Influenza virus haemagglutinin-derived peptides inhibit T-cell activation induced by HLA-DR4/1 specific peptides in rheumatoid arthritis

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Abstract

Objective
To investigate whether influenza virus haemagglutinin (HA)-derived altered peptide ligands (APLs) could abrogate T-cell responses to wild type HA308-317 or type II collagen (CII) 263-272 peptides and explore the potential inhibitory effects of the altered HA308-317 peptides on T-cell activation in rheumatoid arthritis (RA).

Methods
Altered HA308-317 peptides containing substitutions of T-cell receptor (TCR)-contact residues were synthesized. Peripheral blood mononuclear cells (PBMC) were obtained from 27 HLA-DR4/1-positive RA patients. Impact of the altered HA308-317 peptides on T-cell responses and the inhibitory effects on T-cell activation were determined by using PBMC from RA.

Results
The results showed that the altered HA308-317 peptides could bind to HLA-DR4/1 on cell surface and had no effects on T-cell proliferation and CD25 expression. Moreover, all the altered HA308-317 peptides inhibited T-cell proliferative responses to wild type HA308-317 or CII263-272. In addition, Th1 type cytokine profile was found when PBMC were cultured with wild type HA308-317 or CII263-272, but not the altered HA308-317 peptides.

Conclusions
It is suggested that altered HA308-317 peptides bind to the RA-associated HLA-DR4/1 with no stimulating effects on T cells and might be potentially important in inhibition of T-cell activation in RA.

Key words
Altered peptide ligands, T-cell responses, type II collagen, influenza virus haemagglutinin, rheumatoid arthritis.
Introduction
CD4+ T cells recognize antigenic peptides bound to major histocompatibility complex (MHC) class II molecules through their T-cell receptors (TCR). Previous studies have demonstrated that the impact of altered TCR ligands on T-cell activation and differentiation (1-3). Altered peptide ligands (APLs) with the substitutions of TCR-contact residues on the antigenic peptides can induce T-cell responsiveness (4, 5) or affects the profile of cytokine production (6, 7). Recently, there has been an increase in interest in the use of APLs as antigen specific therapeutic agents in autoimmune diseases (8-10).

Rheumatoid arthritis (RA) is a T-cell-mediated autoimmune disease and associated with HLA-DR4 and HLA-DR1 subtypes (11-13). RA-associated HLA-DR4/1 molecules confer disease susceptibility by presenting different antigenic peptides to CD4+ T cells, which may initiate an immune responses leading to the diseases (14, 15). type II collagen (CII) 256-271 peptide and influenza virus haemagglutinin (HA) 306-318 peptide have been proved to bind to DR4/1 molecules. The human CD4+ T-cell responses to CII-derived peptides or HA306-318 peptide in the context of DR4/1 have been extensively studied and CII has been implicated as an autoantigen involved in the pathogenesis of RA (16-18). Antigenic peptides binding to HLA-DR4/1 might be inhibited by APLs with similar or even higher affinity to DR4/1 molecules. Therefore, the APLs might be used to compete with HLA-DR4/1-binding antigenic peptides and inhibit T-cell activation.

Previous studies from our laboratory have suggested that CII263-272 was the important immunodominant determinant and could induce T-cell proliferation, while the altered CII263-272 peptides were able to inhibit CII263-272-induced T-cell activation (19, 20). HA306-318 is high affinity HLA-DR4/1-binding peptide (21) and altered HA306-318 peptides with the substitutions of TCR-contact residues are not recognized by HA specific T-cell clones, although these peptide analogues still bind to DR4/1 (22). Therefore, altered HA peptides might be more efficient antagonist peptides in suppression of T-cell activation compared to altered CII peptides. In this study, we synthesized three altered HA308-317 peptides and explored the role of these APLs in T-cell responses and cytokine differentiation in RA patients.

