



Enhancement of epitope-specific cellular immune responses by immunization with HIV-1 peptides genetically conjugated to the B-subunit of recombinant cholera toxin

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ABSTRACT

As more HIV-1 infected patients receive anti-retroviral drug treatment, the occurrence of drug-resistant variants of the virus is increasing. We have previously shown that mutated HIV peptide sequences represent mutations induced by antiretroviral drugs are equally good and often better immunogens than wild type peptides. The non-toxic B subunit of cholera toxin (CTB) is an active substance in the oral cholera vaccine, and has been shown to bind ganglioside receptors and activate mucosal cells. By fusing mutant epitopes deriving from HIV-1 enzymes with the B subunit of cholera toxin, we aim to induce cellular responses against virus harboring drug-induced mutations. We successfully created conjugates of HIV peptide sequences fused to rCTB. The immune response against the different peptides was strongly enhanced by the fusion to the toxin. Moreover, immunization with sequence containing drug-induced mutation triggered a cross-reactive immune response against the wild type epitope. Long-term follow-up of immunized animals revealed a persistence of cellular immune response for over 4 months, which could readily be boosted with an additional late immunization. By linking HIV-peptides to the B subunit of cholera toxin it is thus possible to stimulate a strong and long-lasting immune response, significantly better than that evoked by the peptide alone.

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1. Introduction

HIV vaccines need to include both drug-resistant variants as well as sequences representing the wild type virus [1,18]. One way of evoking a response against a drug induced mutant is to use a mixture of peptides representing drug resistant variants of HIV [2,3]. Peptides covering key sequences from new emerging HIV variants can readily be added to any vaccine mixture. We have previously shown that sequences representing drug-induced mutations are equally, or more, potent immunogens than peptides representing the wild type sequence [4]. Moreover, since HIV is mainly transmitted over mucosal tissues it is assumed that a strong mucosal immunity needs to be induced in order to lower the risk of sexual transmission of the virus (reviewed in [5]). The non-toxic

B subunit of cholera toxin (CTB) is an active substance in the oral cholera vaccine, which has been shown to stimulate good mucosal immunity against *Vibrio Cholera* (reviewed in [6,7]). By combining HIV-1 epitopes representing drug resistance mutations with rCTB, we assessed the possibility to trigger both a strong systemic and mucosal epitope specific immune response. This type of response may be able to suppress upcoming mutant virus as well as reducing the transmission rate of both wild type and drug resistant HIV-1.

2. Materials and methods

2.1. Preparation and purification of rCTB-HIV peptide conjugate

A fusion protein of recombinant cholera toxin subunit B (rCTB) and a short amino acid fragment corresponding to the drug escape variant of HIV RT epitope amino acids 33–41 (with the methionine to leucine mutation introduced at position 41; M41L) was constructed (rCTB-RT_{33–41}M41L). The fragments were genetically conjugated and cloned in a plasmid in *E. coli* where the fusion protein formed inclusion bodies. These were dissolved with urea in

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Table 1
Immunization protocol and schedule

Group	Antigen	Amount	Immunization schedule (weeks from study start)
RT _{33–41} M41L	Free peptide corresponding to the mutant variant of HIV RT aa 33–41M41L (ALVEICTEL)	50 µg	0, 4, 8, 28. Mice were bled 10–12 days after each injection
rCTB-RT _{33–41} M41L	The mutated epitope variant conjugated to rCTB	50 µg (NB! RT _{33–41} epitope corresponds to app. 10% of conjugate MW)	
rCTB + RT _{33–41} M41L	The mixture of rCTB and peptide	50 µg + 5 µg	
rCTB	rCTB	50 µg	
Untreated	–	–	

RT = reverse transcriptase; rCTB = recombinant cholera toxin B subunit; aa = amino acid.

carbonate buffer and DTT and subsequently dialysed in carbonate buffer to refold the protein and form functional pentamers. Chromatographic purification of the fusion protein was performed. The purity and the binding capacity of the conjugate to the ganglioside receptor 1 (GM1) receptor were verified by Coomassie blue or silver staining of SDS gels, western blot, GM1 affinity column, Biacore GM1 binding, C-terminal amino acid sequencing and electrospray-mass spectroscopy analysis.

