

# Remodeling of Dynamic Structures of HIV-1 Envelope Proteins Leads to Synthetic Antigen Molecules Inducing Neutralizing Antibodies

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A synthetic antigen targeting membrane-fusion mechanism of HIV-1 has a newly designed template with C3-symmetric linkers mimicking N36 trimeric form. The antiserum produced by immunization of the N36 trimeric form antigen showed structural preference in binding to N36 trimer and stronger inhibitory activity against HIV-1 infection than the N36 monomer. Our results suggest an effective strategy of HIV vaccine design based on a relationship to the native structure of proteins involved in HIV fusion mechanisms.

## INTRODUCTION

Antibody-based therapy is one of the promising treatments for AIDS. In recent years, AIDS antibodies have been produced by immunization (1) and by de novo engineering of monoclonal antibodies (mAb) with molecular evolution tactics such as phage display (2). Despite enormous efforts, however, only a limited number of highly and broadly HIV-neutralizing human mAbs have been isolated and characterized. These antibodies include gp41 Abs, 2F5 (3–6) and 4E10 (5–7), and gp120 Abs, 2G12 (8) and b12 (9). gp41 is a transmembrane envelope glycoprotein, which is divided into an endodomain and an ectodomain by the transmembrane region; the latter contains a hydrophobic amino-terminal fusion peptide, followed by amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat domains with helical structures (HR1 and HR2, respectively). In the membrane fusion process of HIV-1, these subunits form a “pre-bundle” complex. The HR1 and HR2 regions are termed the N-terminal helix (N36) and C-terminal helix (C34), respectively. These helices form a six-helical bundle consisting of a central parallel trimeric coiled-coil of N36 surrounded by C34 in an antiparallel hairpin fashion. In design of immunogens that elicit broadly neutralizing antibodies, a useful strategy is to produce molecules that mimic the natural trimer on the virion surface. Previous studies show that these molecules could be proteins expressed as a recombinant form or on the surface of particles such as pseudovirions or proteoliposomes (10–12). The X-ray crystallographic study of gp41 shows that the distances between any two residues at the N-terminus of N-region are almost equal at approximately 10 Å (Figure 1A). A chemically synthetic template could be useful in connection with the design of a peptidomimetic corresponding to the native structure of gp41. To date, several gp41 mimetics have been synthesized as inhibitors or antigens and subjected to inhibition or neutralization assays (13–16). However, the templates for assembly of these helical peptides contain branched peptide linkers, which are not exactly equivalent in length (14). The N-terminal peptides constrained by another threefold linker showed high affinity for

C-terminal peptides, although its biological advantages have not been determined (15). The mimicry can be estimated using the broadly neutralizing mAbs; suitable mimetics will bind neutralizing mAbs efficiently, but they will bind non-neutralizing mAbs poorly. In the present study, we designed and synthesized a novel three-helical bundle mimetic, which corresponds to the trimeric form of N36. We investigated whether mice immunized with the equivalent trimeric form of N36 mimetic can produce antibodies with stronger binding affinity for N36 trimer than for N36 monomer. This approach demonstrates the possibility of producing structure-specific antibodies by immunization of synthetic antigens corresponding to the natural form of viral proteins.

## EXPERIMENTAL PROCEDURES

**Conjugation of N36REGC and the Template to Produce triN36e.** Compound 6 (100 µg, 0.174 µmol) and N36REGC (3.4 mg, 0.574 µmol) were dissolved in a mixture of 300 µL of 200 mM acetate buffer (pH 5.2) and 300 µL of TFE under a nitrogen atmosphere, then TCEP·HCl was added. The reaction was stirred for 72 h at room temperature and monitored by HPLC. The ligation product (triN36e) was separated as an HPLC peak and was characterized by ESI-TOF-MS, *m/z* calcd for C<sub>690</sub>H<sub>1160</sub>N<sub>226</sub>O<sub>201</sub>S<sub>3</sub> 15933.1, found 15933.8. The purification was performed by reverse phase HPLC (YMC-Pack ODS-A column, 10 × 250 mm). Elution was carried out with a 40–50% linear gradient of acetonitrile (0.1% TFA) over 50 min. Purified triN36e, obtained in 16% yield, was identified by ESI-TOF-MS. The detailed synthesis of peptides is described in the Supporting Information (SI).

