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Cross-Neutralizing Activity against Divergent Human Immunodeficiency Virus Type 1 Isolates Induced by the gp41 Sequence ELDKWAS

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Previously we identified the highly conserved amino acids Glu-Leu-Asp-Lys-Trp-Ala (ELDKWA) on the ectodomain of gp41 as the epitope of a neutralizing monoclonal antibody (2F5) directed against human immunodeficiency virus type 1. In the present study, the sequence defining the epitope was introduced into the loop of antigenic site B of the influenza virus hemagglutinin. The resulting chimeric virus was able to elicit ELDKWA-specific immunoglobulins G and A in antisera of mice. Moreover, the distantly related human immunodeficiency virus type 1 isolates MN, RF, and IIIB were neutralized by these antisera. These data suggest that this conserved B-cell epitope is a promising candidate for inclusion in a vaccine against AIDS. The results also show that influenza virus can be used to effectively present the antigenic structure of this B-cell epitope.

Antigenic variation is considered to be a major obstacle to human immunodeficiency virus type 1 (HIV-1) vaccine development. Most neutralizing antibodies elicited by HIV-1-derived immunogens show only very limited cross-neutralizing activity (1, 3, 5, 8, 9, 11, 13, 15, 21, 27, 28). In addition, escape mutants emerge under immune pressure (2, 14, 16, 17, 20, 24, 25). In a previous study, a human monoclonal antibody (2F5) which neutralized different HIV-1 strains was established (4, 22). The 2F5 epitope sequence has been mapped to the conserved amino acids ELDKWA, with DKWA being the core sequence. Antigenic variation of this epitope was shown to be limited (17). In this report, recombinant DNA technology was used to insert this epitope sequence into the antigenic site B of the hemagglutinin (HA) of influenza A/WSN/33 (WSN) virus (Fig. 1). Since it was hypothesized that hydrophilic amino acids might result in better presentation of the epitope, we also inserted the Ser which is located C terminal to the ELDKWA epitope sequence into antigenic site B of the HA. Antigenic site B was previously shown to tolerate foreign antigens (9a). Also, insertion of 12 amino acids of the principal neutralizing domain sequence of HIV-1 into antigenic site B resulted in a chimeric virus that induced both neutralizing antibodies and cytotoxic T cells which recognize the principal neutralizing domain sequence (12). To obtain the chimeric virus containing the gp41 sequence ELDKWAS, plasmid pHA-ELDKWAS was constructed (Fig. 1). RNA derived from plasmid pHA-ELDKWAS was then introduced into the genome of influenza WSN virus by ribonucleoprotein transfection (18).

After isolating an influenza virus containing the ELDKWAS sequence, we first analyzed the antigenic properties of this virus. A plaque reduction assay using the chimeric virus and different dilutions of the 2F5 antibody was performed. As shown in Fig. 2, the influenza-ELDKWAS virus was neutralized by the antibody. Incubation of the chimeric virus in the presence of 25 ng of monoclonal antibody 2F5 per ml resulted in a 50% reduction of plaque number. In contrast, wild-type (wt) WSN virus was not neutralized by a 400-fold-higher concentration of antibody 2F5 (data not shown). This neutralization assay indicates that the antigenicity of the 2F5 epitope sequence expressed on the HA of the chimeric influenza virus mimics that of the 2F5 epitope on HIV.

The immunogenic potential of the chimeric virus was investigated in OF-1 mice. OF-1 mice were immunized with the chimeric influenza-ELDKWAS virus with 10^2 PFU intranasally and then given an intranasal booster immunization with 5×10^5 PFU, an intraperitoneal immunization with 20 μg of sucrose-gradient purified live virus after 3 more weeks, and a final intraperitoneal boost with 20 μg of sodium dodecyl sulfate-denatured virus in incomplete Freund's adjuvant after 3 additional weeks. For intranasal immunizations, mice were under ether anesthesia. The same immunization scheme was used for wt WSN virus. Mice were bled 12 days after the final boost, antisera were inactivated for 1 h at 56°C, and the enzyme-linked immunosorbent assay (ELISA) and neutralizing titers of the antisera were analyzed. As shown in Fig. 3, both ELDKWA-specific immunoglobulin G (IgG) and IgA were detected in the antisera. The specific IgG and IgA titers were in the range of 1:3,200 to 1:25,600 and 1:70 to 1:560, respectively. However, one antiserum induced by the chimeric virus did not show a significant ELISA titer against the ELDKWA epitope (not included in Fig. 3).

