Peptide analogues of a T-cell epitope of ricin toxin A-chain prevent agonist-mediated human T-cell response

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Abstract

The clinical efficacy of immunotoxins (IT) containing ricin toxin A-chain (RTA) can be drastically reduced by anti-toxin-neutralizing antibodies developed by patients. Strategies aimed at epitope-specific modulation of the immune response must be therefore set up to broaden the clinical applicability of RTA-based IT. Prevention or reduction of humoral immune responses against RTA could be achieved by peptide-based down-modulating strategies. Peptide analogues were investigated as candidate antagonist altered peptide ligands (APL) considering the sequence of a previously identified dominant T-cell epitope of RTA (i.e. I175–E185) presented in the context of the HLA-DRB1*0301 allele. Alanine-substituted peptides provided information on the role of individual residues of the wild-type peptide and allowed to identify one antagonist APL corresponding to the double-mutant peptide E177A/A178D. The analogue E177A/A178D not only prevented the agonist from stimulating anti-RTA human T-cell clones but also failed to induce down-regulation of surface-expressed TCR, thus suggesting its possible use for in vivo immune modulation of anti-RTA responses.

Introduction

Potent cytotoxic reagents [immunotoxins (IT)] are obtained by conjugating cell-selective vehicle molecules (e.g. antibodies, ligands, growth factors) to the enzymatic polypeptide ricin toxin A-chain (RTA). RTA-IT yielded promising results in the treatment of human diseases, particularly hematological neoplasias (1, 2). However, injection of RTA-IT often induces the development of high-titer anti-toxin antibodies, affecting IT efficacy and strongly limiting a more general application of RTA-IT in vivo (3, 4). Development of antibodies to the targeting molecules can be circumvented by using reagents of human origin. Prevention or modulation of anti-RTA immune response will instead require a more complex intervention.

Strategies to prevent or reduce the anti-RTA immune response were extensively evaluated in animal models by treating mice with cyclophosphamide (5) or with other immunosuppressive agents (6). These approaches, however, may not be directly applicable in humans because immunosuppressive drugs can increase the risk of concomitant infections and malignancies. To overcome these limitations, development of less aggressive and more selective methods to suppress or reduce the host immune response against RTA would be advisable. The observation that human anti-ricin antibodies belong to the IgG class indicates that RTA is a thymus-dependent antigen able to induce a secondary immune response involving both T cells and B cells (7). Manipulating the response of potentially reactive T cells using altered peptide ligands (APL) represents a very attractive approach (8). Once immunodominant T-cell epitopes are identified, down-modulation of the immune response can be achieved using APL (9). Antagonist peptide analogues are able to inhibit the agonist-mediated T-cell activation and therefore the downstream events leading to a humoral immune response (10). Functional inhibition of T-cell responses by APL has been described in animal models of autoimmune diseases (11, 12) and encouraging results have been obtained also in humans (13, 14).

We have previously described a set of human RTA-specific T-cell clones, obtained by in vitro priming of peripheral blood cells from healthy donors as a source of responder
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T cells (7). Clonal T cells recognized a minimal T-cell epitope spanning the sequence I175–E185 of RTA (7) by preferentially engaging TCR-\(\alpha\)1 and TCR-\(\beta\)8 chains. The epitope I175–E185 is presented to anti-RTA T-cell clones in association with HLA-DRB1*1101 and HLA-DRB1*0301 molecules, which are widely expressed in the Caucasian population (15).

We therefore set out to investigate the reactivity and the modulating properties of alanine-substituted analogues of I175–E185 considering the proliferative response of anti-RTA T-cell clones. By this approach we identified one candidate epitope-derived APL with antagonist properties, which could become a suitable tool to prevent/reduce anti-RTA responses.

Methods

Chemicals and reagents

All the chemicals were purchased from Sigma (St Louis, MO, USA). Peptides based on the sequence of the RTA and point mutant peptides were synthesized by an Applied Biosystem (Monza, Italy) automated synthesizer on solid phase (16). Purity was assessed by HPLC and mass spectrometry and was found to be >90%.