Patients and methods
Patients
Twenty-seven HLA-DR4/1 positive RA patients (21 females and 6 males) were entered into the study. All patients fulfilled the ACR revised criteria for the classification of RA. The age was 53.6 ± 13.3 years (20-82 years), and the disease duration was 10.4 ± 8.4 years (8 months -37 years). The majority of patients were being treated with non-steroidal anti-inflammatory drugs, prednisolone (≤ 10mg/day), and disease modifying anti-rheumatic drugs (methotrexate, sulfasalazine, hydroxychloroquine, and gold salts). Of 27 RA patients, 63.0% (17/27) were positive for DRB1*0405, 14.8% (4/27) for DRB1*0404, 11.1% (3/27) for DRB1*0401 or DRB1*0102, respectively. HLA-DR4/1 subtypes were analyzed by PCR-SSP (DRB1*04 and DRB1*01 SSP UniTray Kit, Dynal, Oslo, Norway).

Peptide synthesis
Altered HA308-317 peptides were synthesized using solid-phase techniques on an Applied Biosystems Peptide Synthesizer (Genomed Synthesis, Inc. San Francisco, CA, USA). The peptides were purified by reversed phase high pressure liquid chromatography with a purity of more than 95%. Sequences of the altered HA308-317 peptides were YVAQNTLKL (APL1), YAKQATLKL (APL2) and YAKQATLALA (APL3). Wild type HA308-317 (YVKQNTLKL) and CII263-272 (FKGEQGPKGE) were used as positive controls.

Peptide binding assays
Direct binding assay of altered HA308-317 peptides for HLA-DR1 on cell surface was performed as previously described (17, 23). Briefly, L cells
transfected with HLA-DR1 molecule (L57.23 cell) or untransfected L cells were suspended in complete RPMI 1640 medium and incubated overnight at 37°C in a humidified 5% CO2 atmosphere. The cells were then washed and incubated at 37°C for 4 hours at a concentration of 3x10^5 well in the presence of FITC-labelled peptides. Following this step, the cells were washed twice with phosphate-buffered saline (PBS) and stained cells (10^5) were detected by flow cytometry on a FACScan analyzer (Becton & Dickson Co, San Jose, CA). Background fluorescence was evaluated by cells incubated only with unlabelled peptides. Dead cells were eliminated by the use of propidium iodide. Under the same conditions, antibody inhibition was performed by coincubation of the FITC-labelled altered HA308-317 peptides (10µg/ml) and various doses (0, 5 and 5µg) of anti-HLA-DR antibodies (TU36, BD PharMingen, San Diego, USA). Peptide competitive binding assays were performed in the presence of FITC-labelled CI1263-272 or HA308-317 (10µg/ml) and various concentrations (0, 5 and 50µg/ml) of unlabelled altered HA308-317 peptides added stimulantaneously.

Cell isolation and T-cell proliferation
Heparinized peripheral blood were collected under sterile conditions and diluted 1:1 with RPMI1640. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Hypaque. Cell viability was > 95% by trypsin blue exclusion assay.

Cells were resuspended in complete medium consisting of RPMI 1640 supplemented with 10% fetal calf serum. T-cell proliferation experiments were performed in 96-well microtiter plates in a total volume of 200µl. PBMC at a density of 2.0x10^5 per well were incubated with peptides (CI1263-272, HA308-317 or altered HA308-317 peptides) at 10µg/ml, phytohemagglutinin (PHA) at 5µg/ml or medium alone at 37°C with 5%CO2 for 5 days. In competitive studies, PBMC (2.0x10^5 cell/well) were preincubated with various concentrations of altered HA308-317 peptides (0, 2, 10 and 50µg/ml) for 2 hours before the addition of CI1263-272 or HA308-317. Cultures were pulsed with [H] thymidine (0.5Ci/well) before the last 12 hours. Plates were harvested and the incorporated radioactivity was counted in a β-scintillation counter. The data are presented as stimulation index (SI), calculated as the ratio of counts per minute in the presence of peptides to cpm without peptides. T-cell proliferative responses were considered positive if the SI values were ≥ 2.