The neutralizing activity of the antisera against HIV-1 strains MN, RF, and IIIB was determined by syncytium inhibition assays. The reciprocal serum dilutions that inhibit syncytium formation by 50% (EC_{50}s) are shown in Table 1. Antisera M1 to M3 neutralized the entire test panel at various serum dilutions. Antiserum M4 neutralized HIV-1 isolates MN and RF but not IIIB. The antisera induced by the WSN wt virus did not neutralize any of the HIV-1 isolates tested at the lowest serum dilution (1:40). Surprisingly, three of the mouse sera showed higher neutralization titers for HIV strain RF than for strain MN. These differences in neutralization sensi-

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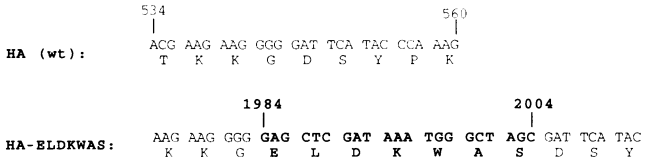

FIG. 1. Construction of the chimeric influenza virus. The gp41 sequence ELDKWAS was inserted into the antigenic site B of the HA of WSN virus. Plasmid pHA-ELDKWAS was obtained by replacing the *Pst*I-*Hin*dIII fragment of pT3/WSN-HAm1 (12) with a PCR product which was obtained by using pT3/WSN-HAm1 as the template and the sense and antisense primers 5′CAGTATCCTGCAGCCA TA3′ and 5′ATTGGTAAGCTTTGGGTATGAATCGCTAGCCCA TTTATCGAGCTCCCCCTTCTTCGTCAGC3′, respectively. The sense primer corresponds to nucleotides 478 to 495 of WSN HAm1. The antisense primer corresponds to nucleotides 569 to 546 of WSN HAm1 and a 21-nucleotide insertion corresponding to positions 1984 to 2004 of gp160 of HIV-1 isolate BH10, followed by nucleotides 545 to 530 of WSN HAm1. The nucleotide numbering for the HA corresponds to that described by Hiti et al. (10), and the nucleotide numbering for gp160 corresponds to that used in the Los Alamos data base (19). Transfection of RNA derived from this plasmid into MDBK cells and selection of chimeric virus were done as previously described (7). HA (wt) shows the nucleotide and amino acid sequence of antigenic site B of the WSN virus; HA-ELDKWAS shows the nucleotide and amino acid sequence of antigenic site B of the chimeric influenza-ELDKWAS virus. Residues derived from gp41 are shown in boldface letters and are inserted at nucleotide position 545 of the HA of WSN virus.

tivity might be due to different conformations of the epitope on different HIV-1 isolates. For example, it was found that the EC_{50} of human monoclonal antibody 2F5 against the RF strain was 9.7 μg/ml and that the EC_{50} against the MN strain was 0.7 μg/ml (6, 22). We also performed a neutralization assay using p24 production as the replication marker for two of the sera. Preincubation of HIV strains MN and RF with both sera resulted in significant reduction of p24 production (Fig. 4). It should be noted that another chimeric influenza virus, which contains amino acids LELDKWAS, was also able to induce

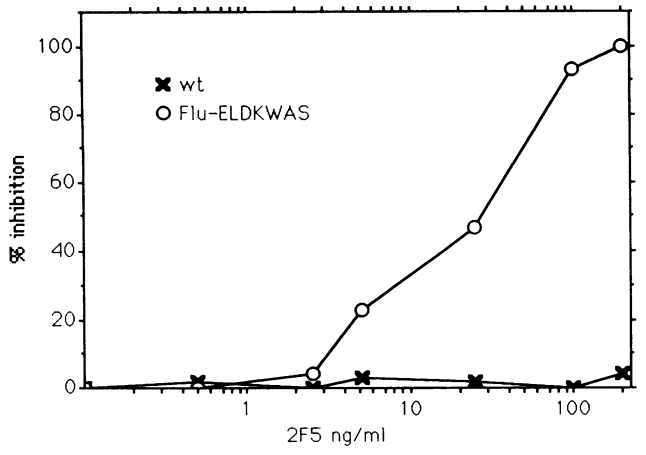

FIG. 2. Neutralization of the influenza-ELDKWAS virus by the human monoclonal antibody 2F5. Approximately 2×10^2 PFU of the chimeric influenza virus (○) or of wt influenza WSN virus (×) were preincubated with different concentrations of antibody 2F5 for 1 h at 4°C, and infectivity was analyzed in plaque assays, using MDBK cells (26). The agar overlay contained the same concentration of antibody 2F5 as used in the preincubation step.