RTA-specific T-cell clones

Antigen-specific T-cell clones were obtained by in vitro priming of PBMC obtained from a normal donor (donor M.C.) with no history of previous sensitization to RTA and no evidence of anti-RTA antibodies in the serum. The RTA-specific T-cell clones used in the present study were partly characterized previously (7), and those retaining a higher proliferative potential were selected for further studies. Here T-cell clones MC44, MC69, MC37 and MC52 were used. All the T-cell clones showed comparable proliferation in response to the stimulating peptide I175–E185, expressed CD4 antigen and recognized the RTA peptide I175–E185 in the context of the MHC class II allele HLA-DRB1*0301 utilizing \(\alpha\)1 and \(\beta\)8 TCR chains (7). Use of different T-cell clones in different sets of experiments is due to the limited availability of clonal responder T cells in some instances.

B-lymphoblastoid cell lines

An autologous B-lymphoblastoid cell line (B-LCL) was obtained from a depleted PBMC population from the donor M.C. as reported elsewhere (7). Briefly, freshly isolated PBMC were depleted of T cells and infected with an EBV-containing supernatant of the marmoset cell line B95-8 (American Tissue Culture Collection, Rockville, MD, USA) and grown under standard culture conditions (see below).

Two EBV-transformed B-LCL (from European Collection for Biomedical Research, Southampton, UK), homozygous for the indicated human MHC class II alleles, were used as antigen-presenting cells (APC) in proliferation assays. WT49, expressing HLA-DRB1*0301, and SWEIG, expressing HLA-DRB1*1101, were chosen based on the HLA typing of the PBMC used as source of anti-RTA T-cell clones (17, 18). Nomenclature of HLA-DR alleles is reported by Robinson et al. (19).

Cell culture conditions

T-cell clones as well as B-LCL were grown under standard culture conditions, i.e. at 37°C in a 5% CO2 atmosphere, in RPMI-1640 medium (Life Technology, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Boehringer, Mannheim, Germany), 2 mM L-glutamine (Seromed, Berlin, Germany) and antibiotics (penicillin–streptomycin, 400 U ml\(^{-1}\)). T-cell clones required the addition of 100 U ml\(^{-1}\) of human recombinant IL-2 (rIL-2) (Chiron, Siena, Italy) and periodic stimulation with either 1% PHA (Life Technology) or 25–50 µg of heat-denatured native RTA (dRTA) in the presence of irradiated (4000 rad) autologous PBMC.

Proliferation assay

RTA-specific T cells were co-cultured with either autologous or homozygous B-LCL as APC to measure antigen-induced T-cell proliferation. T cells were plated at 1–2 × 105 cells per well in 96-well culture plates (Greiner, Longwood, FL, USA), following exhaustive washings to eliminate rIL-2 from cell cultures and overnight incubation in the absence of rIL-2. APC were irradiated (4000–6000 rad) by exposure to a source of \(^{137}\)Cs radiations and plated 1 : 2 with respect to responder T cells. When a pre-pulsing step was required, irradiated APC were incubated for 2–4 h or overnight in the presence of dRTA or RTA-derived peptides and washed twice with RPMI-1640 medium before co-culture with T cells. After 3–4 days, T-cell proliferation was assessed by addition of 0.5 µCi of tritiated thymidine (\(^{3}H\)TdR) (Amersham, Amersham, UK) to the culture and incubation for further 16 h. Following cell harvesting, washing and drying onto glass fiber filters (using a cell harvester, Dynatech, Haverhill, MA, USA), the radioactivity incorporated by the cells was measured in a \(\beta\)-spectrometer (Wallac 2406). The counts per minute (c.p.m.) values obtained were used to calculate the stimulation index (SI), which is defined as the mean c.p.m. of triplicate or duplicate antigen-stimulated samples divided by the mean c.p.m. of control cultures without stimulating antigen (i.e. the mock-treated controls). Proliferation was considered significant when SI > 2. Values of \(^{3}H\)TdR incorporation in control samples were usually <500 c.p.m.