Cytokine assays
The supernatants (150µl) were taken at 48 hours from wells for the detection of IFN-γ and IL-4 production. Enzyme-linked immunosorbent assay kits (ELISA, BD PharMingen, San Diego, USA) were used to detect the levels of IFN-γ or IL-4 in the supernatants according to instructions from the manufacturer. Each sample was tested with duplicated wells. The detection limit was 7.8 pg/ml for IFN-γ or IL-4.

Detection of CD25 expression.
Flow cytometry was performed to detect CD25 expression (T-cell activation marker) on cell surface. PBMC (2.0x10^5 cell/well) were incubated with 10µg/ml of CI1263-272, HA307-318 or APLs respectively for 72 hours at 37°C with 5%CO2. Cells were washed twice with 0.2M cold PBS, then stained with FITC-labeled anti-CD4 (RPA-T4, Biosciences, San Diego, CA, USA) and PE-labeled anti-CD25 (BC96) at 4°C for 30 min. After washing, the cells were resuspended in PBS and analyzed using CellQuest™ Software version 7.5.3 on a FACS Calibarator.

Statistical analysis
Unless otherwise stated, SI and cytokine concentrations were indicated as mean ± SD. Student’s t-tests were used for the comparisons of cytokine levels and SI. All analysis were performed using SPSS (11.5 version). P-values less than 0.05 were considered statistically significant.

Results
Specific binding of altered HA308-317 peptides to HLA-DR1/4 on the cell surface
Two different experiments were performed to confirm the direct interaction of HLA-DR1/4 with the FITC-labelled altered HA308-317 peptide (APL3). Firstly, L57.23 cells or untransfected L cells were incubated respectively with a FITC-labelled APL3. A strong fluorescence staining was noticed on L57.23 cells, while there was little fluorescence staining on untransfected L cells (data not shown). In addition, binding specificity was further assessed using anti-DR mAb directed against the HLA-DR1 molecules. When the concentrations of anti-HLA-DR was increased, FITC-labelled APL3 staining on L57.23 cells was dramatically reduced (Fig. 1). The observations indicated that altered HA308-317 peptides specifically bound to HLA-DR1/4 molecules and the binding was competed by mAb against HLA-DR1/4 molecules.

![Fig. 1](image_url)
Binding inhibition of CII263-272, HA308-317 to HLA-DR1 molecules by altered HA308-317 peptides.

As shown previously, CII263-272 peptides could bind to HLA-DR1 molecules on cell surface (17, 19-21). To evaluate whether altered HA308-317 peptide (APL3) competed with CII263-272 for binding to HLA-DR1, competitive binding assay was carried out with FITC-labelled CII263-272 (10 µg/ml) and various concentrations of APL3 as indicated. It was shown that a strong fluorescence signal was observed on L57.23 cells when only FITC-labelled CII263-272 was added. While the concentration of APL3 increased, the binding ability of CII263-272 to HLA-DR1 on cell surface was decreased in a dose response manner (Fig. 2). Similar results were shown in the binding inhibition of HA308-317 to HLA-DR1 molecules by APL3 (data not shown).

Inability of altered HA308-317 peptides on T-cell proliferation in RA.

The ability of the altered HA308-317 peptides to stimulate T cell responses in RA patients was measured. As shown in Figure 3, T-cell proliferative responses to altered HA308-317 peptides in PBMC from RA were 7.4% for APL1, 3.7% for APL2 or APL3, which were lower than CII263-272 (62.2%) and HA308-317 (52.9%). In addition, the SI values for T-cell responses to altered HA308-317 peptides were 1.23 ± 0.43 for APL1, 1.26 ± 0.38 for APL2 and 1.11 ± 0.41 for APL3, respectively, which were significantly lower than CII263-272 (2.03 ± 0.79) and HA308-317 (1.84 ± 0.57). The results indicated that altered HA308-317 peptides were weak stimulators, compared to CII263-272 or HA308-317 and failed to stimulate T-cell responses.

