



Evaluation of a synthetic C34 trimer of HIV-1 gp41 as AIDS vaccines

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ABSTRACT

An artificial antigen forming the C34 trimeric structure targeting membrane-fusion mechanism of HIV-1 has been evaluated as an HIV vaccine. The C34 trimeric molecule was previously designed and synthesized using a novel template with C3-symmetric linkers by us. The antiserum produced by immunization of the C34 trimeric form antigen showed 23-fold higher binding affinity for the C34 trimer than for the C34 monomer and showed significant neutralizing activity. The present results suggest effective strategies of the design of HIV vaccines and anti-HIV agents based on the native structure mimic of proteins targeting dynamic supramolecular mechanisms in HIV fusion.

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1. Introduction

Highly active anti-retroviral therapy (HAART) involving new anti-HIV drugs such as protease inhibitors and integrase inhibitors has been brought a great success to us. Antibody-based therapy is also promising, and several AIDS antibodies have been developed by normal immunization¹ and by de novo techniques of monoclonal antibodies (mAb) using molecular evolution methods such as phage display.² Antibodies including anti-gp41 and anti-gp120 have been identified as human mAbs capable of highly and broadly neutralizing HIV. A transmembrane envelope glycoprotein, gp41 is hidden beneath an outer envelope glycoprotein gp120 and its ecto-domain contains helical amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat (HR) domains HR1 and HR2. These HR1 and HR2 regions are designated as the N-terminal helix (N36) and C-terminal helix (C34), respectively. In the membrane fusion of HIV-1, these helices join to form a six-helical bundle consisting of a central parallel trimer of N36 surrounded by C34 in an antiparallel hairpin fashion. A useful strategy to produce broadly neutralizing antibodies is therefore to synthesize molecules that mimic the natural trimer as it appears on viral surface proteins. Walker et al. reported that antibody recognition for the trimer form is important in HIV vaccine strategies, because antibodies that specifically recognize the trimer formation might have broad and

potent neutralizing activity.³ To date, several gp41 mimetics, especially for N36 regions, which assemble these helical peptides with branched peptide-linkers have been synthesized as antigens.^{4–7} Previously, we synthesized a three-helix bundle mimetic, which corresponds to the trimeric form of N36, with a novel template with C3-symmetric linkers of equal lengths.⁸ Immunization with the equivalent trimeric form of N36 mimetic produced antibodies with stronger binding affinity for N36 trimer than for N36 monomer. The structure-specific antibodies produced in this way showed significant neutralization activity against HIV-1 infection. Several potent anti-HIV-1 peptides based on the gp41 C-terminal HR2 region have been discovered^{9,10} and an HR2-peptide, T20, has subsequently been developed into a clinical anti-HIV-1 drug, enfuvirtide (Roche/Trimeris).^{11–14} The C-terminal helix C34 is also an HR2-derived peptide containing the amino acid residues required for docking into the hydrophobic pocket of the trimer of the N-terminal HR1 region, and potently inhibits HIV-1 fusion in vitro.¹⁵ Recently, we also synthesized a three-helix bundle mimetic, which corresponds to the trimeric form of C34, with a novel different template with C3-symmetric linkers of equal lengths.¹⁶ The C-terminal ends of three peptide strands are assembled in the C34 trimer, while the N-terminal ends of three peptide strands are assembled in N36 trimer. As an anti-HIV agent, the C34 trimer peptide showed two orders of magnitude higher inhibitory potency than the C34 monomer peptide. This study demonstrates a useful strategy for the design of effective inhibitors against viral infections that proceed by membrane fusion with host cells. In the present study, we have investigated the activity of the equivalent trimeric

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form of C34 as an antigen peptide producing structure-specific antibodies. We have performed comparative studies of antisera isolated from mice immunized with the C34 trimer in binding affinity for the C34 trimer and for the C34 monomer.

