essentially any amino acid [29, 37]; capable of neutralizing clade B primary isolates of HIV-1 [12]; from S. Zolla-Pazner and S. Koenig), (iii) mouse MAb (MuMAb) 59.1 (mapped serologically [67] and structurally [26] to V3 loop sequence GPGR, from immunization with cyclic V3 loop peptides with the HIV-1_{MN} sequence; from A. Profy, Repligen Corporation), and (iv) MuMAb NM-01 (mapped to V3 loop sequence GPGR, from immunization with HIV-1_{MN} [48]; from M. Terada).

Construction and generation of an HRV14:HIV-1_{MN} V3 loop library. Mutagenic cassettes encoding V3 loop sequence IGPGRFYTTKN and zero to three flanking randomized residues were generated as described previously and ligated into the p3IIST plasmid (60). Mutagenized plasmids were electroporated into JS4 cells with a Gene Pulser System (Bio-Rad Laboratories), using conditions recommended by the manufacturer. Transformed cells were grown in bulk liquid cultures at 30°C, and the plasmids were isolated via the alkaline lysis method (57). Plasmid pools were used for in vitro transcription and transfection reactions. RNA was transfected into H1-HeLa cells via DEAE-dextran-mediated transfection (44) or electroporation. RNA electroporation was done by mixing 0.1 to 10 µg of RNA with 10⁷ H1-HeLa cells in 0.4 ml of serum-free minimum essential medium (Gibco catalog no. 1-1090-081) and pulsing with 250 volts at 960 µF. Pulsed cells were subsequently plated with twice as many unpulsed cells for the harvesting of virus. Chimeric viruses were harvested from cultures as previously described (51). To determine the number of transfectants obtained,

pulsed cells were mixed with unpulsed calls at various ratios and the mixtures were then plated to generate isolated plaques.

Screening of the library with MAbs that neutralize HIV-1. Chimeric HRV14:HIV-1 viruses were selected with a panel of MAbs (shown below) as described previously (51), with the following modifications. The coating concentration of antibody varied between 0.1 and 0.2 µg/ml depending on the antibody used. The concentration of virus added to the wells was 1×10^6 to 3×10^6 PFU/ml.

Propagation, purification, and sequencing of immunocaptured chimeric viruses. Pools of immunoselected chimeric viruses were propagated and purified (72), and individual isolates were obtained following two rounds of plaque purification. To confirm the presence of the HIV V3 loop sequence and to determine the number and composition of the randomized residues, PCR products derived from cDNA copies of the viral RNA were sequenced with the fmol sequencing kit (Promega, Madison, Wis.).

Microtiter neutralization assays. Fifty microliters of medium M containing 10⁴ PFU of chimeric viruses was added in quadruplicate to wells containing 50 µl of twofold dilutions of anti-HIV-1 MAbs (694/98-D, 447-52-D, 59.1, or NM-01) in medium M. After 1 h at room temperature, 50 µl containing 10⁴ H1-HeLa cells was added. After 2 to 3 days at 34.5°C and 2.5% CO₂, virus neutralization was assessed by a cytotoxicity assay (45). Fifteen microliters of a 5-mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in phosphate-buffered saline (PBS) was added to all wells. After 1.5 h at 34.5°C, 150 µl of 20% sodium dodecyl sulfate in 50% *N,N*-dimethylformamide was added to all wells. The absorbance at 570 nm was then determined and expressed as a percentage of the average absorbance from wells that received cells only (corresponding to 100% viability). Titers are expressed as the reciprocals of the dilutions of antibodies that reduced cell death by 50%.

Plaque reduction assay. Approximately 50 to 100 PFU of chimeric virus (in medium M) was mixed with a fixed concentration of antibody (HuMAb 447-52-D at 10 ng/ml in medium M) in a total volume of 250 µl. The virus antibody mixtures were incubated for 1 h at room temperature. Then, 200 µl of each mixture was plated onto 60-mm-diameter tissue culture dishes containing approximately 1.5×10^6 H1-HeLa cells. After 1 h, the inoculum was aspirated and the monolayers were washed with Dulbecco's PBS+ (D-PBS+; Gibco catalog no., 14080-022), overlaid with 5 ml of PA medium containing 0.5% Noble agar (Difco, Detroit, Mich.) and incubated at 34.5°C for approximately 72 h. The monolayers were then fixed with formalin and stained with crystal violet. Plaques were counted, and the results were expressed as percentages of the control receiving no antibody.