Inhibition of antigen processing

T-cell proliferation assays were performed essentially as described above, by adding 1 × 105 cells per well from a T-cell clone to 2 × 105 cells per well of pre-pulsed WT49 cells, which were either irradiated or fixed after antigen pulsing. The irradiated WT49 cells were used as positive control of competent APC, whereas inhibition of intracellular processing of antigen was achieved by treating WT49 cells as follows: after extensive washings, cells were re-suspended in 100 µl of complete medium supplemented with 80 µM chloroquine. After 10 min incubation at room temperature, peptides (10 µg ml\(^{-1}\)) were added and cells were incubated for further 4 h at 37°C. Excess antigen was removed by washing the cells twice in complete medium and once in PBS. Cells were then fixed with 0.25% glutaraldehyde for 60 s and fixation was blocked by adding 0.2 M glycine for 10 min.
TCR antagonism assay

Antagonist activity of two RTA-based peptides (E177A and E177A/A178D) was assayed by proliferative assays, in which the APC were pre-pulsed with the agonist peptide I175–E185, essentially by following the method described by Frasca et al. (8). Autologous B-LCL cells were pre-pulsed overnight with a fixed dose of I175–E185, washed and then incubated for further 5 h with either E177A or E177A/A178D at various concentrations. In parallel, equal amounts of the same antagonist peptides alone were incubated overnight with the autologous B-LCL. The peptide I175–E185 was used at 10 μg ml⁻¹, corresponding to the concentration resulting in 50% of maximum T-cell stimulation, according to dose–response experiments performed previously. Following incubations with peptides, the APC were irradiated (6000 rad) and added to T-cell clones in 96-well round-bottom microplates (5 × 10⁴ cells per well). After 3 days, T-cell proliferation was measured by following the standard protocol described above. The data are presented as percent inhibition of T-cell proliferation with respect to the maximum c.p.m. values obtained with the stimulating peptide alone or in the absence of other antigenic stimuli.

Down-modulation of TCR expression

Down-modulation of TCR expression was evaluated by measuring the surface expression of the CD3 co-receptor by T-cell clones, pre-incubated separately with RTA-based peptides (L161–T190, I172–M188, I175–E185) and with mutants of the I175–E185 peptide (E177A and E177A/A178D). The assay was performed essentially as described elsewhere (20). Briefly, autologous B-LCL cells (2 × 10⁵ cells per well) were re-suspended in 100 μl of 5% FBS-containing RPMI-1640 medium and pulsed for 3 h with peptides (at 50 μg ml⁻¹ concentration). A mock-treated sample was also included as control. Following three washings with medium to eliminate unbound peptides, pre-pulsed B-LCL cells were mixed with resting T cells (1 × 10⁵ cells per well) in round-bottom microplates and centrifuged to allow the formation of conjugates at 37°C. After 4 h incubation, cells were re-suspended and washed in PBS containing 0.5 mM EDTA to break the conjugates. Cells were then stained with a PE-conjugated anti-CD3 mAb (Becton-Dickinson, San Diego, CA, USA) and the CD3-associated fluorescence was analyzed with a FACScan (Coulter, Hialeah, FL, USA). A sample stained with an irrelevant PE-labeled mAb (Becton-Dickinson) was considered as the negative control. Expression of cell-surface CD3 was evaluated by considering the mean fluorescence intensity. Data are presented as percent TCR down-regulation (i.e. reduction of CD3 surface expression) with respect to the maximum CD3 level (100% expression) measured in T-cell clones that were incubated with non-pulsed APC.

Results and Discussion

In the present study, the T-cell epitope I175–E185 was investigated in detail by assaying single-substituted analogs for their ability to induce and modulate RTA-specific T-cell activation. A set of high-responder human anti-RTA T-cell clones were used which recognized the relevant epitope in the context of the widely distributed MHC class II allele HLA-DRB1*0301. Ricin is a member of the ribosome-inactivating proteins (RIP) family which also includes a number of single-chain RIP-I, such as diathin, momordin, saporin, pokeweed anti-viral protein and gelonin. The enzymatic subunit of ricin (RTA) and toxins belonging to the RIP-I group are all of similar size, all carry out the same N-glycosidation reaction and show a high degree of sequence similarity (21, 22). The T-cell clones used by us recognize a region of RTA containing the enzymatic site of action of the toxin which also shows a high degree of similarity with domains belonging to other RIP-I (7). However, they do not recognize a panel of RIP-I showing stretches similar but not identical to fragment L161–T190 (7).