2. Materials and methods

2.1. Immunization and sample collection

Six-week-old male BALB/c mice, purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan), were maintained in an animal facility under specific pathogen-free conditions. The experimental protocol used was approved by the ethical review committee of Tokyo Medical and Dental University. Freund incomplete adjuvant and PBS 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 (C34 monomer C34REG) was dissolved in PBS (50 μ L) and DMSO (1 μ L). The antigen C34 trimer triC34e (100 μ g) was dissolved in PBS (50 μ L). This solution was mixed with Freund incomplete adjuvant (50 μ L) and the mixture was injected subcutaneously under anesthesia on days 0, 7, 14, 21 and 28. Mice were bled on days 5, 12, 19, 26 and 33. Serum was separated by centrifugation (1500 rpm) at 4 °C for 10 min, and inactivated at 56 °C for 30 min. Sera were stored at –80 °C before use.

2.2. Serum titer ELISA

Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) was purchased from Sigma–Aldrich. Anti-mouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninety-six well microplates were coated with 25 μ L of a synthetic peptide in a 10 μ g/mL solution in PBS at 4 °C overnight. The coated plates were washed 10 times with deionized water and blocked with 150 μ L of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °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 μ L of twofold serial dilutions of sera from 1/200 to 1/409600 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were again washed 10 times with deionized water. HRP-conjugated anti-mouse IgG, diluted 1:2000 in 0.02% PBST (25 μ L), was added to each well. After incubation for 45 min, the plates were washed 10 times and 25 μ L of HRP substrate, prepared by dissolving ABTS (10 mg) in 200 μ L of HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H₂O₂ (3 μ L), and H₂O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μ L/well 0.5 M H₂SO₄, and optical densities at 405 nm were recorded.

2.3. Virus preparation

For virus preparation, 293FT cells in a 60 mm dish were transfected with 10 μ g of the pNL4-3 construct by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 μ m filter, and stored at –80 °C as the virus stock.

2.4. Neutralizing assay (P24 assay)

For viral neutralizing assay, the NL4-3 virus (5 ng of p24) was bound to MT-4 cells (5×10^4 cells/200 μ L) by spinoculation at

2100 g for 20 min at 4 °C. After removal by washing out of unbound virus, cells were resuspended with 200 μ L of medium containing 10 μ L sera from immunized or pre-immunized mice and were cultured. Half of the culture medium was changed every 2 or 3 days. At 7 days after infection, the level of p24 in the culture supernatant was determined by the p24 ELISA kit (PerkinElmer, MA).¹⁷

3. Results and discussion

In the C34 trimer, triC34e, which was previously synthesized,¹⁶ the triplet repeat of arginine and glutamic acid (RERERE) was added to the C-terminal end of the C34 sequence to increase solubility in buffer solution, and glycine was fused to the C-terminus (Fig. 1A and B). The C3-symmetric template with three hydrophilic branches of equal length was adopted to assemble three peptide strands. As a control peptide, which corresponds to the monomeric form of C34, C34REG having RERERE and Gly in the C-terminus was used (Fig. 1C).¹⁶

To investigate whether antibodies are efficiently produced, mice were immunized with C34REG and triC34e and the increase in the titer in 5 weeks' immunization was observed (Fig. 2). Titers and specificity of antisera isolated from mice immunized with C34REG or triC34e were evaluated by serum titer ELISA against coated synthetic antigens. In each case, the increase in antibody production was observed as time passed. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Fig. 3). The C34REG-induced antibody showed approximately 1.2 times higher antibody titer against the coated C34REG than against the coated triC34e; the serum dilutions at 50% bound are 1.06×10^{-3} and 1.30×10^{-3} , respectively (Fig. 3A and B). The triC34e-induced antibody showed approximately 23 times higher titer against the coated triC34e than against the coated C34REG; the 50% bound serum dilutions are 3.15×10^{-4} and 7.30×10^{-3} , respectively (Fig. 3A and B). C34REG-induced or triC34e-induced antibody did not show any significant binding titer against an unrelated control peptide (Fig. 3C and D). Although purified monoclonal antibodies were not used for this evaluation, the antibodies produced exploited specific affinity for each antigen of the monomer or the trimer. These results suggest the synthesis of structure-involving antigens leads to the production of antibodies with structural specificity.