Immunization of guinea pigs. Approximately 50 µg of purified chimeric virus emulsified in Complete Freund's adjuvant was used for the primary subcutaneous inoculations of three guinea pigs (Dunkin Hartley; Cocalico Biologicals, Reamstown, Pa.) for each chimera. Fifty micrograms of purified chimeric virus emulsified in incomplete Freund's adjuvant was used for all boosts. The following inoculation schedule was employed: week 0, prebleed and first immunization; week 4, second immunization; and week 10, third immunization. Sera were collected by femoral bleeds 2, 3, and 4 weeks following the second and third immunizations (i.e., at weeks 6, 7, and 8, and 12, 13, and 14). The immune responses of the guinea pigs were monitored by enzyme-linked immunosorbent assays (ELISAs) using an immobilized octameric peptide containing the V3 loop sequence of HIV-1_{MN} (66).

HIV-1 neutralization assay. The neutralization assay used was a modification of the standard serum neutralization assay described by White-Scharf et al. (67). Test samples consisted of either unfractionated guinea pig sera or the immunoglobulin G (IgG) fraction obtained by purification with a protein A-agarose column (Pharmacia). The eluted antibodies were equilibrated with D-PBS (without calcium and magnesium), pH 6.5, by using a 30,000-molecular weight-cutoff Centricon filter (Amicon, Beverly, Mass.). The serum equivalents of the purified IgG fraction were determined by ELISAs. Reverse transcriptase (RT) activity was determined on day 4 by the method of Willey et al. (68), and the results were quantitated with a Molecular Dynamics phosphorImager. Reciprocal neutralization titers were determined from data averaged over at least two experiments. The antibody concentrations that gave 50 and 90% reductions in RT activity were derived with the Kaleidagraph (Synergy Software, Reading, Pa.) curve-fitting program.

RESULTS

Construction of an HRV14:HIV-1_{MN} V3 loop library. Previous work from our laboratory has demonstrated that it is possible to create libraries of viable HRV14:HIV-1 chimeras that present V3 loop sequences or mimotopes thereof by random systematic mutagenesis (51, 60). To further investigate the potential of this system, a library of chimeric human rhinoviruses displaying a core sequence derived from the V3 loop of the MN isolate of HIV-1 was produced.

The core HIV-1 sequence, IGPGRFYTTKN, was flanked by zero to three randomized residues. The randomized residues act as linkers or adaptors connecting the HIV-1 sequence

FIG. 1. Chimeric virus pools generated by selection with anti-V3 loop antibodies. Chimeric viruses were immunoselected initially with one of three anti-V3 loop MAbs. Recovered viruses were propagated and then subjected to a second round of selection employing an antibody not used in the first round. The sequence was repeated to generate pools of chimeric viruses immunoselected with up to four different anti-V3 loop antibodies. Numbers in boxes indicate the antibodies used for selection as follows: 1, HuMAb 694/98-D; 2, MuMAb NM-01; 3, MuMAb 59.1; and 4, HuMAb 447-52-D. The order of numbers corresponds to the order of immunoselection. For example, designation 12 signifies that the pool was selected with HuMAb 694/98-D in the first round of selection and MuMAb NM-01 in the second round. MN designations indicate the selected pools that were chosen, on the basis of their neutralization profiles, as sources from which to obtain individual isolates to be further characterized.

to HRV14. The site chosen for insertion is between alanine 159 and asparagine 160 of the surface loop connecting β strands E and F of the VP2 coat protein. This loop is the largest of three loops that constitute neutralizing immunogenic site II (NIm-II site) of HRV14 (4, 53). A total of 7.1×10^7 possible unique members can be encoded by this library.

E. coli JS4 cells were electroporated with ligated plasmids encoding chimeric viruses, yielding 8.0×10^7 transformants. DNA obtained from these transformants was transcribed to create a pool of infectious chimeric viral RNAs that were used to transfect H1-HeLa cells. Approximately 1.5×10^6 transfectants were obtained.

Immunoselection of chimeric viruses. To enrich for chimeric viruses with V3 loop sequences transplanted in immunologically relevant conformations, a sequential immunoselection procedure using four different anti-V3 loop MAbs was employed (Fig. 1; antibodies described in Materials and Methods). The first round of selection was performed with either HuMAb 694/98-D, MuMAb NM-01, or MuMAb 59.1. The second round of selection was performed with each of the other MAbs. HuMAb 447-52-D was used for selection in the third round. The fourth round of selection was conducted with MAbs 694/98-D, NM-01, or 59.1 to include the missing antibody from each set.