2.2. Immunization

Immunograde purified peptide of the mutant peptide variant RT_{33–41}M41L was ordered from Thermo Hybaid, Germany. The peptide was dissolved in 10% DMSO in PBS to a concentration of 10 mg/mL and stored at –20 °C until use. HLA-A0201 transgenic [8,9] female mice were divided into groups of five to six mice each. The mice were given free peptide in PBS, rCTB-peptide conjugates, rCTB together with the peptide, rCTB alone or were left untreated, Table 1. The immunizations were performed subcutaneously at the root of the tail at weeks 0, 4, 8 and 28 and blood was drawn 10–12 days after each injection at which serum was collected and peripheral blood mononuclear cells (PBMCs) were purified and subjected to IFN-γ ELISpot (Mabtech, Nacka, Sweden).

2.3. IFN-γ ELISpot assay

200,000 Ficoll-paque (GE Healthcare, Sweden) gradient purified PBMCs or splenocytes were incubated together with 1 µg of test

peptide (ALVEICTEM; ALVEICTEL; SLYNTVATL; rCTB; Medium; Con A). After 20 h incubation at 37 °C and 5% CO₂ in air, IFN-γ secretion was stained according to manufacturer's protocol. Plates were read with an AID ELISpot reader system.

2.4. rCTB ELISA

ELISA plates (NUNC Maxisorp Odense, Denmark) were pre-coated with 0.45 µg/mL GM-1 (Sigma–Aldrich, Sweden) and incubated at room temperature over night. The plates were overlaid with 0.1% BSA–PBS for 30 min at 37 °C. rCTB was diluted to a concentration of 0.5 µg/mL and 100 µL was added per well. The plates were incubated at room temperature for 1 h, the wells were emptied and washed three times in 0.05% Tween 20 in saline. Blocking was performed with 1% BSA–PBS at 37 °C for 1 h. Serum from immunized or untreated animals was diluted in PBS-T (0.05%) and 100 µL was added per well. Goat-anti-mouse Ig conjugated to HRP (DAKO, Denmark) was used as detection antibody (at a dilution of 1/2000) followed by the substrate *O*-phenylene diamine (Sigma–Aldrich, Sweden). The color reaction was stopped with 100 µL of 2.5 M H₂SO₄ per well. Plates were read at 490–650 nm. Cut off was calculated as geometric mean of serum from untreated animals +3 standard deviations at the dilution of 1/100. One animal was excluded from the calculation due to background stimulation in the assay (and in the ELISpot).

