Chimeras from a Human Rhinovirus 14–Human Immunodeficiency Virus Type 1 (HIV-1) V3 Loop Seroprevalence Library Induce Neutralizing Responses against HIV-1

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A chimeric virus library was designed whereby sequences corresponding to the V3 loop of human immunodeficiency virus type 1 (HIV-1) were presented on the surface of human rhinovirus 14. The V3 loop sequences consisted of a relatively conserved segment of seven amino acids and five adjacent residues that were allowed to vary in proportion to their seroprevalence among HIV-1 isolates of North America and Europe. A technique called random systematic mutagenesis was used to incorporate the composite V3 loop sequences flanked by zero to two randomized amino acids. This library could contain 2.7×10^8 members having diverse sequences and conformations. Immunoselection of a portion of this library by using two neutralizing V3 loop-directed monoclonal antibodies followed by selection for desirable growth and purification characteristics yielded a set of chimeric rhinoviruses, five of which are described. The inserted sequences in the five chimeras do not match those of any known isolate of HIV-1. Nonetheless, all five chimeras were neutralized by antibodies directed against different strains of HIV-1 and were able to elicit the production of antibodies that bind V3 loop peptides from diverse HIV-1 isolates. Moreover, antisera derived from four of the five chimeras were capable of neutralizing one or more strains of HIV-1 in cell culture. This study demonstrates that random systematic mutagenesis in conjunction with antibody screening is a powerful and efficient means to obtain antigenic chimeras with relevant immunogenic properties.

Human rhinovirus (HRV), a picornavirus responsible for approximately 50% of common colds, can be engineered to present foreign immunogens, making it a candidate for use as a live-virus vaccine vector against human immunodeficiency virus type 1 (HIV-1) and other dangerous pathogens. Attributes of HRVs (reviewed in references 6 and 10) that are useful in vaccine design include their ability to elicit robust and long-lasting mucosal and serum-mediated immunity, their mild pathogenicity, and their numerous serotypes, suggesting that vaccination would be practical even in adults. Moreover, knowing the three-dimensional structure (2, 39) and the precise locations of neutralizing immunogenic (NIm) regions (39, 42, 43) of HRV14 facilitates engineering of foreign epitopes into sites well recognized by the immune system.

Transplantation of foreign epitopes onto picornaviral surfaces has been demonstrated previously for poliovirus (1, 4, 9, 12, 19, 24, 27, 29), coxsackievirus (35), rhinovirus (3), and the plant virus cowpea mosaic virus (34). While the number of foreign amino acids that can be incorporated onto the surfaces of picornaviruses may be limited, protein epitopes are quite small, typically consisting of only 14 to 21 amino acids (reviewed in reference 8). Thus, HRVs and other picornaviruses have the capacity to display complete foreign epitopes on their surfaces, making them potentially valuable vaccine vehicles.

Here we describe HRV14 chimeric viruses that display sequences from the primary target of vaccines designed against HIV-1, the principal neutralizing determinant. This epitope has been mapped to the V3 loop of the gp120 envelope glycoprotein (17, 33, 40). Initial attempts to transplant this determinant onto the surfaces of poliovirus (7) and rhinovirus (3) met with significant difficulty. Subsequently, live-virus presentations of HIV-1 V3 loop sequences have been reported for poliovirus (9), influenza virus (22), and rhinovirus (44).

In an attempt to generate a broadly reactive immune response to a virus as antigenically diverse as HIV, we have opted to represent composite sequences of the V3 loop. The sequences chosen for presentation in this study include a relatively conserved seven-amino-acid segment flanked by the five adjacent amino acids, which were each varied in proportion to their prevalence among North American and European isolates. These composite HIV-1 sequences were, in turn, flanked by linkers or adapters of zero to two randomized amino acids and transplanted onto the surface of HRV14, resulting in a library with 2.7×10^8 potential members.