To evaluate the effects of immunoselection on the characteristics of the resulting chimeric virus pools, microtiter neutralization assays were used. The MAbs used in the selection process were evaluated for their abilities to neutralize the original unselected (US) pool or the pools resulting from the various stages of the selection process. While mechanisms of neutralization of chimeric viruses are likely to be different from mechanisms of neutralization of HIV-1, this assay was chosen in preference to the less-stringent measures afforded by antibody-binding assays (e.g., in ELISAs, Western blotting, or immunoprecipitation assays).

Figure 2 illustrates two examples of the ability of a particular antibody, HuMAb 447-52-D, to neutralize various pools of virus. Antibody selection effectively resulted in the generation of chimeric virus pools with antigenic profiles that were significantly altered compared to those of the unselected pool. Immunoselection of chimeric viruses with HuMAb 694/98-D (Fig. 2A) but not with MuMAb 59.1 (Fig. 2B) resulted in an increase in the average degree of neutralization by HuMAb 447-52-D. Increases in the ability of HuMAb 447-52-D to neutralize virus pools were seen after subsequent immunoselection steps until a maximum gain was achieved (Fig. 2). While only two examples are shown, the overall pattern seen was that, for each immunoselection series (except for the series initiated with MAb NM-01), the ability of any given antibody to neutralize a chimeric virus pool increased with multiple rounds of selection.

To confirm these results, plaque reduction assays were also performed on various pools with fixed concentrations of antibodies. Results for the selection series shown in Fig. 2 are shown in Fig. 3. A comparison of the plaque reduction data with the microtiter neutralization assay data indicates that the same trends and conclusions could be made on the basis of the results of either method.

Based in part on their differing antigenicity characteristics and extents of immunoselection, the six pools designated with an MN code in Fig. 1 were selected for additional characterization. These pools were propagated and purified. Two rounds of plaque purification were used to obtain 59 isolates for further characterization (14 from MN-I, 15 from MN-II, 15 from MN-III, and 5 each from MN-IV, MN-V, and MN-VI).

Analysis of chimeric virus sequences. The sequences of 30 clones from the unselected pool were determined (Table 1). Twenty-nine of the 30 sequences were unique, and none matched those of the final 10 immunoselected clones characterized in the greatest detail. Five of the unselected clones had alterations to the transplanted HIV-1 V3 sequence (US3, US5, US6, US23, and US27). Four of these had deletions (US3,

FIG. 2. Neutralization of chimeric virus pools by HuMAb 447-52-D following different selection schemes. Pools resulting from sequential selection of HRV14: HIV-1 chimeras with anti-HIV MAbs were tested for their susceptibilities to neutralization by HuMAb 447-52-D. Neutralization is expressed as the percentage of protection from infection (measured as cell viability for virus-treated cells relative to that for untreated cells).

FIG. 3. Plaque reduction of chimeric virus pools by HuMAb 447-52-D. Pools resulting from sequential selection of HRV14:HIV-1 chimeras with anti-HIV MAbs (in the order shown) were tested for their susceptibilities to neutralization by MAb 447-52-D. Fifty to 100 PFU of virus from the indicated pools was incubated with a fixed concentration of HuMAb 447-52-D and then plated onto H1-HeLa cell monolayers. After 3 days, the numbers of plaques were determined. Plaque reduction is expressed as the percentage of the number of plaques observed in the presence of HuMAb 447-52-D relative to the number observed in the absence of antibody.

US5, US6, and US23); two had mutations (US6 and US27). The remaining 25 unselected chimeras all contained the full-length, unmutated HIV-1 V3 loop sequence.

The number of randomized positions in this library was designed to vary from zero to three residues on each side of the fixed core HIV-1 V3 loop sequence. Even in the absence of immunoselection, the numbers of residues in the N-terminal (left) and C-terminal (right) linkers are strongly asymmetric. Nineteen of the 30 unselected chimeras (63%; Table 1; Fig. 4) and 7 of the final 10 immunoselected chimeras (70%; Table 2) contained three amino acids in the randomized N-terminal linkers, but only 2 of 30 unselected chimeras (7%) and 1 of the final 10 immunoselected chimeras (10%; having part of the V3 loop sequence deleted) had three amino acids in the C-terminal randomized linker. In contrast, 15 of the 30 unselected chimeras (50%) did not have any randomized residues on the C-terminal side, as was also the case for 7 of the 10 final immunoselected chimeras (70%). In fact, there was only 1 chimera, MN-III-10, with an intact V3 loop insert among the final immunoselected 10 that had any C-terminal linker at all.