Role of individual amino acid residues in T-cell activation

It was reported that the most striking effects on the binding of peptides to the HLA-DRB1*0301 allele (23) are caused by hydrophobic and aromatic residues in position 1 (i.e. the primary anchor) and position 2 within the nonapeptide core that typically characterizes class II-restricted T-cell epitopes. The role of isoleucine 175 as the primary anchor of the RTA epitope I175–E185 was indeed confirmed on the basis of several experimental findings (7). Peptide positions 4, 6, 7 and 9 of the HLA-DRB1*0301-specific binding motif are usually defined as secondary anchors devoted to peptide stabilization, whereas there is typically a TCR-contact residue in position 3. Anchor IV (in position 4) is dominated by aspartic acid; positions 5 and 6 have preference for basic and hydrophilic amino acids and position 7 for hydrophobic and aromatic side chains (23). Finally, positions 8 and 9 are occupied by structurally different residues (23). Based on this background information, we observed that the sequence of the epitope I175–E185 satisfies the main chemical restrictions for a productive presentation by HLA-DRB1*0301 molecules, with the exception of position 4 that is occupied by alanine. According to this, the region I175–Y183 is likely to represent the nonapeptide core of the epitope I175–E185, fitting the MHC groove and including most of the crucial residues involved in interactions with both TCR and MHC. To evaluate the relative contribution of individual amino acids of the epitope I175–E185, the reactivity of single-substituted peptides was evaluated in proliferation assays. The contribution of the amino acid side chains in either MHC binding or TCR engagement was abrogated by replacing each peptide position with an alanine.

Table 1 shows the sequence and the stimulation effect of the alanine-substituted peptides (used at the fixed concentration of 20 μg ml⁻¹) in comparison with the wild-type epitope I175–E185. The same set of mutated peptides was also assayed in dose–response experiments using different T-cell clones. Figure 1 shows the results obtained with one representative T-cell clone (MC44). Abrogation of the epitope’s reactivity provided by almost every substitution within the sequence I175–Y183 strongly supported its identification as the nonapeptide core of the epitope. In fact, only the mutation S176A in peptide position 2 did not affect the stimulatory efficiency of the epitope (Fig. 1) and can be therefore considered an ‘indifferent substitution’ (23). A slight stimulatory effect was observed only when the mutated peptides substituted in R180, F181 and Y183 were used at very high doses.
Peptides I–IX. The one-letter code for amino acids is used. 

The nonapeptide core of the epitope (I175–Y183) is shown in bold. The anchor residues are labeled by roman numbers I–IX. The one-letter code for amino acids is used.

<table>
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<th>Peptides</th>
<th>I175</th>
<th>I176</th>
<th>I177</th>
<th>R180A</th>
<th>F181A</th>
<th>Q182A</th>
<th>Y183A</th>
<th>I184A</th>
<th>E185A</th>
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<td>S</td>
<td>E</td>
<td>F</td>
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<td>11</td>
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</table>