It is important to know if the antisera produced have inhibitory activity against HIV-1 infection. Accordingly, the inhibitory activity of the antisera was assessed by p24 assays utilizing the antisera bled from three mice that showed antibody production for each antigen (Fig. 4). The experiments using HIV-1 was performed in the biosafety level 3 laboratory #5 in the National Institute of Infectious Diseases. Sera from mice immunized with the monomer C34REG and the trimer triC34e antigens contained antiviral activities compared to control sera. Any significant difference of inhibitory effects was not observed between the sera isolated from C34REG-immunized mice and those from triC34e-immunized mice. The synthetic C34 trimeric antigen induces antibodies with a structural preference, but the levels of neutralization activity of sera from mice immunized with the C34 trimer were similar with those of sera from the C34 monomer-immunized mice. This suggests that antibodies with structural specificity against the gp41-C34-derived region do not always have more potent neutralization activity. The difference of recognition mechanism of two types of antibodies might cause different neutralizing mechanism although their levels of neutralization activity are almost the same. This result is not consistent with the data of the synthetic antigen molecules derived from N36, in which the N36 trimer-specific antibodies showed higher neutralization activity against HIV-1 infection than the N36 monomer-specific antibodies.⁸ In any case,

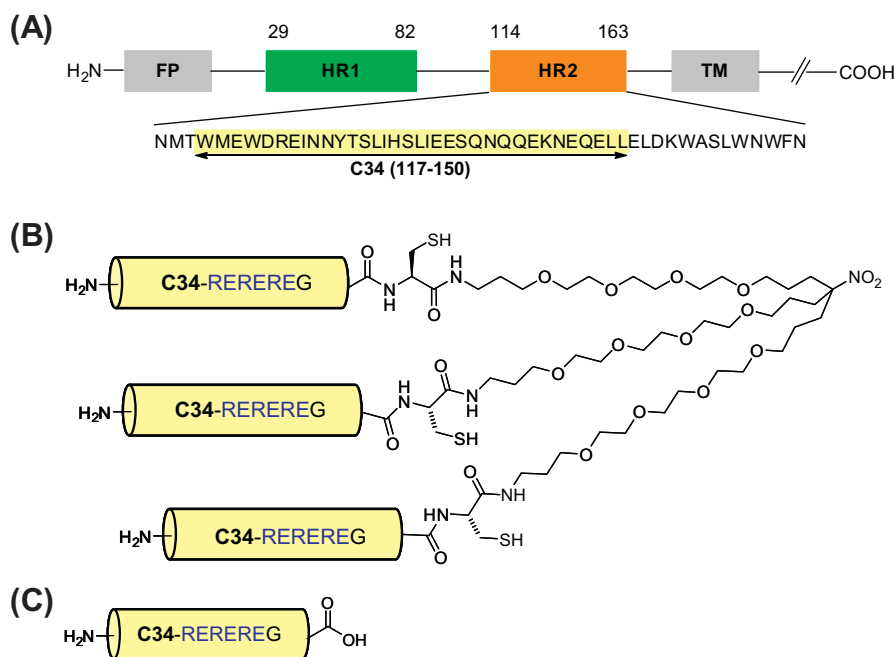


Figure 1. The sequence of C34 in gp41 (NL4-3) (A). FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively. Structures of C34-derived peptides, the C34 trimer with a C3-symmetric linker, triC34e (B), and the C34 monomer, C34REG (C).