The percentages of occurrence of specific amino acid residues within the N- and C-terminal randomized linkers of unselected chimeras were also asymmetric (Fig. 4). At the N-terminal linkers, 37 of 70 (53%) residues were negatively charged. In fact, every N-terminal linker that had any residues had at least one negatively charged amino acid. This percentage is significantly different from what would be expected from random chance (6%) and contrasts with the distribution of negatively charged residues found at the C-terminal linkers (4 of 23; 17%) and of residues encoded by the plasmid DNAs used to produce the chimeric virus library (4 of 22 [18%] at the

TABLE 1. Sequences and charge characteristics of unselected (US) HRV14:HIV-1 chimeras

Virus	Sequence ^a					Charge ^b
	HRV14	NL	Core	CL	HRV14	
HRV14	...SSA				NEV...	-1
US1	SSA	EN	IGPGRAFYT TKN		NEV	0
US2	SSA	E	IGPGRAFYT TKN	EI	NEV	-1
US3	SSA	E	IGPGRAF Y		NEV	-1
US4	SSA	TDN	IGPGRAFYT TKN	L	NEV	0
US5	SSA		TKN		NEV	0
US6	SSA		IGPGRDL TTKN	YE	NEV	0
US7	SSA	TD	IGPGRAFYT TKN	V	NEV	0
US8	SSA	GSE	IGPGRAFYT TKN	F	NEV	0
US9	SSA	GTD	IGPGRAFYT TKN	EPT	NEV	-1
US11	SSA	PE	IGPGRAFYT TKN	AI	NEV	0
US12	SSA	GDS	IGPGRAFYT TKN	Q	NEV	0
US13	SS	E	IGPGRAFYT TKN		NEV	0
US14	SS	E	IGPGRAFYT TKN		NEV	0
US15	SSA	GSD	IGPGRAFYT TKN	DIP	NEV	-1
US16	SSA	ERD	IGPGRAFYT TKN		NEV	0
US17	SSA	ADD	IGPGRAFYT TKN		NEV	-1
US18	SSA	DER	IGPGRAFYT TKN		NEV	0
US19	SSA	D	IGPGRAFYT TKN		NEV	0
US20	SSA	GED	IGPGRAFYT TKN	L	NEV	-1
US21	SSA	EEA	IGPGRAFYT TKN		NEV	-1
US22	SSA	DTN	IGPGRAFYT TKN	T	NEV	0
US23	SSA	QSD	IGPGRAFYT TKN		NEV	0
US24	SSA	NGE	IGPGRAFYT TKN		NEV	0
US25	SSA	SEE	IGPGRAFYT TKN		NEV	-1
US26	SSA	SQE	IGPGRAFYT TKN	Y	NEV	0
US27	SSA	SEP	IGPGEHF IQQKI	TG	NEV	-2
US28	SSA	EHD	IGPGRAFYT TKN		NEV	-1
US29	SSA	EDS	IGPGRAFYT TKN	L	NEV	-1
US31	SSA	ED	IGPGRAFYT TKN	S	NEV	-1
US32	SSA	NSE	IGPGRAFYT TKN		NEV	0

^a Sequences are (in order) those of HRV14 VP2 residues (HRV14), variable length and sequence N-terminal linker residues (NL), core HIV-1_{MN} V3 loop residues (Core), variable length and sequence C-terminal linker residues (CL), and HRV14 VP2 residues (HRV14).

^b Net charge of loop for HRV14 residues SSA through NEV.

N-terminal linkers and 3 of 18 [17%] at the C-terminal linkers; data not shown). In addition, only 2 of 70 (3%) residues of the N-terminal linkers were positively charged, in contrast with an expected 13% and an occurrence of 5 of 22 (23%) positively charged residues in the plasmid DNAs.

FIG. 4. Lengths and amino acid compositions of N-terminal and C-terminal linkers of unselected HRV14:HIV-1 chimeras. (A) The percent occurrences at which 0, 1, 2, and 3 amino acids appear in the amino- and carboxy-terminal linkers are indicated along with the expected occurrences based on the molar quantities of oligonucleotides used in the construction of the library. (B) The percentages of linker residues that are charged (Arg, Asp, Glu, Lys), polar (Asn, Gln, His), or hydrophobic (Ile, Leu, Met, Phe, Tyr, Trp, Val) or that have the propensity to form turns (Gly, Pro, Ser, Thr) are shown for the amino- and carboxy-terminal linkers as are the expected occurrences due to chance.