Chimeras whose composite V3 loop sequences were presented in conformations that mimicked those of HIV-1 were immunoselected to enrich for antigenically relevant members of the library. Two neutralizing anti-HIV-1 V3 loop monoclonal antibodies (MAbs) were used for the immunoselection. Immunoselection methods have been used previously to identify HIV-1 mimotopes derived from segments of fully randomized amino acids, using phage display libraries (5, 18). The chimeric rhinovirus subset that was immunoselected was then propagated and purified to select for chimeras with favorable

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TABLE 1. Amino acid sequences of NIm-II inserts^a

Linker	Varied HIV-1 sequences, 93%	HIV-1 core, 53%	Varied HIV-1 sequences				Linker
			95%	94%	97%	89%	
XX	H (50, 45)	IGPGRAF	Y (80, 72)	T (55, 60)	T (88, 81)	G (75, 81)	XX
X	T (10, 5)		V (6, 1)	A (39, 40)	A (4, 9)	E (6, 9)	XX
None	P (10, 15)		H (5, 9)		I (4, 5)	R (4, 9)	XX
XX	R (10, 15)		F (2, 8)		R (1, 4)	K (4, 1)	X
X	N (7, 15)		L (2, 1)		G (0, 0.5)		X
None	S (6, 5)		D (0.3, 9)		V (0, 0.5)		X
XX							None
X							None
None							None

^a The approximate percentages of sequenced HIV-1 isolates containing any of the residues encoded are shown. In parentheses, the first number corresponds to the percentage among the 354 known sequences, and the second number corresponds to the percentage encoded in the HRV14-HIV-1 library.

growth and purification properties. Immunological characterization of five of the resulting chimeras is described.

MATERIALS AND METHODS

Cells, viruses, and media. H1-HeLa cells (21) were used for the production of HRV14 (43) and HRV14-HIV-1 chimeras. *Escherichia coli* JS4 (Bio-Rad Laboratories) was used for electroporation of plasmid DNA. H9/FDA cells (23) were used for propagation of HIV-1 (47). The HIV-1 strains used were MN (14, 41) (NIH AIDS Research and Reference Reagent Program, from R. Gallo) and IIB (14) and ALA-1 (11) (from S. Zolla-Pazner). Medium M is minimal essential medium supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.3), 0.1 mM nonessential amino acids, 10 mM L-Gln, and 100 U of ml penicillin-streptomycin per ml. PA medium is minimal essential medium supplemented with 20 mM HEPES (pH 7.3), 10 mM MgCl₂, 4 mM L-Gln, 100 U of penicillin-streptomycin per ml, and 10% fetal bovine serum.

Construction of an HRV14-HIV-1 V3 loop seroprevalence library. Mutagenesis was performed with plasmid p3IIST (44). Cassettes encoding HIV-1 V3 loop sequences, the flanking randomized amino acids, and *Apa*I and *Cla*I sites were generated by hybridizing oligonucleotides complementary at their 3' ends and completing second-strand synthesis. To generate amino acids in proportion to their observed distributions, the phosphoramidites used for DNA synthesis were mixed in ratios calculated to yield the desired codon frequencies (Table 1). Cassettes were digested with *Apa*I and *Cla*I and ligated to dephosphorylated *Apa*I-*Cla*I-digested p3IIST.

Generation of chimeric viruses. The ligated products were electroporated into *E. coli* with a Gene Pulser system (Bio-Rad Laboratories). Transformed cells were grown in liquid cultures, and the mutagenized plasmids were isolated in pools. The plasmids were used for in vitro transcription and transfection reactions (28). Viable chimeric viruses were obtained from H1-HeLa cell cultures exhibiting cytopathic effects.

Screening of the library with MAbs that neutralize HIV-1. Chimeric viruses were captured on microtiter plates (Nunc Immunosorb) coated with 40 µl of a 0.1-µg/ml concentration of mouse MAb (MuMAb) NM-01 (mapped to the V3 loop sequence GPGR [32], from M. Terada) or human MAb (HuMAb) 694/98-D (mapped to the V3 loop sequence GRAF [16], from M. Gorny and S. Zolla-Pazner) in 50 mM sodium borate (pH 8.5). After an overnight incubation at room temperature, the plates were blocked with 3% gelatin in phosphate-buffered saline (PBS) for 1 h at 37°C and then washed with PBS containing 0.05% Tween 20 (PBS-T). Then 2.5×10^4 PFU of chimeric HRV14-HIV-1 was added to each well in 25 µl of PBS-T with 20% normal goat serum. After a 1-h incubation at room temperature, the plates were washed with PBS-T followed by PBS alone. Then 2×10^4 H1-HeLa cells in 200 µl of medium M with 10% fetal bovine serum were added to each well. Plates were incubated at 34.5°C and 2.5% CO₂ until cells exhibited 60 to 100% cytopathic effects (~72 h), after which they underwent three cycles of freezing (~80°C) and thawing (23°C).