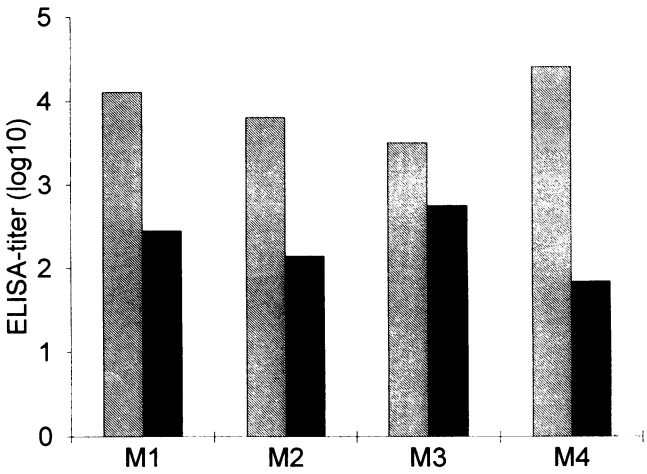

FIG. 3. Antibody response of mice immunized with influenza-ELDKWAS. Reactivity of antisera to the sequence ELDKWA was determined by ELISA. Ninety-six-well microtiter plates were coated with the glutathione *S*-transferase–ELDKWA fusion peptide at 4 μg/ml (100 μl per well) in carbonate buffer (pH 9.6) for 4 h at room temperature. Plates were then washed with phosphate-buffered saline (PBS)–0.1% Tween 20. Antiserum diluted in PBS–1% bovine serum albumin–0.1% Tween 20 was added, and the mixture was incubated for 1 h at room temperature. After washing, antibodies were detected by incubation with a goat anti-mouse IgG γ-chain-specific antibody conjugated with horseradish peroxidase. Following an additional washing step, the plates were stained with *o*-phenylenediamine dihydrochloride as the substrate. The reaction was stopped with 2.5 M H_2SO_4, and the plates were measured (measure wavelength, 492 nm; reference wavelength, 620 nm). For detection of IgA antibodies, the same protocol was followed except that an anti-mouse IgA α-chain-specific antibody conjugate was used. M1 to M4 represent antisera induced by the chimeric virus. IgG titers are represented by shaded columns, and IgA titers are represented by solid columns. An absorbance value of 0.11 (IgG) or 0.02 (IgA) was used as the cutoff value (mean value of the absorption of negative control sera plus 2 standard deviations).

TABLE 1. Inhibition of syncytium formation by antisera induced by influenza-ELDKWAS virus[a]

Antiserum	Neutralization titer (EC_{50})		
	MN	RF	IIIB
M1	106	190	40
M2	48	80	320
M3	68	320	134
M4	58	48	—
wt	—	—	—

[a] The indicator cell line AA-2 and, as virus inoculum, frozen stocks of HIV-1 strains MN, RF, and IIIB were used. All virus stocks were diluted to $10^{1.9}$ to $10^{2.5}$ 50% tissue culture infective doses per ml. Mouse antisera were diluted twofold in medium and distributed in 96-well microtiter plates (four replicas of each dilution); 50 μl of virus was added to 50 μl of diluted antisera, and the virus-antibody mixture was preincubated for 2 h at 4°C. For infection, 100 μl of AA-2 cells (5×10^6 cells per ml) was added to each well. Presence of syncytia was recorded after 5 days as an indication of HIV-1 infection. The reciprocal serum dilution that inhibits syncytium formation by 50% is shown as the neutralization titer and corresponds to the dilution of antiserum in the final test volume (200 μl). The EC_{50} was estimated as described by Reed and Muench (23) and is defined as the reciprocal of the serum dilution producing a 50% reduction in the number of wells in which syncytia were observed. —, no neutralization titer at the lowest dilution tested (1:40).