TABLE 2. Neutralization of HRV14:HIV-1 chimeras by anti-V3 loop antibodies and neutralization of HIV-1 by antichimera antisera

Chimera ^c	Sequence inserted into NIm-II site ^d	Neutralization of chimeric HRVs ^a				Neutralization of HIV-1 ^b				
		694/98-D	447-52-D	59.1	NM-01	Guinea pig no.	Titer vs HIV-1 _{ALA-1}		Titer vs HIV-1 _{MN}	
							90%	50%	90%	50%
MN-I-4	<u>DNG</u> IGPGRAFYT ^f TKN	100	40	10	30	42	950	2,580	30	ND
						43	<10	50	110	ND
						44	<10	30	<10	150
MN-II-11	<u>SA</u> IGPGRA <u>LN</u> ^f <u>Y</u>	>1,750	20	410	300	60	<10	<10	<10	<10
						61	<10	<10	<10	<10
						62	<10	<10	<10	<10
MN-III-2	<u>ADT</u> IGPGRAFYT ^f TKN	40	110	20	10	45	380	ND	<20	130
						46	50	ND	100	530
						47	10	200	<10	40
MN-III-6	<u>DDS</u> IGPGRAF <u>SKN</u> ^f	>1,000	>1,000	200	120	ND	ND	ND	ND	ND
MN-III-8	<u>EER</u> IGPGRAFYT <u>S</u> ^f	20	20	10	10	48	20	150	<10	50
						49	140	ND	<10	160
						50	70	ND	<10	<10
MN-III-10	<u>NES</u> IGPGRAFYT ^f TKN <u>L</u>	80	50	10	10	63	60	ND	10	ND
						64	50	ND	<10	140
						65	90	ND	90	ND
MN-III-11	<u>EE</u> IGPGRAFYT ^f TKN	210	80	45	20	66	50	ND	<10	240
						67	<10	40	<10	10
						68	190	ND	100	920
MN-III-12	<u>GES</u> IGPGRAFYT ^f TKN	190	30	40	20	51	40	260	200	ND
						52	490	ND	320	1,030
						53	150	ND	<10	200
MN-III-15	<u>DD</u> IGPGRAFYT ^f TKN	240	>1,000	40	20	ND	ND	ND	ND	ND
MN-V-2	<u>TEG</u> IGPGRAFYT ^f TKN	100	50	20	10	54	<10	280	<10	40
						55	<10	40	<10	1,200
						56	<10	30	<20	310

^a Anti-chimera neutralization titers are expressed as concentrations of antibody (in ng/ml) that resulted in a 50% reduction in cell viability in treated wells compared to control wells receiving no virus or antibody. HRV, human rhinovirus.

^b Anti-HIV-1 neutralization titers are expressed as the reciprocal of the dilution of serum that results in a 90 or 50% reduction in RT activity in culture supernatants compared to controls receiving virus only.

^c Abbreviations correspond to chimeric virus isolates obtained from pools, subjected to sequential immunoselection by the following antibodies: MN-I: 694/98-D, NM-01, 447-52-D, and 59.1; MN-II: NM-01, 59.1, 447-52-D, and 694/98-D; MN-III: 59.1, NM-01, 447-52-D, and 694/98-D; MN-V: 694/98-D and 59.1; and MN-VI: 59.1 and NM-01.

^d Residues appearing at randomized positions are underlined.

^e ND, not determined.

^f Part of the common core HIV sequence is deleted for this sequence.

One result of the asymmetric distribution of negatively charged residues (Asp and Glu) between the N- and C-terminal linkers is that the mutagenized loop maintains a net neutral or negative charge. Normally, the HRV14 loop connecting β strands E and F of VP2 has a net charge of -1 (between serine 157 and valine 162). The V3 loop chosen for transplantation contains two positively charged residues and no negatively charged residues. All of the chimeras sequenced were found to have a net charge of either -1 or 0 in this region (with the exception of US27, which has a net charge of -2 and six amino acid mutations in its core HIV sequence) (Table 1). Similar results have been obtained with two other HRV14:HIV-1 V3 loop libraries in which 29 of 30 viable chimeras had net loop charges between -2 and 0 (51, 60). The asymmetric distribution of negatively charged residues and the bias toward overall neutral or negative loop charge suggest that these asymmetric distributions may reflect constraints on virus viability.