Propagation, purification, and sequencing of immunocaptured chimeric viruses. Immunoselected and purified chimeric viruses were plaque purified twice, harvested by up to three cycles of freezing and thawing, and used to infect H1-HeLa cells, yielding passage 1 viruses. Passage 2 viruses derived from the subsequent passage were used as inocula for further propagations. Viruses were purified by differential high-speed centrifugation (49). PCR products derived from cDNA copies of the viral RNA were sequenced by using standard techniques.

Plaque reduction assays using neutralizing anti-HIV-1 antibody preparations. Chimeric viruses were incubated with diluted antibody preparations for 1 h at room temperature and added to H1-HeLa cell monolayers (25 PFU with antibodies per 1.8×10^6 cells). After 1 h at 34.5°C and 2.5% CO₂, they were overlaid with 0.5% Noble agar (Difco) in PA medium and incubated for 72 h. Anti-HIV-1 antibody preparations used were MuMAb NM-01 (mapped to the V3 loop

sequence GPGR), HuMAb 694/98-D (mapped to the V3 loop sequence GRAF), MuMAbs 59.1 (mapped serologically [47] and structurally [15] to the V3 loop sequence GPGR) and 50.1 (mapped serologically to the V3 loop sequence RIHIG [47] and structurally to the sequence KRIHIGP [38], from A. Profy, Repligen Corporation), polyclonal antibodies (PABs) anti-MN octamer p200M and anti-IIB octamer p127 (guinea pig polyclonal antisera raised against the octameric peptide preparations corresponding to the V3 loop sequences from HIV-1_{MN} and HIV-1_{IIB}, respectively [45], from M. Li and C. Y. Wang, United Biomedical, Inc.), and MuMAb 0.5β (mapped to the V3 loop of HIV-1_{IIB} [25, 26], from A. Profy).

Immunization of guinea pigs. Purified chimeric virus (30 to 90 µg) was inoculated subcutaneously into each of three guinea pigs (Dunkin Hartley; Cocalico Biologicals, Reamstown, Pa.). The following inoculation schedules (given in weeks) were used: for D6-4, 0, 3, and 6; for DN-1, 0, 4, and 8; for DN-6, 0, 5, and 9; for DN-7, 0, 3, and 6; and for DN-9, 0, 5, and 8. Sera were collected prior to immunization, 2 weeks after the primary boost, and 2 and 4 weeks after the final boost. Purified HRV14 (40 µg) was inoculated subcutaneously into each of two guinea pigs (Dunkin Hartley; United Biomedical) on weeks 0, 5, and 10. Sera were collected on weeks 11.5 and 13. Complete Freund's adjuvant was used for all primary immunizations; incomplete Freund's adjuvant was used for all boosts.

ELISAs against peptides containing sequences of the V3 loop. The peptides used in the enzyme-linked immunosorbent assays (ELISAs) are shown in Table 2. Conditions for the ELISAs were modified from those described by Wang et al. (46).

HIV-1 neutralization assays. Neutralization assays were based on the Std. SN assay described by White-Scharf et al. (47). Reverse transcriptase activity was determined on day 4 as described by Willey et al. (48).

RESULTS

Design and construction of a HRV14-HIV-1 V3 loop seroprevalence library. We designed a library of chimeric HRV14-HIV-1 viruses that would display many V3 loop sequences in a vast array of conformations. The site chosen for insertion was the major immunogenic portion of the NIm-II site of HRV14, loop 2 of the VP2 puff. Asn-160 was removed, and amino acid residues were inserted between Ala-159 and Glu-161 (Table 1). The central element of the V3 loop sequence is an invariant core sequence, IGPGRAF, represented in 53% of 354 HIV-1 isolates predominantly from North America and Europe (20, 30). Adjacent to this HIV-1 core are five residues that were represented in proportion to their seroprevalence among these isolates. The six residues most commonly found in the position N terminal to the invariant core (His, Thr, Pro, Arg, Asn, and Ser) represented 93% of amino acids found at this position. Likewise, the amino acids chosen for the four positions C terminal to the HIV core represented 89 to 97% of amino acids for each of these positions. Zero to two completely randomized positions were engineered on each side of the HIV-1 insert, resulting in 2.7×10^8 possible unique members associated with a vast array of lengths, sequences, and conformations. We obtained 5.4×10^6 colonies after transformation, indicating that $\leq 2\%$ of the possible mutagenized plasmids

FIG. 1. Optical density measurements from ELISA experiments binding antichimera antisera from three guinea pigs per chimera to immobilized V3 loop peptides derived from seven strains of HIV-1. All antisera were diluted 1:8,000 and are shown in the same order for each peptide-binding experiment. OD 490, optical density at 490 nm.

each of the preimmunization antisera did not react with any of the peptides (at dilutions of 1:1,000).