Impact of the altered HA308-317 peptides on CD25 expression.

To further analyze the effects of the altered HA308-317 peptides on T-cell activation in RA patients, we evaluated CD25 expression on T-cells stimulated with the altered HA308-317 peptides, wild type HA308-317 and CII263-272, respectively. As shown in Figure 4, altered HA308-317 peptides did not increase CD25 expression on T-cells of RA. In contrast, CD25+ T-cell was significantly increased when the cells were stimulated with wild type HA308-317 or CII263-272 peptides.

Fig. 2. Altered HA308-317 peptides (APL3) competitively inhibited the binding of CII263-272 to HLA-DR1. L57.23 cells were incubated with unlabelled-APL3 alone (A) or coincubated with FITC-labelled CII263-272 (10 µg/ml) and unlabelled-APL3 at increasing concentrations of 0 µg/ml (B), 5 µg/ml (C) and 50 µg/ml (D). As the concentration of APL3 was increased, the binding ability of CII263-272 to HLA-DR1 on cell surface was reduced in a dose response manner from 52.4% to 27.4%.

Fig. 3. T-cell proliferative responses to altered HA308-317 peptides, CII263-272 and wild type HA308-317 in PBMC from RA. A. T-cell proliferative responses to altered HA308-317 peptides were 7.4% (APL1), 3.7% (APL2) and 3.7% (APL3) in 27 RA patients, respectively, which were much lower than CII263-272 (62.2%) and HA308-317 (52.9%). B. The SI values for T-cell responses to altered HA308-317 peptides were 1.23 ± 0.43 (APL1), 1.26 ± 0.38 (APL2) and 1.11 ± 0.41 (APL3), and which were lower than wild type CII263-272 (2.03 ± 0.79) and HA308-317 (1.84 ± 0.57). *p < 0.01.

Fig. 4. Impact of the altered HA308-317 peptides on CD25 expression. Cells were double-stained with FITC-labeled CD4 mAb and PE-labeled CD25 mAb in presence of medium only (A). CII263-272 (B), HA308-317 (C), APL1 (D), APL2 (E) or APL3 (F). Compared to medium, altered HA308-317 peptides did not increase CD25 expression in PBMC of RA. In contrast, CD25+ T-cell was significantly increased when the cells were stimulated with wild type HA308-317 or CII263-272 peptides.
HA308-317 peptides have no effects on T-cell activation.

**Effect of the altered HA308-317 peptides on cytokine production of T-cells from RA patients.**

To find whether the altered HA308-317 peptides affected cytokine profile secreted by T-cells from RA, supernatants of PBMC were evaluated for the IFNγ production from 13 RA patients with the altered HA308-317 peptides, wild type HA308-317 or CII263-272 stimulation. The levels of IFNγ were significantly increased in cultures stimulated with wild type HA308-317 (62.78 ± 17.51 pg/ml) or CII263-272 (73.27 ± 18.44 pg/ml), compared to the unstimulated culture (34.38 ± 10.22 pg/ml). In contrast, the altered HA308-317 peptides had no effects on IFNγ production in PBMC from RA with the levels of 34.99 ± 9.11 pg/ml for APL1, 39.26 ± 11.3 pg/ml for APL2 and 34.39 ± 7.65 pg/ml for APL3, which were similar as unstimulated control (34.38 ± 10.22 pg/ml).

IL-4 levels in the supernatants of PBMC from 11 RA patients were measured to find whether the altered HA308-317 peptides were able to trigger Th2 cytokine profile. The results did not show significant differences in IL-4 productions when PBMC from RA patients were stimulated with the altered HA308-317 peptides or wild type antigenic peptides (Fig. 5).