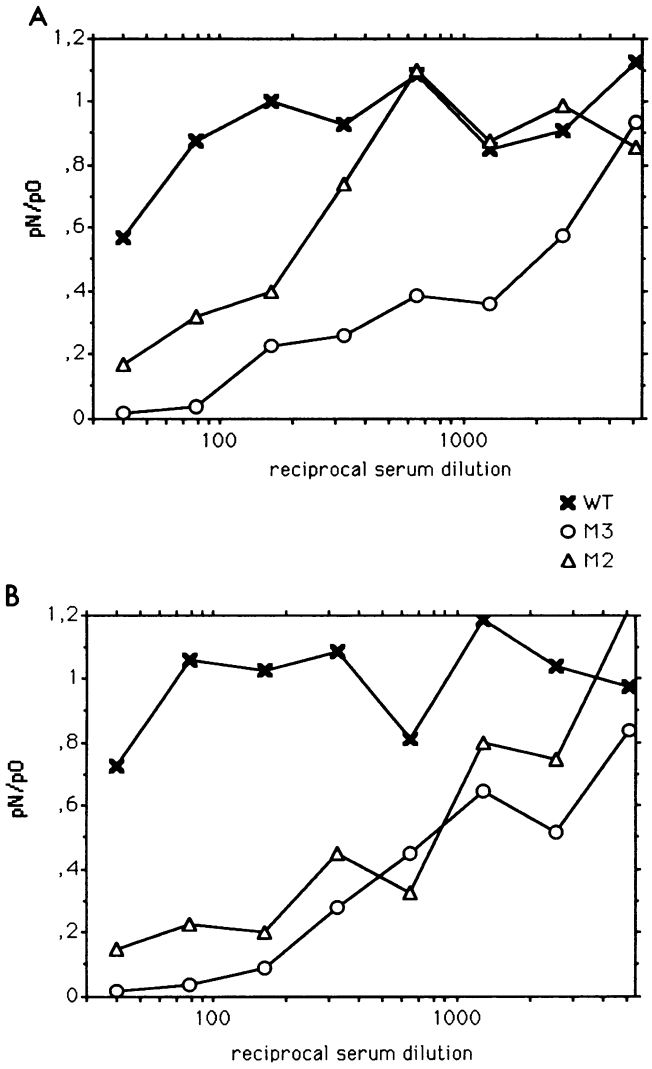

FIG. 4. Inhibition of p24 production by immune mouse sera. Two mouse sera induced by the chimeric virus (M2 and M3) and one serum induced by the wt virus were tested with HIV-1 strains RF (A) and MN (B). Neutralization assays were performed as described for Table 1 except that culture supernatants of each serum dilution were analyzed for p24 production. Neutralization was determined by the reduction in virus-induced p24 production in the presence of two-fold dilutions of serum (pN) divided by virus-induced p24 production in the absence of serum (p0). The culture supernatants were monitored at day 5. The average amounts of p24 in the supernatant in the absence of serum were 13 ng/ml for HIV-1 strain MN and 11 ng/ml for HIV-1 strain RF. For detection of p24, six wells of each serum dilution were pooled and p24 was determined by using a double-antibody sandwich ELISA. Briefly, a murine monoclonal antibody against p24 was bound to Maxisorp ELISA plates (Nunc) at a concentration of 2 μg/ml in 0.1 M sodium carbonate buffer (pH 9.5). Plates were then incubated for 1 h with samples that were treated with 2% Nonidet P-40. Captured p24 was detected with a horseradish peroxidase-labeled human monoclonal antibody against p24. The reaction was developed with 1,2-*o*-phenylenediamine dihydrochloride solution containing 0.03% H_2O_2 and stopped with 1.25 N sulfuric acid. Optical density was read at 492 nm (reference wavelength, 620 nm).

neutralizing anti-HIV antibodies. These antibodies had titers similar to those found for mice immunized with the ELDKWAS virus (data not shown).

These results suggest that the conserved gp41 sequence ELDKWAS is sufficient to elicit neutralizing antibodies against HIV-1. Most importantly, the antisera induced by the chimeric virus were able to neutralize the distantly related HIV-1 strains RF and MN, which belong to the subtype B group of HIV-1. It should be noted that both strains contain the gp41 sequence ELDKWAS and that the ELDKWAS sequence is also found in other subtype groups of HIV-1. However, restriction of antigenic variation for this epitope is not absolute (17).

Since sequence variability of neutralizing epitopes and emergence of escape mutants are major obstacles in HIV-1 vaccine development, the efficient presentation of this highly conserved gp41 sequence, which is only minimally immunogenic in the context of the entire gp160, is highly desirable. Although only moderate neutralization titers were obtained, influenza virus appears to be an appropriate candidate for the presentation of this epitope. Booster immunizations using the ELDKWAS sequence in different vectors such as other subtypes of influenza virus should induce antisera with improved neutralization titers. In addition, the influence on immunogenicity of amino acids adjacent to the ELDKWAS sequence needs to be investigated further.

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