While the N- and C-terminal randomized positions did not exhibit unusual distributions of polar residues (Asn, Gln, and His) and residues often associated with turns (Gly, Pro, Ser, and Thr), the distribution of hydrophobic residues (Ile, Leu, Met, Phe, Tyr, Trp, and Val) was strikingly different for the two termini (Fig. 4). No hydrophobic residues were observed at the 70 N-terminal randomized positions, while 10 of the 23 (43%) C-terminal randomized positions were found to be hydropho-

bic. An asymmetric distribution of hydrophobic residues were also found in a previous HRV14:HIV-1 V3 loop library in which no hydrophobic residues were found at the 48 N-terminal randomized positions but in which 23 of the 48 (48%) C-terminal randomized positions were hydrophobic (60). The sequence distributions of the plasmid DNAs used to produce infectious RNA transcripts did not exhibit these asymmetries and, instead, appeared to be random.

Only a marginal loss of diversity was seen after one round of selection. However, with an additional round of selection, half of the pools examined were homogeneous. By the fourth round of selection, only one of three pools examined (MN-III) remained heterogeneous (data not shown).

In addition to the 30 unselected isolates, 47 of the 59 immunoselected and purified isolates were sequenced. All 5 of the isolates characterized from the pool designated MN-IV (immunoselected with HuMAb 694/98-D and MuMAb NM-01), as well as all 10 isolates sequenced from the derivative MN-I pool (immunoselected with HuMAb 694/98-D, MuMAb NM-01, HuMAb 447-52-D, and MuMAb 59.1) turned out to have one sequence (represented in Table 2 as the MN-I-4 sequence). Likewise, all nine isolates sequenced from the MN-II pool (immunoselected with MuMAb NM-01, MuMAb 59.1, HuMAb 447-52-D, and HuMAb 694/98-D) have a common sequence (represented in Table 2 as the MN-II-11 sequence). The MN-

III, MN-V, and MN-VI pools had 7 of 13, 3 of 5, and 4 of 5 unique sequences, respectively (some of which are shown in Table 1). In total, 10 immunoselected isolates that contained unique sets of randomized residues were identified (Table 2). In three of these isolates (MN-II-11, MN-III-6, and MN-III-8) part of the HIV-1 sequence was deleted.

The sequences of a number of chimeras were determined after as many as 12 rounds of viral replication. In each case, the inserted sequences were found to be unchanged (data not shown).

Antigenic characteristics of the chimeric virus isolates. Microtiter neutralization assays were employed to evaluate the antigenic characteristics of the HRV14:HIV-1 chimeras. Four anti-HIV-1 V3 loop antibodies (MuMAbs 59.1 and NM-01 as well as HuMAbs 447-52-D and 694/98-D) were tested for their ability to neutralize the individual chimeric viruses.

All four antibodies used in the selection process were tested for their ability to neutralize the individual immunoselected HRV14:HIV-1 chimeras. As indicated in Table 2, all four antibodies were able to neutralize all but 3 (i.e., MN-II-11, MN-III-6, and MN-III-15) of the 10 chimeras tested. Chimeras MN-II-11 and MN-III-6 had deletions within the V3 loop insert sequence; MN-III-15 had an intact V3 loop insert. Based in part on the antigenic character of the chimeric viruses, 8 of the 10 unique chimeras were used in immunogenicity studies with guinea pigs (resulting in the elimination of MN-III-6 and MN-III-15). MN-II-11 was chosen for immunogenicity studies to contrast with the other chimeras.

Immunogenicity of HRV14:HIV-1 chimeras. Eight chimeric viruses were used to immunize three guinea pigs each. Serum antibody responses were initially evaluated with ELISAs using an immobilized octameric peptide corresponding to the V3 loop of HIV-1_{MN} (66). All of the chimeras (with the exception of MN-II-11) were effective at eliciting the production of antibodies reactive with the MN peptide (e.g., at serum dilutions of 1:1,000; data not shown).

All of the serum samples were then tested at a few fixed concentrations for their abilities to neutralize the ALA-1, MN, and IIB strains of HIV-1 in cell culture. The serum sample from each animal that exhibited the most potent neutralization in the screening assay was then further characterized. Sera from guinea pigs immunized with MN-II-11 (guinea pigs 60 to 62) did not exhibit any neutralization activity in the screening assay.

Neutralization titers were determined by using a modification of the standard neutralization assay of White-Scharf et al. (67) (Table 2). Where possible, the dilution of antisera required to reduce RT levels by 90% was determined. In many cases where 90% titers could not be determined, 50% reduction values could be determined. While animal-to-animal variation is commonly a complication and a challenge in the development of effective immunogens, 90% inhibition titers of approximately 100 or greater against both the ALA-1 and MN strains were seen for at least one guinea pig for each of the chimeras tested with the exception of the chimeras known to be missing HIV-1 residues (MN-II-11 and MN-III-8) and only one other chimeric virus (MN-V-2). None of the guinea pigs produced antisera that were able to potently neutralize the IIB strain of HIV-1, although a few samples exhibited weak neutralization activity (50% inhibition titers of ≤ 80 ; data not shown).