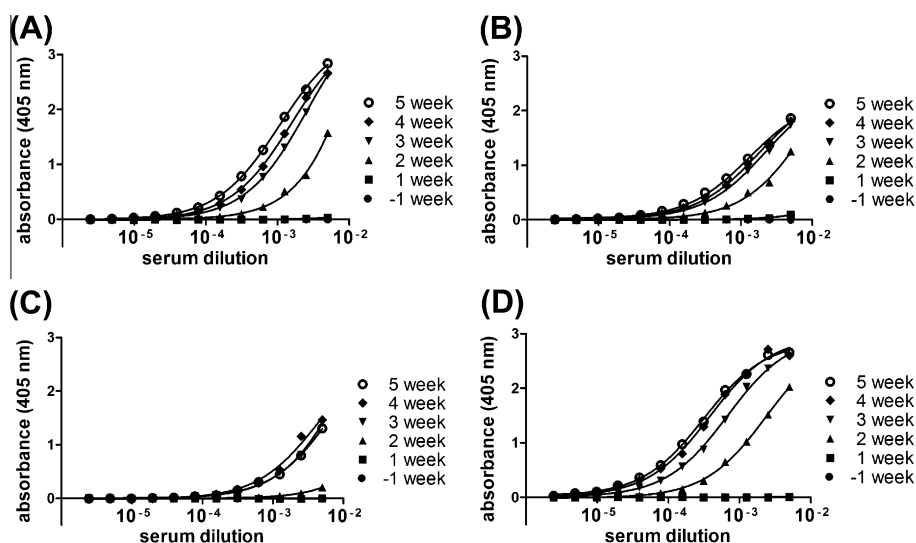


Figure 2. Results of serum titer ELISA of antisera collected during immunization (from one week before start to five weeks after immunization start) to determine the immunogenicity of designed antigens. The titers were evaluated as follows; antiserum against C34REG binding to C34REG (A); antiserum against C34REG binding to triC34e (B); antiserum against triC34e binding to C34REG (C); antiserum against triC34e binding to triC34e (D).

the synthetic C34 trimeric antigen induces antibodies with a structural preference and potent neutralization activity. In case antibodies bind to the gp41 C-terminal HR2 region and suppress membrane fusion, they may recognize the primary amino acid sequence of the C34 region or its structural conformation, because the C34 region is located outside in the formation of a six-helical bundle. It is suggested that suppressant potencies of these types of antibodies are almost similar. In addition, the action of these antibodies might be orthogonal and supplementally effective.

Recently, broadly active and potent neutralization antibodies, PG-9 and PG-16, were isolated from sera of HIV-1 infected individuals.³ The antibodies can neutralize ~80% of HIV-1 isolates across all clades with approximately one order of magnitude higher po-

tency than those of broad neutralizing mAbs reported previously. It is interesting that the epitopes for these mAbs are quaternary, and preferentially displayed on Env trimers, as expressed on the surface of virions and transfected cells. These results suggest that there may be production mechanisms for antibodies recognizing epitope structures.^{18–20} The sera obtained from immunization of the C34 trimer antigen have structural specificity and neutralization activity. Thus, our trimer antigens, including the N36 trimer,⁸ could work efficiently as a new class of HIV-1 vaccines.

Concerning inhibitory activity of these C-region peptides against HIV-1 entry, the potency of triC34e is one hundred times higher than that of C34REG.¹⁶ It indicates that a trimeric form is critical as the active structure of the inhibitor, although as vaccines