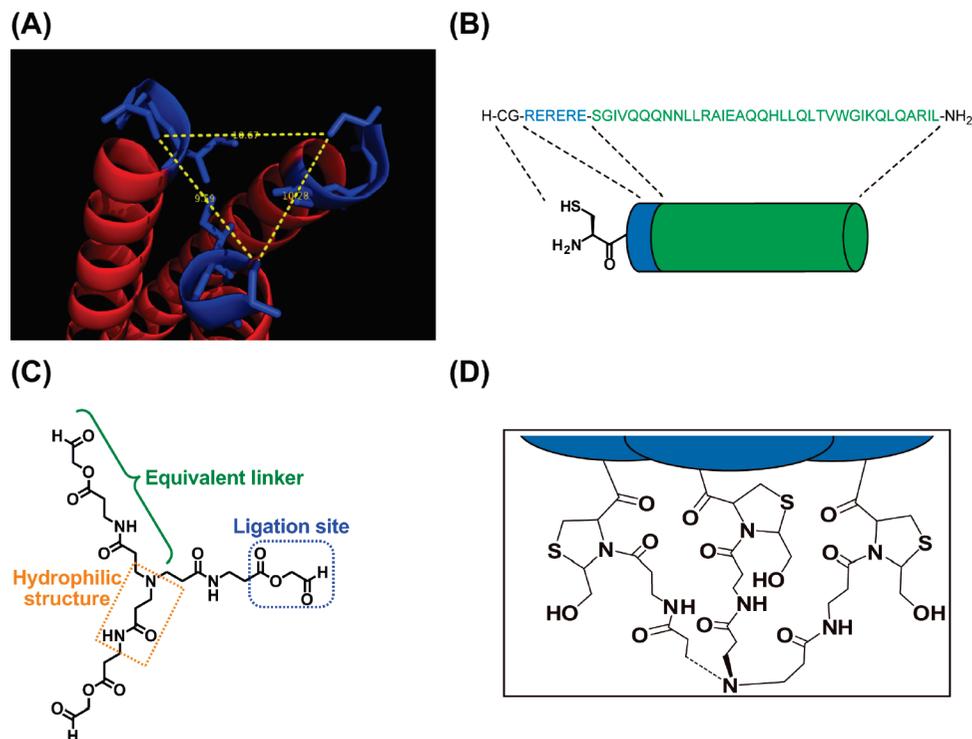
**CD Spectra.** CD measurements were performed with a J-720 circular dichroism spectropolarimeter equipped with a thermoregulator (JASCO). The wavelength dependence of molar ellipticity [ $\theta$ ] was monitored at 25 °C from 190 to 250 nm. Peptides were dissolved in 20 mM acetate buffer (pH 4.0) containing 40% MeOH (23, 24). The experimental helicity was calculated as reported previously (17–19).

**Immunization and Sample Collection.** Six-week-old male BALB/c mice were purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan) and maintained under specific pathogen-free conditions in an animal facility. The experimental protocol was approved by the ethical review committee of Tokyo Medical and Dental University. Freund incomplete adjuvant and PBS

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**Figure 1.** (A) Distances between hydrogen atoms for hydroxyl groups in N-terminal serine residues of N36 helices in trimeric form. The distances were evaluated by PyMOL (21). (B) Cartoon presentation of each N36 derived peptide, N36REGC. (C) Design of a C<sub>3</sub>-symmetric template. The amino acid residues are described in single letters. (D) Conjugated structure of trimeric N36 after thiazolidine ligation.

were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMSO (endotoxin free) was purchased from Sigma-Aldrich (St. Louis, MO).

All mice were bled one week before immunization. One hundred micrograms of antigen was dissolved in 1  $\mu$ L of DMSO. The solution was mixed with 50  $\mu$ L of PBS and 50  $\mu$ L of Freund incomplete adjuvant. The mixture was injected subcutaneously under anesthesia on days 0, 14, 28, 42, and 58. Mice were bled on days 21, 35, 49, and 65. Serum was separated by centrifugation (15 000 rpm) at 4  $^{\circ}$ C for 15 min and inactivated at 56  $^{\circ}$ C for 30 min. Sera were stored at  $-80^{\circ}$ C before use.