The MN-I-4 chimera induced the most potent neutralizing antibody response against the ALA-1 strain of HIV-1 (albeit in only one of three guinea pigs), with a 90% inhibition titer of 950. Two of three animals immunized with the chimera produced measurable 90% inhibition titers (of 30 and 110) against

the MN strain of HIV-1 as well. Chimeras MN-III-2, -III-8, -III-10, and -III-12 each elicited significant 90% inhibition titers against ALA-1 in three of three guinea pigs (ranging from 10 to 490). Significant 90% inhibition titers were less frequently observed against MN; however, MN-III-12 was able to elicit potent neutralizing responses in two of three guinea pigs against both the ALA-1 (90% inhibition titers of 150 and 490) and the MN (90% inhibition titers of 200 and 320) strains.

DISCUSSION

In this study, we demonstrate the value of generating a combinatorial library of V3 loop presentations and sequentially immunoselecting with multiple antibody preparations to generate HRV:HIV-1 V3 loop chimeras that elicit potent anti-HIV-1 neutralization activity in cell culture. By expanding the diversity and extent of the immunoselection used to isolate chimeras with HIV-like immunogenicity relative to previous efforts (51), we were able to obtain chimeras that were more uniformly and more potently able to elicit anti-HIV-1 activity. We have operated on the assumption that, if a reconstructed epitopic region can be recognized by multiple neutralizing anti-HIV antibodies directed against overlapping-but-distinct epitopes (i.e., that have somewhat different recognition sites and binding requirements), then its features should bear significant similarity to those of the epitope in the context of HIV. Furthermore, if a reconstructed epitope resembles the native epitope, then it is expected to elicit the production of neutralizing antibodies like the native epitope (and conceivably more so).

An examination of the HIV immunogens being developed for AIDS vaccines reveals that there are few capable of eliciting the production of antisera that can inhibit 70 to 90% of the infectivity in cell culture at dilutions on the order of 400-fold and higher. To our knowledge, immunogens of this potency are limited to some mixtures of gp160 with peptides or proteins (6, 27, 28), a gp120 construct (7), some V3 loop peptides (1, 2, 64, 66), an influenza:V3 loop chimera (40), a Ty:V3 loop chimera (31), and several HRV14:HIV-1 chimeras (this work and reference 51). While the assays involved have their limitations, it is noteworthy that three of the eight chimeric HRV14:HIV-1 viruses described here stand out as being among the most potent HIV immunogens described.

In general, the immunoselected chimeric viruses were found to be more potently neutralized by anti-HIV-1 antibodies with successive rounds of immunoselection as evidenced by the results of microtiter neutralization assays (Fig. 2) and plaque reduction assays (Fig. 3). This indicates that chimeric viruses that were less well neutralized by the anti-HIV-1 antibodies were being eliminated from the pools with multiple rounds of selection.

It is remarkable that, of the eight chimeric viruses used to immunize guinea pigs, only one, MN-II-11, failed to elicit an anti-HIV-1 neutralizing response. This chimera had a deletion of 6 of the 12 V3 loop residues (Table 2). The lack of production of neutralizing antibodies to HIV-1 by MN-II-11 correlated with a lack of reactivity in an ELISA with an immobilized HIV-1_{MN} V3 loop octameric peptide (data not shown). In addition, MN-II-11 was significantly less sensitive to neutralization by anti-HIV-1 antibodies (with the exception of neutralization by HuMAb 447-52-D; Table 2). Since this chimeric virus elicited comparably high anti-self neutralization titers compared to other chimeric viruses (data not shown), it appears that the loss of the six V3 loop residues in this construct results in the impaired ability of the chimera to both react with and elicit anti-HIV-1 neutralizing antibodies.

The other seven immunoselected chimeras chosen for im-

munization studies were able to elicit the production of significant titers of anti-HIV-1 neutralizing antibodies against the ALA-1 and MN strains of HIV-1 in at least one of three guinea pigs, although MN-V-2 was only able to elicit significant 50% inhibition titers. The MN-V-2 chimera was the only one used in the immunogenicity studies that was subjected to only two rounds of immunoselection. The other seven chimeric viruses chosen for immunization studies were immunoselected four times.

The immunoselection procedure appeared to influence the nature of the chimeric viruses obtained in a favorable way. As with panning for multiple rounds with one antibody, panning for multiple rounds with different antibodies in each round resulted most typically in a marked reduction in the diversity of the sequences remaining. While diversity was decreasing, the remaining chimeric viruses were found to be more effectively neutralized by the various anti-HIV antibodies tested.