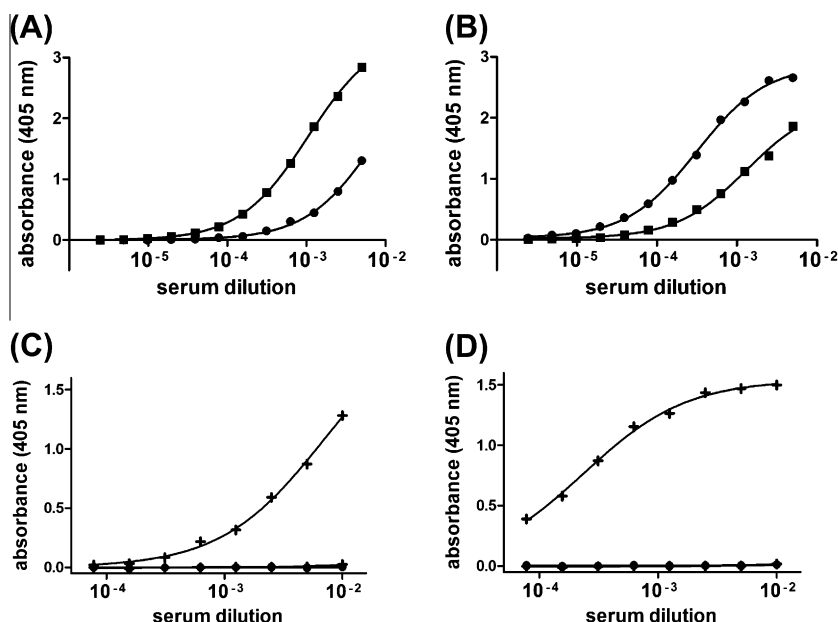


Figure 3. Serum titers of the antibodies produced by the fifth immunization of the C34REG antigen and the fourth immunization of the trIC34e antigen. These titers were evaluated against ELISA templates of C34REG (monomer) (A) and trIC34e (trimer) (B), using sera obtained from a C34REG-immunized mouse (■) and a trIC34e-immunized mouse (●) as each representative. Titers of C34REG-induced antibodies were evaluated against ELISA templates of C34REG (+) and an unrelated control peptide (●) (C), and titers of trIC34e-induced antibodies were evaluated against ELISA templates of trIC34e (+) and an unrelated control peptide (●) (D). Unrelated control peptide: $\text{CH}_3\text{CO-GELDKWEKIRLRPGGGC}(\text{CH}_2\text{CONH}_2)\text{-NH}_2$.

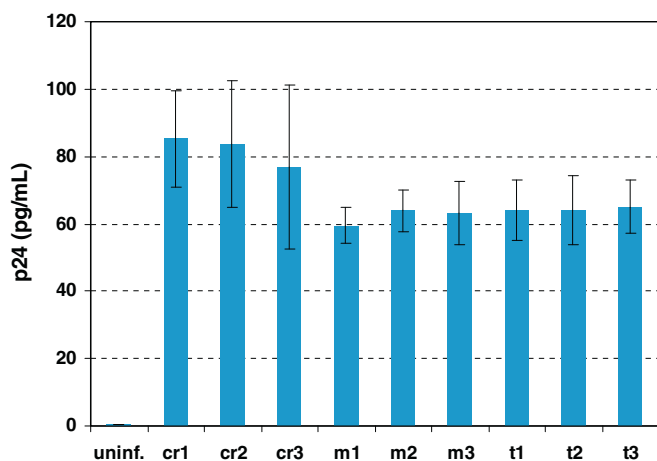


Figure 4. Determination of neutralization activity of the antibodies produced by immunization of C34REG and trIC34e antigens. Inhibition of HIV-1 (NL4-3 strain) infection by produced antibodies was evaluated by the p24 assay in MT-4 cells. Y-axis shows the amount of p24 production. Uninf. means uninfected cells. Pre-immunization sera (–1 week) were used as controls (cr1–3). C34REG- and trIC34e-immunization sera (5 weeks) were used (m1–3 and t1–3, respectively). Experiments were conducted in triplicate. Error bars show standard error of mean.

there is no significant difference in neutralization activity of induced antibodies between the monomer and the trimer.

The exposed timing of epitopes of the helical region trimers is limited in the fusion step,²¹ and carbohydrates are not included in the amino acid residues of the regions. The effectiveness of the vaccine design based on the gp41 helical regions is supported by the critical advantages cited above. Our developed N36 and C34 trimer-form specific antibodies might have the above properties. The designs of antigens and inhibitors targeting the dynamic supramolecular mechanism of HIV-1 fusion will be useful for future studies on AIDS vaccines and inhibitors.

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