**Serum Titer ELISA.** Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako. ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was purchased from Sigma-Aldrich. Antimouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninety-six-well microplates were coated with 25  $\mu$ L of a synthetic peptide at 10  $\mu$ g/mL in PBS at 4  $^{\circ}$ C for overnight. The coated plates were washed 10 times with deionized water and blocked with 150  $\mu$ L of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37  $^{\circ}$ C for 1 h. The plates were washed with deionized water 10 times. Mice sera were diluted in 0.02% PBST with 1% skim milk, and 50  $\mu$ L of 2-fold serial dilutions of sera from 1/200 to 1/102400 were added to the wells and allowed to incubate at 37  $^{\circ}$ C for 2 h. The plates were washed 10 times with deionized water. Twenty-five microliters of HRP-conjugated antimouse IgG, diluted 1:2000 in 0.02% PBST, was added to each well. After 45 min incubation, the plates were washed 10 times and 25  $\mu$ L of HRP substrate, prepared by dissolving 10 mg ABTS to 200  $\mu$ L of HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H<sub>2</sub>O<sub>2</sub> (3  $\mu$ L), and H<sub>2</sub>O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25  $\mu$ L/well 0.5 M H<sub>2</sub>SO<sub>4</sub>, and optical densities were measured at 405 nm.

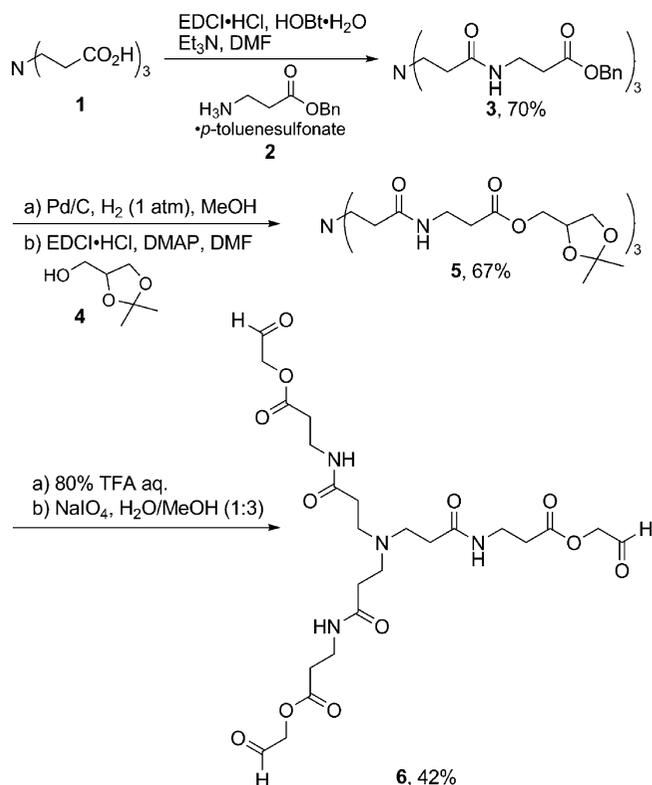
**Virus Preparation.** The pNL4-3 construct (8  $\mu$ g) was transfected into 293T cells by Lipofectamine LTX (Invitrogen,

Carlsbad, CA) followed by changing medium at 12 h after transfection. At 48 h after changing medium, the supernatant was collected, passed through a 0.45  $\mu$ m filter, and stored at  $-80^{\circ}$ C as HIV-1<sub>NL4-3</sub> strain before use. For titration, MT-4 cells were infected with serially 3-fold diluted virus from 1/10 to 1/196830, and cultured for 7 days. HIV-1 p24 levels in supernatants were measured, and then the titer of virus solution was calculated.