The stimulatory ability of the wild-type peptide I175–E185 and point mutants is compared and reported as SI. The T-cell clone MC44 was used as a source of responder cells. Dashes represent identity with mutants is compared and reported as SI. The T-cell clone MC44 was used. WT49 cells, either untreated or fixed, were added at the concentration of 10^5/ml. Fig. 2, panel B) concentration of the I175–E185 peptide were used. For comparison, the other alanine-substituted peptides were also assayed in the presence of a sub-optimal dose of I175–E185 (2.5 µg/ml). Use of sub-optimal doses of wild-type peptide allowed to maximize the possible displacing effect of a competitor ligand. Three main profiles of the competitive effect of the mutants emerged from our results (represented in Fig. 3, panels A and B). Peptides S176A (shown as an example in panel A), I184A and E185A stimulated anti-RTA T-cell clone MC69 with comparable efficiency in the presence or absence of the wild-type I175–E185. Peptides R180A, F181A, Q182A and Y183A (the latter reported in panel B) failed to activate T-cell clones (except at very high concentrations) if tested alone, whereas they revealed a synergetic effect on T-cell activation when combined with the wild-type epitope. This finding could be explained as the recruitment of a threshold number of TCR leading to a productive T-cell activation only when both wild-type and mutant peptides are present, which is not obtained in the presence of the mutants alone or in the presence of a sub-optimal concentration of the agonist peptide (25). Finally, E177A was the only mutant which was unable to activate T-cells or to improve the epitope’s reactivity at each concentration assayed (Fig. 2, panel A). More interestingly, however, E177A inhibited the epitope-mediated T-cell stimulation when the wild-type I175–E185 was added at the concentration of 10 µg/ml, corresponding to a 50% stimulation (Fig. 2, panel B).

E177A and E177A/A178D as antagonist peptides

Taking into account the restrictions set forth for a suitable binding of the peptide to HLA-DRB1*0301 molecules (23),
the residue A178 of the epitope I175–E185 was replaced with aspartic acid, allowing to obtain a peptide (A178D) endowed with greater stimulatory ability than the wild-type peptide. The mutant activated all T-cell clones assayed ~40% more effectively than I175–E185 (data not shown), possibly because of an improved peptide anchoring to MHC (21). The mutation A178D was then combined with the substitution of E177 in position 3, thus obtaining the double-mutant E177A/A178D. Comparable to the peptide E177A, the double-mutant E177A/A178D failed to activate anti-RTA T-cell clones due to the substitution at a TCR-contact site (E177).

We then evaluated the ability of E177A and of E177A/A178D to interfere with agonist-mediated T-cell activation and therefore to act as antagonists of the wild-type peptide. Figure 4 shows the results of two representative TCR antagonism assays performed with the anti-RTA T-cell clones MC37 and MC52. After pre-pulsing the autologous B-LCL with a stimulating concentration of the I175–E185 peptide (10 μg ml⁻¹), APC were washed to remove unbound peptide. The subsequent incubation in the presence of competitor peptide, either E177A or E177A/A178D, allowed the bound epitope to be displaced on the surface of APC. T-cell activation was finally measured in a proliferation assay after addition of responder clonal T cells. Results illustrated in Fig. 4 demonstrated that the double mutant behaved as a more effective competitor in displacing the native I175–E185 epitope than E177A. Thus, E177A/A178D can act as an efficacious antagonist APL and might be considered as a suitable tool for negatively interfering with agonist-induced T-cell activation also in vivo.

**TCR down-modulation analysis**

Peptides displaying antagonist features not only fail to activate T cells but they also prevent the generation of a TCR-mediated activation signal by agonist peptides (26). It has been reported that antagonist APL fail to induce down-regulation of surface-expressed TCR at concentrations much higher than those required for inducing the partial tyrosine phosphorylation of CD3-ζ chains (27). These observations indicate that antagonist peptides can interfere with the very initial steps of the T-cell activation, i.e. at the level of TCR engagement (28). The effect of antagonist APL can be therefore ascribed to competitive recruitment of TCR and subsequent prevention of TCR oligomerization (29) and productive engagement by agonist peptides (30).

The effect of E177A and E177A/A178D on surface-expressed TCR was compared with the effect induced by
the sequence could in some cases depend on the existence of 
has been reported that the induction of antibodies to a given 
region of RTA (corresponding to the stretch L161–E185). It 
(i.e. L161–I175) (31) allows to define a highly immunogenic 
maps immediately downstream to a linear B-cell determinant 
relevance. The observation that the epitope I175–E185 
sion of humoral responses against molecules of therapeutic 
reagents for immunological manipulations, such as suppres-
Antagonist APL can therefore be considered as suitable 
and in the inhibition of agonist-induced T-cell responses (8). 
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mutations which enhance the interaction with MHC class II 
to displace binding of the agonist as compared with the 
mutant E177A/A178D might be ascribed to a superior ability 
to a control sample (non-pulsed APC).