It can be seen that the shapes of the curves representing the neutralization of chimeric virus pools by anti-HIV antibodies (Fig. 2) correlate with the amounts of diversity observed in the pools. For example, in Fig. 2A, the curves derived for the US pool and for the pool obtained from selection with MAb 694/98-D are essentially linear. In contrast, the curves derived for the pools that underwent three or four rounds of selection are distinctly biphasic. The sequence data obtained revealed that linear neutralization curves reflected the presence of diverse sequences, whereas sharply biphasic curves were characteristic of pools composed of chimeric viruses with only one or a few sequences. Thus, it should be possible to estimate the extent of sequence diversity in selected pools simply by examining the shapes of their neutralization curves.

It is quite apparent that among the immunoselected chimeric viruses, only some chimeras are both recognized and neutralized by anti-HIV antibodies. This phenomenon is exemplified by chimeras MN-II-11, MN-III-6, and MN-III-15. These three chimeras were captured by all four antibodies used for immunoselection. Nonetheless, for each of them, at least one of the four antibodies proved ineffective in neutralizing the captured virus at the concentrations tested.

A number of nonrandom distributions of amino acids were seen for the randomized linkers, highlighting the value of allowing for unexpected preferences. In particular, it is quite striking that, although the original DNA library showed no specific bias toward either the number or type of residues appearing in the randomized linkers on either side of the core HIV-1 V3 loop epitope (data not shown), asymmetric distributions of both were observed among the viable chimeric viruses produced (Table 1; Fig. 4). A greater number of randomized residues was found in the N-terminal linker than in the C-terminal linker at the protein level. This may reflect a chemical or biological benefit from positioning the GPGR sequence more centrally within the context of the loop or may reflect structural constraints caused by the propensity of the V3 loop sequence to adopt a preferred conformation (9, 10, 26, 32, 52). In addition, the distribution of types of residues found at the N-terminal versus the C-terminal sites is quite nonrandom in nature. There is a complete lack of hydrophobic residues at the N-terminal linker and an unexpected preponderance of hydrophobic residues at the C-terminal linker, which is normally fully solvent exposed in HRV14 (4, 53). A similar asymmetric distribution of hydrophobic residues was observed in a previous V3 loop library containing the sequence IGPGR flanked by two randomized residues on either side (60). In addition, there seems to be a preference for an overall net charge of -1 or 0 for the foreign insert (V3 loop and linkers) along with the three HRV14 residues on either side

(with the exception of US27, having a net charge of -2) (Table 1). Previous work from our laboratory also supports this correlation. Nearly all of the viable chimeras sequenced to date have a net charge of 0 or -1 (51, 60; data not shown), whereas the majority of nonviable chimeric constructs engineered at both the NIm-IA and NIm-II sites have a net positive charge (5). In addition, a similar correlation between viability of PV-1_{Sabin} HIV-1 chimeras and a decrease in the net positive charge of the mutagenized β B- β C loop of the N-AgI site of VP1 has been reported (15). Altogether, these results suggest that the overall loop charge may be an important determinant of chimera viability.

Use of the random systematic mutagenesis approach allows for the generation and identification of valuable chimeras, despite the reduction in the number of potentially viable chimeras that can result from unpredictable sequence preferences. It would have been difficult to predict *a priori* any of these biases, and it would have been unlikely that chimeric viruses would have been designed with these specific sequences.

It is clear from these results and from previous work in our laboratory (5, 51, 60) that chimeric rhinoviruses can present foreign sequences in immunologically relevant conformations capable of stimulating robust neutralizing anti-HIV-1 immune responses in guinea pigs. Although some antibody cross-reactivity between MN-like strains of HIV-1 was observed, neutralization of more divergent strains, such as the IIIB strain, was less successful. Further efforts will focus on identifying chimeric virus constructs that can elicit more broadly neutralizing anti-HIV-1 responses. This will entail the use of diverse HIV sequences, anti-HIV neutralizing antibodies, and linker sequences.

To date, all of these studies have been conducted with animals nonpermissive for HRV14 replication. Studies are under way to evaluate the abilities of some of these chimeric viruses to act as live-virus vectors in chimpanzees, which are permissive for HRV replication. The chimeric virus system described is a potential source of vaccines against a wide variety of infectious diseases and, conceivably, cancer because it results in the presentation of relevant antigens to target the immune destruction of antigen-bearing pathogens, cells, or sources of cancer.

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