**Anti-HIV Assay.** Virus was prepared as described above except that the transfection of pNL4-3 was performed by the calcium phosphate method. Anti-HIV-1 activity was determined on the basis of protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of AZT, N36RE, and triN36e (The starting concentrations are 100, 10, and 1  $\mu$ M, respectively) were added to HIV-1-infected MT-4 cells (MOI = 0.01) by 2-fold serial dilution and placed in wells of a flat-bottomed microtiter plate (2.0  $\times$  10<sup>4</sup> cells/well). After 5 days' incubation at 37  $^{\circ}$ C in a CO<sub>2</sub> incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (EC<sub>50</sub>). Cytotoxicity of compounds was determined on the basis of viability of mock-infected cells using the MTT method (CC<sub>50</sub>). Each experiment was performed three times independently.

**Neutralizing Assay.** MT4-cells (1  $\times$  10<sup>5</sup> cells/100  $\mu$ L) were incubated in 100  $\mu$ L medium containing 10  $\mu$ L sera from immunized or preimmunized mice for 1 h at 37  $^{\circ}$ C, then pretreated MT-4 cells were infected with HIV-1<sub>NL4-3</sub> (MOI = 0.05). At 3 days after infection, cells were collected by centrifuge at 4000 rpm for 10 min at 4  $^{\circ}$ C. After discarding supernatant, pellets were lysed with 30  $\mu$ L of lysis buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% NP-40), then 30  $\mu$ L of 2  $\times$  SDS buffer (125 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-ME, 0.004% BPB) were added and boiled for 10 min. The samples (5  $\mu$ L) were subjected to SDS-page to perform Western blotting. The HIV-1 gag p24 was detected by using Western lightning ECL kit (PerkinElmer, MA) according to manufacturer's instruction after treatment of HIV-1 p24

## Scheme 1. Synthesis of the Equivalently Branched Template 6



antibody (2C2; 1:2000 dilution) (20) and anti- mouse IgG (H+L)-HRP (Millipore, MA). The band intensity of p24 was calculated with post/pre-immunized samples by using *ImageJ* image analyzing software.

## RESULTS AND DISCUSSION

The N-region of gp41 is known to be an aggregation site involving a trimeric coiled-coil conformation. In design of an N36-derived peptide (N36RE), the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase the solubility in buffer solution (Figure 1B). In order to form a triple helix corresponding precisely to the gp41 prefusion form, we designed the novel C3-symmetric template depicted in Figure 1C. This designed template linker has three branches of equal length and possesses the hydrophilic structure and ligation site for coupling with N36RE. The template was synthesized from the commercially available 3-[bis(2-carboxyethyl)amino]propanoic acid **1** as shown in Scheme 1. Coupling of **1** with  $\beta$ -alanine benzyl ester **2** gave the corresponding triamide **3** in 77% yield. Cleavage of three benzyl esters by hydrogenation and coupling with solketal **4** produced the corresponding triester **5**. Deprotection of the acetonides with aqueous 80% TFA

followed by oxidative cleavage of diol group led to the desired template **6**. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected N36RE (N36REGC) with a three-armed aldehyde scaffold producing triN36e (Figure 1D). Thiazolidine ligation is a peptide segment coupling strategy which does not require side chain protecting groups (22–26). The reaction consists of three steps: (i) aldehyde introduction, in which a masked glycolaldehyde ester is linked to the carboxyl terminus of an unprotected peptide by reverse proteolysis; (ii) ring formation, in which the unmasked aldehyde reacts at acidic pH with the  $\alpha$ -amino group of an N-terminal cysteine residue of the second unprotected peptide forming a thiazolidine ring; and (iii) rearrangement at higher pH in which O-acyl ester linkage is converted to an N-acyl amide linkage forming a peptide bond with a pseudoproline structure (Figure 2).

Circular dichroism (CD) spectra of triN36e and N36RE, which is a monomer form without N-terminal Cys-Gly residues, are shown in Figure 3A. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, suitable for measurement of CD spectra of membrane proteins (27, 28). Both spectra display double minima at 208 and 222 nm and showed high molar ellipticity as absolute values (Table 1). The results indicate that these peptides form a highly structured  $\alpha$ -helix and that the helical content of the trimer triN36e is higher than that of the monomer N36RE. Furthermore, to assess the interaction of triN36e with C34, CD spectra of the peptide mixture with C34-derived peptide, C34RE, were measured (Figure 3B,C). The spectrum of triN36e and C34RE mixture showed high molecular ellipticity as an absolute value comparable with that of triN36e alone. This supports the conclusion that C34RE interacts with tri36e and thereby induces a higher helical form as shown previously (29).