three stimulating peptides L161–T190, I172–M188 and I175–
E185 (of 30-mer, 17-mer and 11-mer residues, respectively, 
all containing the minimal epitope I175–E185) using T-cell 
clone MC52. Autologous B-LCL cells were pre-pulsed with 
each peptide and co-cultured with anti-RTA T-cell clones to 
allow the formation of cell–cell conjugates. TCR expression 
the T-cell surface was then evaluated by CD3 
fluorescent staining and cytofluorometric analysis. As shown 
in Fig. 5, the treatment with each agonist peptide resulted in 
a marked decrease in the surface expression of the TCR/ 
CD3 complex to 25–50% of normal levels, whereas treatment 
with the antagonists (E177A or E177A/A178D) failed to 
induce any appreciable TCR down-modulation. The lack of 
a productive TCR engagement by the antagonists, both 
displaying the E177A substitution, can be therefore hypothe-
sized as a possible mechanism leading to the inhibition of 
the agonist-induced T-cell stimulation. The total absence of 
TCR down-regulation observed in the presence of the double-
mutant E177A/A178D might be ascribed to a superior ability 
to displace binding of the agonist as compared with the 
single-mutant E177A due to the effect of the A178D sub-
stitution which enhances the interaction with MHC class II 
molecules (23). Small changes in the sequence of a T-cell 
epitope can result in the loss of activation signals in T cells 
and in the inhibition of agonist-induced T-cell responses (8). 
Antagonist APL can therefore be considered as suitable 
reagents for immunological manipulations, such as suppres-
ion of humoral responses against molecules of therapeutic 
relevance. The observation that the epitope I175–E185 
maps immediately downstream to a linear B-cell determinant 
(i.e. L161–I175) (31) allows to define a highly immunogenic 
region of RTA (corresponding to the stretch L161–E185). It 
has been reported that the induction of antibodies to a given 
sequence could in some cases depend on the existence of 
T<sub>T</sub> cells specific for a stretch proximal to the antibody 
epitope. This so-called ‘T–B reciprocity’ (32) could thus 
explain the high antigenic potential of a limited RTA domain 
containing both a T-cell and a B-cell epitope. This RTA region 
is therefore an ideal target for rational antigen modifications 
aiming at preventing/reducing the triggering of an anti-RTA 
immune response. Indeed, it was shown in animal models 
(33, 34) that inhibition of immune response to an immunodom-
inant epitope prevents spreading of immune recognition 
and response to secondary/sub-dominant epitopes. More-
over, single conservative amino acid substitutions prevented 
the induction of responses to both the immunodominant and 
sub-dominant epitopes, which corresponded to the loss of 
response to the whole protein molecule (35). It could thus 
be hypothesized that in vivo application of the APL 
described by us might result in failure to respond to injected 
RTA. This of course must be verified in appropriate in vivo 
models.

We have here demonstrated that APL are able to prevent 
the native epitope from activating RTA-specific T-cell clones 
in vitro, in particular the whole cascade of biochemical 
signals that normally result in T-cell proliferation. Thus, 
treatment strategies exploiting the properties of the antagonist 
peptide discovered by us could also be envisaged; for 
example, the inoculation of the APL before or at the same 
time of a treatment cycle with RTA-based IT may prevent 
the development of potentially anti-RTA reactive T cells and 
allow more effective treatment regimens. In vivo confirmation 
of such a hypothesis is of course warranted. The MHC class II 
allele which is the target of the inhibitory effect of the APL 
described by us has a broad distribution in the Caucasian 
population (15); thus, a considerable number of subjects could 
in principle benefit from such immune-modulating strategies.
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Abbreviations

APC  antigen-presenting cells
APL  altered peptide ligands
B-LCL  B-lymphoblastoid cell line
c.p.m.  counts per minute
dRTA  heat-denatured native RTA
FBS  fetal bovine serum
[^3H]TdR  tritiated thymidine
IT  immunotoxins
rIL-2  recombinant IL-2
RIP  ribosome-inactivating proteins
RTA  ricin toxin A-chain
SI  stimulation index

References