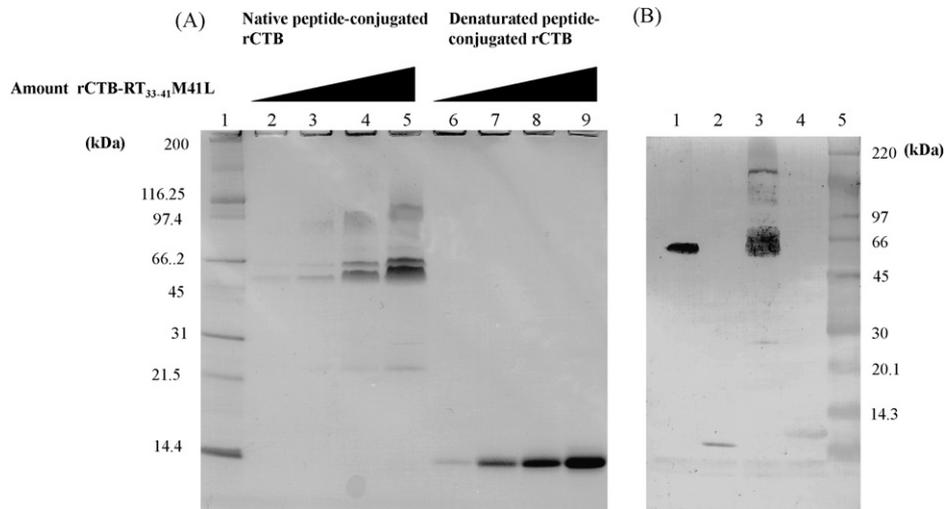


Fig. 1. Purification and control for pentamerization of rCTB-RT_{33–41}M41L conjugate. (A) Coomassie blue stained SDS gel. Lanes (1) Broad range molecular weight, BioRad; (2) 0.5 µg rCTB-RT_{33–41}M41L; (3) 1.0 µg rCTB-RT_{33–41}M41L; (4) 2.0 µg rCTB-RT_{33–41}M41L; (5) 4.0 µg rCTB-RT_{33–41}M41L; (6) 0.5 µg rCTB-RT_{33–41}M41L; (7) 1.0 µg rCTB-RT_{33–41}M41L; (8) 2.0 µg rCTB-RT_{33–41}M41L; (9) 4.0 µg rCTB-RT_{33–41}M41L. (B) Western blot analysis of the rCTB-RT_{33–41}M41L conjugate. A monoclonal antibody, specific for pentameric rCTB was used for detection of natively folded protein. Lanes (1) 1 µg rCTB 401 (RC40201) native; (2) 1 µg denaturated rCTB 401 (RC40201); (3) 8 µg native rCTB-RT_{33–41}M41L; (4) 8 µg denaturated rCTB-RT_{33–41}M41L; (5) rainbow molecular weight, Amersham.