**Inhibition on HLA-DR4/1 restricted T-cell responses by altered HA308-317 peptides**

We next assessed whether the HA308-317-derived APLs were able to inhibit T-cell responses to the wild type HA308-317 or CII263-272 in RA patients. It was shown that the proliferative responses to wild type HA308-317 or CII263-272 were suppressed by the altered HA308-317 peptides dose-dependently in a range from 2.0 µg/ml to 50 µg/ml (Fig. 6). These results suggested that the HA308-317-derived APLs might be potentially antagonists to inhibit specific T-cell responses to HLA-DR4/1-binding peptides in RA.

**Discussion**

The susceptibility to RA is strongly associated with the expression of HLA-DR4/1, which share the common sequence QKRAA/QRRAA at position 70-74 within the third hypervariable region of the HLA-DRB1 chain (12, 13). The region is critical for antigenic peptides presented to CD4+ T-cells (14, 15). Inhibition of antigenic peptides presentation by HLA-DR4/1 molecules might be effective in immunotherapy of RA.

Almost ten years ago, Mayo Clinic investigators showed that transgenic mice carrying the protective genes (B10.RQ3.Eb and B10.RQ3-DRB1 I502) against the development of arthritis exhibited a significant reduction in T-cell proliferation against arthritogenic CII compared to the transgenic negative mice. Interestingly, these transgenic mice that had protection against collagen-induced arthritis were able to mount in T-cell proliferative response against self-antigenic peptides (24, 25). These observations suggest that some endogenous self-
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derived peptides may play a role in the protection against the development of RA. This protection might be due to the role of these peptides to interfere with the presentation of antigenic peptides by MHC class II molecules. APLs that possess affinity for HLA-DR4/1 have been designed to competitively bind to HLA-DR4/1 with antigenic peptides and block T-cell activation because of removing the amino acids at TCR-contact positions (26, 27). Furthermore, APLs represent an antigen-specific method of manipulating T-cell responses and have been used in an animal model of autoimmune disease, experimental allergic encephalitis (EAE), to prevent responses to the disease-causing peptide in myelin basic protein (28). In the present study, we assessed T-cell responses to DR4/1-binding peptides HA308-317 or CII263-272 in RA patients and demonstrated that T-cell responses to these peptides could be inhibited by the newly designed altered HA308-317 peptides with substitutions of the TCR-contact residues. The mechanism which APLs antagonize T-cell responses cannot be based on HLA-DR blockade only. Alternatively, APLs may alter the cytokine production profile of T-cells (3, 6, 7). Studies have suggested an imbalance between T lymphocytes producing Th1 and Th2 cytokines at sites of inflammation in RA patients and chronic inflammatory process in RA might be driven by activated Th1 cells without Th2 cells differentiation to downregulate the inflammation (29, 30). Previous studies indicated that APLs could strongly affect cytokine production by T-cells (31, 32). In the present study, we revealed that altered HA308-317 peptides downregulated the production of IFN-γ compared to wild type HA308-317 or CII263-272 which promoted IFN-γ secretion. These results suggested that altered HA308-317 peptides are not Th1 stimulator. It is not clear whether they regulate Th2 cells since no effects on IL-4 production was found in the present study. We have demonstrated in this study that the altered HA308-317 peptides inhibited T-cell responses induced by wild type HA308-317 or CII263-272. These findings are consistent with previous studies showing that altered CII peptides inhibited T-cell activation induced by wild type HA peptide (2). The cross-inhibition of HLA-DR4/1 restricted T-cell response by the altered HA308-317 peptide is of interest in the treatment of RA since a number of HLA-DR4/1-binding peptides are involved in RA, such as CII and Bip (18, 33, 34). These antigenic peptides share similar three dimensional structures and can fit in the antigen binding groove of HLA-DR4/1 molecules, even though their sequences are different (35). Therefore, the altered HA308-317 peptides may act as “consensus” blockers, resulting in suppression of autoimmune T-cell responses to other RA-associated HLA-DR4/1-binding antigenic peptides. Further studies are necessary to investigate the precise mechanism of their inhibitory effects and whether the altered HA308-317 peptides are effective in inhibition of T-cell activation in vivo, such as in HLA-DR4/1 transgenic animal models.

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