Mice were immunized with these synthetic gp41 mimetics and antibody production was successfully induced (the detailed titer increase in 5 weeks' immunization is given in the Supporting Information). Two out of three mice showed induction of antibodies against either antigen (N36RE or triN36e). Antibody titers and selectivity of antisera isolated from mice immunized with N36RE or triN36e were evaluated by serum titer ELISA against coated synthetic antigens. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Figure 4). The N36RE-induced antibody showed approximately 5 times higher affinity for N36RE than for triN36e, as 50% bound serum dilutions are  $3.88 \times 10^{-4}$  and  $2.14 \times 10^{-3}$  to N36RE and triN36e, respectively. It is noteworthy that the triN36e-induced antibody showed approximately 30 times higher preference in binding affinity for triN36e antigen than for N36RE (serum dilutions at 50% bound are  $3.83 \times 10^{-3}$  to N36RE and  $1.33 \times 10^{-4}$  to triN36e). Although this evaluation was not determined with purified mAbs, it is clear that the antibodies produced exploit a structural preference for antigens. The mechanism of induction

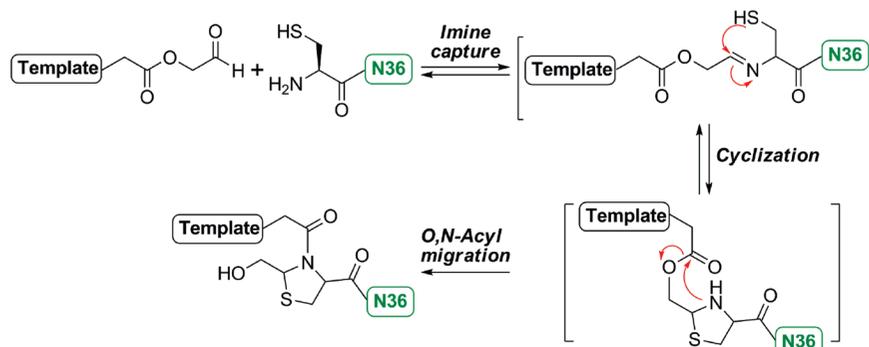
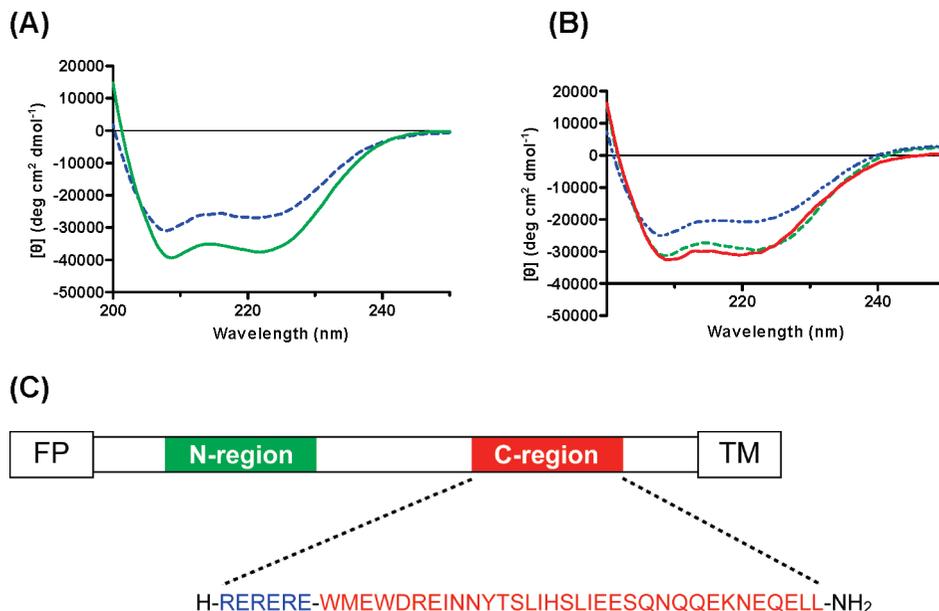
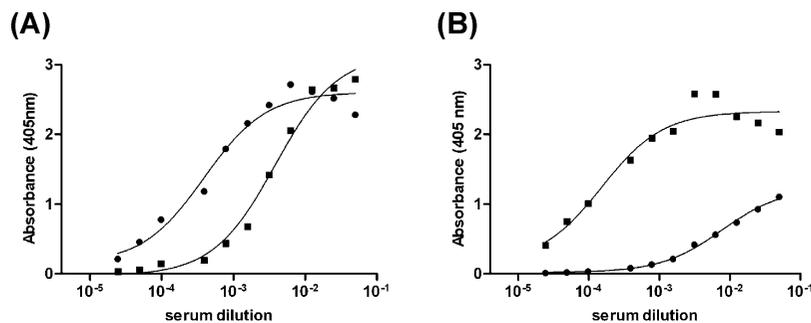