HIV-1 neutralization. Antisera raised against each of the chimeras were tested for the ability to neutralize the HIV-1 strains ALA-1, MN, and IIIB in cell culture (Table 4). Chimeric virus DN-6 elicited the most potent antiserum obtained, with one of three antisera able to neutralize the ALA-1 and MN strains of HIV-1 at 90% inhibition titers of 550 and 40, respectively, and 60% inhibition titers of 2,600 and 1,125, respectively. DN-1 was able to elicit a neutralizing response against ALA-1 in one guinea pig, with a 90% inhibition titer of 40 and a 60% inhibition titer of 80, as well as a neutralizing response against ALA-1 in another guinea pig, with a 60% inhibition titer of 22. One antiserum directed against DN-7 neutralized ALA-1, with a 90% inhibition titer of 17 and a 60% inhibition titer of 77, and cross-neutralized IIIB, with a 90% inhibition titer of 5 and a 60% inhibition titer of 22. D6-4 elicited a neutralizing response against ALA-1 in one guinea pig, with a 60% inhibition titer of 19. DN-9 elicited only marginal neutralizing responses. Antisera raised against wild-type HRV14 were unable to neutralize any of the HIV-1 isolates with a 90 or 60% inhibition titer of ≥ 4 .

TABLE 4. Neutralization of HIV-1 by chimera antisera

Chimera antiserum	Titer ^a					
	ALA-1		MN		IIIB	
	90%	60%	90%	60%	90%	60%
D6-4	—	19	—	—	—	—
DN-1	40	80	—	—	—	—
	—	22	—	—	—	—
DN-6	550	2600	40	1125	—	—
	9	38	—	—	—	—
DN-7	17	77	—	—	5	22
DN-9	—	5	—	7	—	—
HRV14	—	—	—	—	—	—

^a Expressed as the reciprocal of the dilution that inhibited reverse transcription by 90 or 60% relative to value obtained with normal guinea pig serum. —, lack of neutralization at reciprocal dilutions of ≥ 4 . Each line of data corresponds to titers for one guinea pig. Three guinea pigs were immunized with each chimeric virus; two guinea pigs were immunized with HRV14.

DISCUSSION

In this study, random systematic mutagenesis was used to produce a library of chimeric human rhinoviruses displaying diverse V3 loop sequences in proportion to their seroprevalence among North American and European isolates (Table 1). A small subset of this library was generated. HuMab 694/98-D and MuMab NM-01 were used to immunoselect the chimeric viruses that displayed their composite V3 loop sequences in conformations that mimic those of native HIV-1. This antigenically enriched fraction of the library was propagated and purified to select for stable chimeras with hardy growth properties. Five chimeric viruses were characterized. A preliminary description of the experimental approach has been presented elsewhere (37).

All of the chimeric viruses studied were neutralized by a diverse set of anti-HIV-1 antibody preparations (Table 3). All five chimeric viruses selected by MuMab NM-01, which is reported to recognize the sequence GPGR, were strongly neutralized by this antibody, possibly as a function of the selection conditions used. The same chimeras were neutralized over a 10-fold-broader range by HuMab 694/98-D, which immunoselected the D6-4 chimera but not necessarily any of the other four chimeras tested. Two of the five chimeras were neutralized with titers comparable to those obtained with MuMab NM-01; the other three had lower titers. This broader range of virus-antibody interactions could reflect that each of the chimeras was immunoselected with NM-01 and not 694/98-D and/or that the varied amino acids positioned directly adjacent to the GRAF recognition sequence of 694/98-D affected antibody recognition. Each of the chimeric viruses contains the MuMab 59.1 recognition sequence, GPGR, and was neutralized potently, except for D6-4, which was neutralized less effectively by greater than an order of magnitude. Neutralization of each of the chimeric viruses by PAb anti-MN octamer and PAb anti-IIIB octamer provided further confirmation that the desired sequences were presented in an antigenic manner. It appears that the immunoselection procedure led to the identification of chimeras that display their composite V3 loop sequences in a manner comparable to those of at least some of the strains neutralized by the antibodies used (i.e., MN, IIIB, ALA-1, SF2, WMJ2, and RF). Alternatively, the antigenicity of the five chimeras could have resulted from the presence of the common HIV-1 core sequence. In this regard, it is worth not-