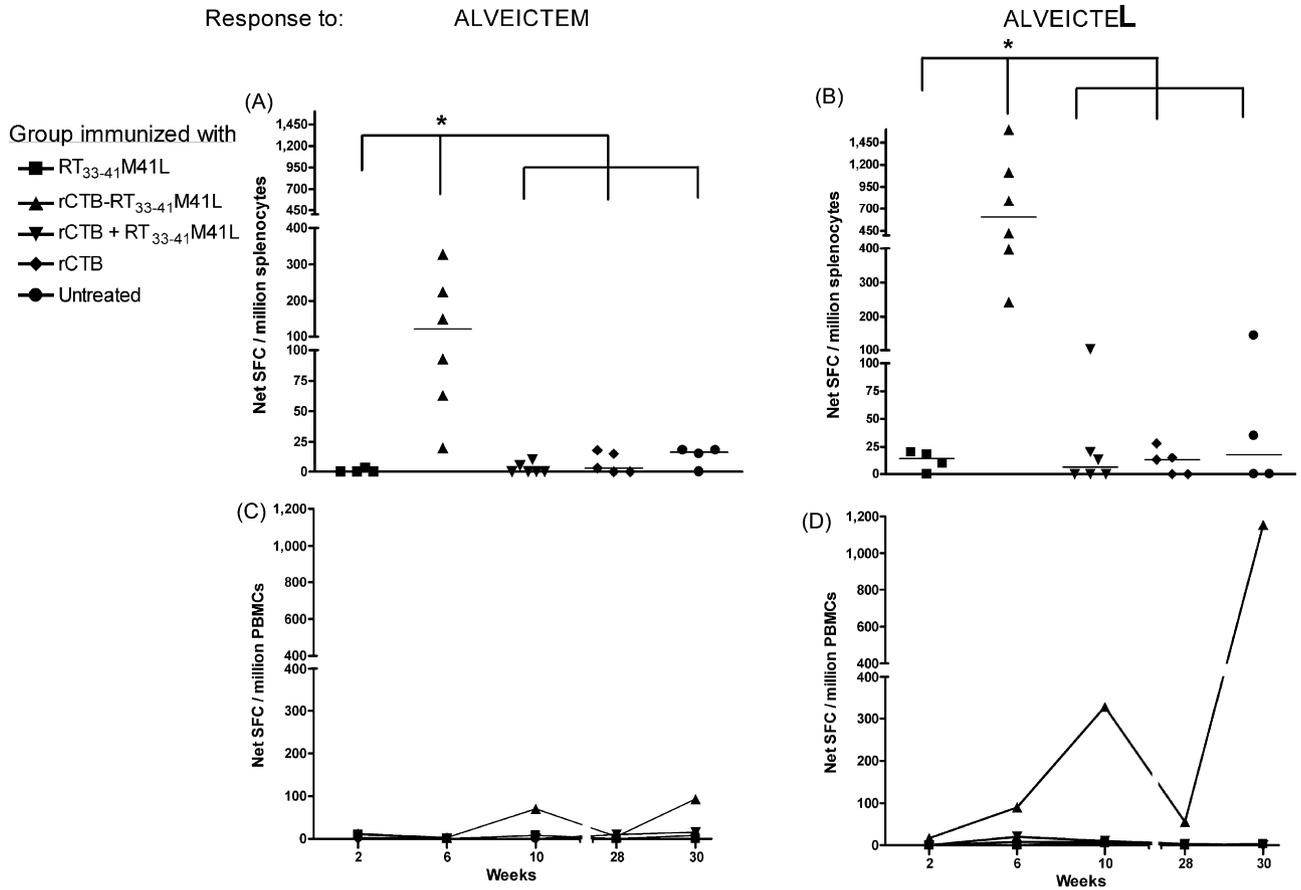


Fig. 2. Immune response and kinetics in spleen and blood cells against RT₃₃₋₄₁ and the mutant variant RT₃₃₋₄₁ M41L. (A) Cross-reactive immune response triggered against the wild type epitope. (B) Immunity induced against the drug resistant mutated epitope variant of RT₃₃₋₄₁. (C) Response to the wild type RT epitope ALVEICTEM. (D) Immune response stimulated against the mutant variant used in immunization. SFC = spot forming cells, PBMC = peripheral blood mononuclear cells. (*) Indicates significant immune responses.

2.5. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 4 (GraphPad Software Inc.). The criterium for statistical significance was $p = 0.05$. The non-parametric Kruskal–Wallis test was used to detect differences among the groups and Mann–Whitney U -test for pair-wise post hoc comparison between groups following a positive Kruskal–Wallis test [10].

3. Results

3.1. Purification of rCTB–ALVEICTEL conjugates

After rCTB–peptide conjugation, the constructs were shown to have a natural folding by SDS–PAGE analysis, followed by Coomassie blue staining of the gels (Fig. 1A). The rCTB fusion protein could also be identified by a monoclonal antibody targeting pentameric rCTB, whereas a denaturated form of the conjugate construct was not detectable (Fig. 1B). These data show that the rCTB–peptide fusion conjugate is correctly folded and thus forms CTB pentamers, which are stable throughout the purification process. Specific binding assays further revealed that the rCTB conjugates bound to GM-1, and thus have an active cell-binding configuration (data not shown).

3.2. Conjugation of peptide significantly enhanced immunogenicity of the epitope

Immunization with the rCTB–RT₃₃₋₄₁ M41L conjugate triggered a strong HIV-specific CTL cellular response against both heterologous wild type and the homologous mutant peptide variants, as analyzed by peptide-specific IFN- γ secretion by splenocytes (Fig. 2A and B). This response was significantly stronger than the responses in any other group ($p < 0.05$). When the peptide instead was delivered as a mixture with rCTB (rCTB + RT₃₃₋₄₁ M41L) no IFN- γ secretion was detected. Immunization with peptide only did not induce a detectable immune response, in spite of a 10-fold