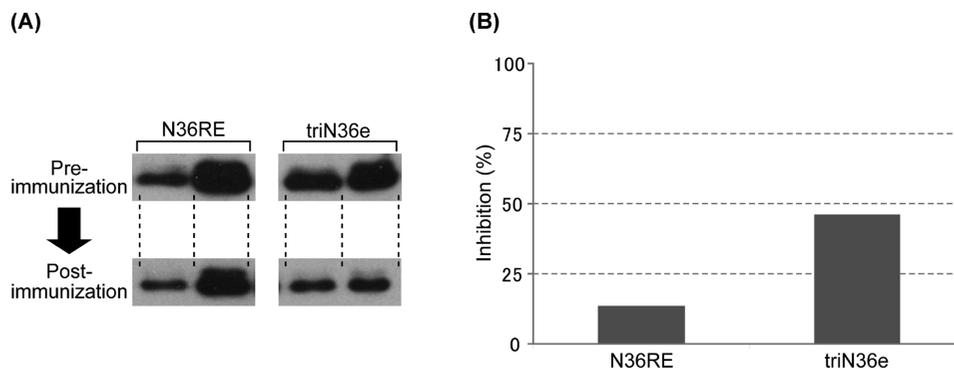
Figure 2. Reaction mechanisms of thiazolidine ligation utilized for assembly of N36RE helices on the template.



**Figure 3.** (A) Circular dichroism (CD) spectra of N36RE and triN36e. In the spectra, a blue dashed line and a green line show N36RE (monomer) and triN36e (trimer), respectively. Concentrations of the peptides are 10 and 3.3  $\mu$ M for N36RE and triN36e, respectively. (B) CD spectra in the presence or absence of C34RE peptide. The spectra show the following: a dashed green line, triN36e; a dashed blue line, C34RE; a red line, triN36e+C34RE, respectively. The concentrations of peptides were as follows: triN36e (2.3  $\mu$ M), C34-derived peptide C34RE (7  $\mu$ M), and mixture of both peptides (3.5  $\mu$ M each). (C) The amino acid sequence of C34RE described in single letters. FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively.



**Figure 4.** Serum titers of antibodies produced by N36 monomer and conformationally constrained N36 trimeric antigen. The titers were evaluated against N36RE (monomer) (A) and triN36e (trimer) (B). The plots indicate the results of sera obtained from N36RE-immunized mouse (•) and triN36e-immunized mouse (■).



**Figure 5.** Determination of neutralization activity of the antibodies produced by immunization of peptidomimetic antigens. (A) Results of p24 assay to evaluate inhibition for HIV-1 infection by produced antibodies. Preimmunization sera were used as control. Experiments were duplicated. (B) Average % inhibition of p24 production calculated from the band intensities in panel (A).

of structure-specific antibody is still not clear, but the results could suggest the efficacy of producing antibodies with structural specificity and that the synthesis of structure-involving antigens is an effective strategy when higher specificity is required.