ing that the chimeric viruses from another HRV14-HIV-1 library described by Smith et al. (44), which were not immunoselected (and also contained a shorter HIV-1 core sequence), displayed generally less favorable antigenic profiles. One of 24 chimeric viruses from that library was neutralized by multiple anti-HIV-1 antibodies directed against diverse strains.

It is not surprising that the anti-HIV-1 MuMAbs 50.1 and 0.5 β did not neutralize any of the HRV14-HIV-1 chimeras. The epitope of MuMAb 50.1, determined serologically to be RIHIG and structurally to be KRIHIGP, was only partially present in any of the chimeras. Likewise, MuMAb 0.5 β , the epitope of which has been mapped only approximately, has been shown to potentially neutralize IIIB in a type-specific manner. As none of the chimeras have IIIB sequences beyond the GPGRF part of the core, it appears either that MuMAb 0.5 β requires interaction with additional amino acids for neutralization (25) and/or that other residues of the chimeras disrupted the preferred contacts between this antibody and its antigen.

Each of the chimeras elicited antibody recognition of V3 loop peptides from the MN, IIIB, SF2, SC, WMJ2, and Uganda strains of HIV-1 (Fig. 1). Additionally, guinea pig antisera against DN-1 and DN-6 reacted with a V3 loop peptide corresponding to the Thai isolate used. The ELISA data suggest that the central GPGRF motif is critical for recognition by these antisera. This motif is common to the most highly reactive sequences (i.e., peptides with sequences from the MN, SC, and WMJ2 strains) and less intact in the poorly recognized sequences (i.e., peptides with sequences from the Thai, Brazil, and RF isolates). The peptides that showed intermediate recognition in these assays (i.e., peptides with sequences from the IIIB and SF2 strains) contain the GPGRF motif but might have been affected by adjacent residues.

Although none of the chimeric viruses encode V3 loop sequences of known HIV-1 isolates, four of the five chimeras studied were able to elicit the production of antisera capable of neutralizing HIV-1 in cell culture (Table 4). DN-6, strongly neutralized by each of the anti-HIV-1 antibody preparations tested, was able to elicit the production of antisera that potentially neutralized the ALA-1 strain of HIV-1, and in one case, substantially neutralized the MN strain as well. Antisera directed against DN-1 and D6-4 were both able to neutralize the ALA-1 strain. In addition, an antiserum directed against DN-7 was able to neutralize both the ALA-1 and IIIB strains of HIV-1.

One of three guinea pigs immunized with DN-6 yielded neutralization titers against HIV-1 that were substantially higher than those for the animal with the next-highest titer. Animal-to-animal variation in immune response has been extensively documented. In this regard, it is worth noting that the purified immunoglobulin G fraction of this most potent antiserum, obtained by using *Staphylococcus aureus* protein A chromatography, neutralized the ALA-1 strain of HIV-1 with titers comparable to those of the untreated antiserum (unpublished results). This finding demonstrated that neutralization of HIV-1 was a direct result of the antibody fraction and not some other component of the antiserum. The neutralization titer of this antiserum is in the range of those elicited by the most immunogenic 10 to 12 HIV-1 vaccine candidates reported (36).

Crystals of two HRV14-HIV-1 chimeras, DN-6 and D6-4, that diffract X-rays beyond 3-Å (0.3-nm) resolution were obtained. The determination of the crystal structures of such chimeras will reveal the conformation of V3 loop epitopes capable of eliciting neutralizing immunogenic responses against HIV-1. Structure determination of chimeras that are

comparably recognized or neutralized by anti-V3 loop antibodies but that differ in the ability to elicit a neutralizing response against HIV-1 may help identify features responsible for immunogenicity and illuminate how such features relate to antigenicity.

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