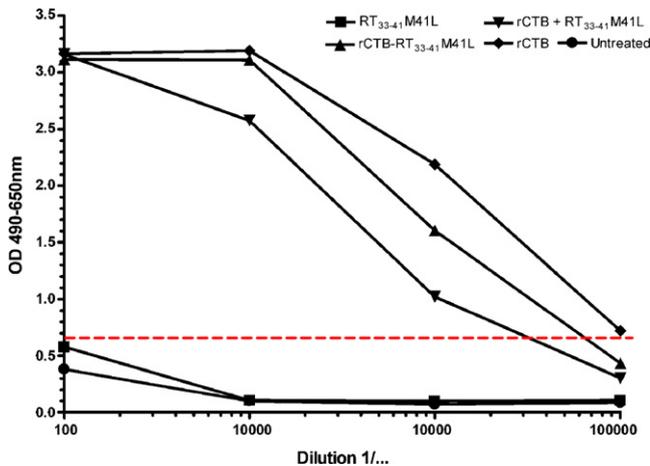


Fig. 3. Humoral response against rCTB. Cut off was calculated as geometric mean of serum from untreated animals +3 standard deviations at the dilution of 1/100, and is shown as dashed line.

higher concentration of peptide than in the group receiving rCTB-RT_{33–41}M41L conjugate. Similar cellular responses were observed in PBMC (Fig. 2C and D). The immune responses were augmented by every additional injection of conjugated peptide. During the interval between the third and fourth injection, the immune response decreased to undetectable levels, but could be boosted by a late fourth injection (Fig. 2C and D), indicating that a vaccine-specific memory was induced by the priming immunizations. Moreover, following immunization with the conjugate (rCTB-RT_{33–41}M41L), cross-reactivity against the wild type epitope was detected both in spleen and in blood (Fig. 2A and C). We could also detect a cellular immune response against rCTB in groups that received it as an antigen component (rCTB-RT_{33–41}M41L, rCTB + RT_{33–41}M41L, and rCTB; data not shown).

3.3. Strong humoral response could be detected to rCTB upon immunization

All animals injected with rCTB (either alone, mixed with the peptide or as a conjugate) induced a strong systemic humoral response against rCTB. The strongest response was detected in the group of mice immunized with rCTB alone, in which the titers exceeded 100,000 (Fig. 3). The anti-rCTB titers dropped significantly during the interval between the third and fourth immunization (data not shown) but were boosted by the fourth immunization. No antibodies directed against rCTB were found in animals immunized with HIV peptide.

4. Discussion

As more HIV-1 infected patients have access to anti-retroviral drug treatment, there is an enhanced risk for development and transmission of drug resistant HIV [1,11–13]. We have addressed this issue by using peptides covering parts of HIV-1 proteins where drug resistance mutations are known to emerge and used those peptide variants for immunization [14]. Furthermore, we explored fusing various peptides to the B subunit of cholera toxin, and used these recombinant conjugates as immunogens, of which one is shown here. The immunogenicity of these conjugates was significantly better than peptide alone or the peptide mixed with rCTB. The enhanced immunogenicity of the conjugated peptide may be due to a better delivery of the peptide to immune cells and/or to an adjuvant effect by rCTB [15]. Even though some early reports have shown adjuvant effects on mucosal responses when rCTB is delivered mixed with peptide, these results are most likely linked to contamination of the CTB preparation by the cholera toxin holoenzyme [15–17]. Encouragingly, the conjugate was superior to a ten-fold higher dose of free peptide; a dose that is highly immunogenic if the peptide is formulated in incomplete Freund's adjuvant (unpublished observation).

Our results indicate that the immunization with HIV peptides linked by molecular conjugation to a natively folded CTB pentamer induces an immunological memory directed against both wild type and mutated HIV epitopes. This is of importance if the vaccine strat-

egy is to be translated into use in therapy naïve HIV-1 infected persons. Ideally, the vaccine should induce the development of a memory T cell population that effectively suppresses drug-resistant viruses as they emerge after the onset of antiviral therapy. Consequently, the same drug combination might be effective for longer periods before a change in therapy is needed.

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