Neutralizing activity of sera against HIV-1 infection was assessed by p24 assays utilizing antisera from two mice that showed antibody production for each antigen (Figure 5). Sera

**Table 1.** Differences of  $\alpha$ -Helicities between N36RE and triN36e Calculated from CD Spectra in Figure 3

	$[\theta]_{222}$	$[\theta]_{222}/[\theta]_{208}$	$\alpha$ -helicity
N36RE	-30 957	0.87	73%
triN36e	-38 998	0.96	95%

**Table 2.** EC<sub>50</sub> and CC<sub>50</sub> Values Calculated from Inhibition Assays of Peptidomimetics

	AZT	triN36e	N36RE
EC <sub>50</sub> (μM) <sup>a</sup>	0.047	0.49	1.4
CC <sub>50</sub> (μM) <sup>b</sup>	>50	>1	>10

<sup>a</sup>EC<sub>50</sub> values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. <sup>b</sup>CC<sub>50</sub> values are based on the reduction of the viability of MT-4 cells. All data are the mean values for at least three experiments.

from mice immunized with the same antigen showed similar inhibitory activity against viral infection (12.5% and 14.8% for N36RE, 40.3% and 52.1% for triN36e). A trend was observed that the sera from triN36e immunization shows higher inhibition than those from N36RE immunization. This suggests that the synthetic antigen corresponding to the N36 trimeric form induces antibody with neutralization activity superior to that of the monomer peptide antigen and implies a restricted response of B-cells upon immunization to the trimeric form of N36RE. In order to assess the compatibility of induced antibodies in HIV-1 entry inhibition, the HIV-1 inhibitory activities of peptidomimetics (N36RE and triN36e) have been evaluated by viral infection and cytotoxicity assays. A C-terminal region peptide known as Enfuvirtide (T20, Roche/Trimeris) has been used clinically as a fusion inhibitor, and its success indicates that gp41-derived peptides might be potent inhibitors, useful against HIV-1 infection (30). In the development of anti-HIV peptides, several mimetics such as Enfuvirtide, CD4 binding site of gp120 (31), and protein-nucleic acid interactions (32), which disrupt protein-protein interactions, have been produced. As indicated in Table 2, N36 and triN36e showed modest inhibitory activity as reported in previous studies (33–35). The potency of triN36e was three times higher than that of N36RE indicating that the active structure of monomer N36RE is a trimeric form. Cytotoxicity of the antigens was not observed at concentrations of 1 μM of triN36e and 10 μM of N36RE.

## CONCLUSIONS

In summary, a mimic of HIV-1 gp41-N36 designed as a new vaccine has been synthesized utilizing a novel template with three branched linkers of equal lengths. Thiazolidine-forming ligation attached the ester aldehyde of three-branched template with N-terminal cysteine of peptides in an aqueous medium. The resulting peptide antigen successfully induces antibodies with neutralization activity against HIV-1 infection. It is of special interest that the antibody produced acquires structural preference to antigen, which showed 30 times higher binding affinity for trimer than for monomer. This indicates the effectiveness of the design based on the structural dynamics of HIV-1 fusion mechanism of an antigen which could elicit neutralizing antibodies. In a design based on the N36 region of gp41, the exposed timing of epitopes is limited during HIV-1 entry (36), and carbohydrates, which could make accession of antibodies to epitopes difficult, are not associated with the amino acid residues of the native protein. These two advantages could further enhance the potential of a vaccine design based on the N36 region. During preparation of the manuscript, a new HIV vaccine strategy was reported by Burton's group (37). The report describes the importance of antibody recognition for the trimer form of surface protein. The trimer-specific antibodies indicate broad and potent neutralization. The gp41 trimer-form specific antibody produced in this study could also obtain the corresponding properties. The elucidation of antibody-producing mechanisms and epitope recognition mode of antibodies in antiserum during HIV-1 entry will be addressed in future studies.

## ACKNOWLEDGMENT

The authors deeply thank Prof. K. Akiyoshi (Tokyo Medical and Dental Univ.) for allowing access to CD spectropolarimeter.

**Supporting Information Available:** HPLC chromatograms and NMR charts of compounds **3**, **5**, and **6**. Results of ESI-TOF-MS, and HPLC chromatograms of peptides N36RE, N36REGC, and triN36e. Results of serum titer ELISA of antisera collected during immunization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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BC900502Z