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# Rat MHC class I peptides are immunogenic

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**Abstract** We postulated that indirect recognition of MHC-derived peptides might modulate the alloresponse to donor antigen. In this study, we looked at the potential of two class I peptides derived from the α1 and α2 regions of the DA RT1A$^a$ molecule. Lew responder rats were immunized by varying concentrations of two 25mer peptides covering residues 56–81 and 96–120. The injections were under the footpad and were repeated on day 14. The thickness of the footpads was measured to control delayed-type hypersensitivity (DTH). The animals were sacrificed on day 16 and the splenocytes were tested in mixed lymphocyte culture as responders against DA stimulator cells and CAP third-party splenocytes. In addition, the phenotype of the cells was measured using flow cytometry with antibodies against CD4, CD8, CD5, MHC class II, CD25, CD14 and CD19. Peptide 96–120 induced strong sensitization of the Lew recipient animals at concentrations of 200–500 μg ($n = 4$). The stimulation index was 2–3 times higher than that of untreated animals. Peptide 56–81 failed to induce sensitization at the concentrations used, but surprisingly induced a concentration-dependent immunosuppression that was highest at 400 μg ($n = 4$). In proliferation experiments responder Lew rats proliferated only to peptide 56–81 in vitro.

## Introduction

Indirect presentation of alloantigen can have beneficial or detrimental effects on graft outcome. It has become well established that antigen-presenting cells of recipient origin pick up soluble donor antigen, process it and induce anti-donor reactivity [6]. The antigen-presenting cells present antigen to T cells that are either CD4 or CD8 and to B cells that produce antibodies against the graft. Since the structure of the HLA-A2 molecule was published [1] it has become much easier to better define the polymorphic regions of human and murine MHC class I molecules, and also those of class II. Peptides derived from these regions have been shown to sensitize and induce accelerated rejection [3].

Class II derived 25mers have been shown to induce tolerance after intrathymic injection [5]. Nisco et al. [4] have shown prolonged survival induced by a human class I peptide in rats and mice. Up to now there have been no systematic studies on rat class I peptides to investigate their efficacy in prolonging graft survival. In the current study, we looked at the potential of two DA-derived class I peptides to induce sensitization and unresponsiveness in Lew responder rats.

## Materials and methods

### Peptides

Peptides of the DA RT1A$^a$ strain were synthesized using the Fmoc technique. Peptides were analysed by reversed phase high-pressure liquid chromatography (HPLC) and mass spectrometry. In these experiments peptides with the sequences 56–81 and 96–120 were used. Sequences were confirmed by mass spectrometry. The amino acid sequences of the two peptides were as follows:

56–81: NH2-Gly-Pro-glu-Tyr-Trp-Glu-Gln-Gln-Thr-Arg-Ile-Ala-Lys-Glu-Trp-Glu-Gln-Ile-Tyr-Arg-Val-Asp-Leu-Arg-Thr-COOH
96–120: NH2-Gln-Glu-Met-Tyr-Gly-Cys-Asp-Val-Gly-Ser-Asp-Gly-Ser-Leu-Leu-Arg-Gly-Tyr-Arg-Gln-Asp-Ala-Tyr-Asp-Gly-C-OOH

### Immunization

Lew rats were immunized under the footpads with various peptide concentrations: 200, 300, 400 and 500 μg/animal. In preliminary studies, we had been able to show that 100 μg of either peptide was ineffective, so that the concentrations used in the current studies were higher. The immunizations were repeated on day 14. On day 16 the thickness of the footpads was measured to detect any delayed-type hypersensitivity (DTH), and the animals were subsequently sacrificed. Splenocytes were used for both mixed lymphocyte reactions and phenotyping studies. Four animals were immunized for each concentration.

### Mixed lymphocyte reaction and proliferation assays

Irradiated DA stimulator cells (10 Gy) were added to $2 \times 10^5$ Lew responder splenocytes at 1:1 and 1:2 cell ratios in RPMI supplemented with 10% foetal calf serum (Gibco, UK) and 1% rat normal serum. In addition, untreated Lew rats were used as controls. As third-party stimulator cells the rat strain CAP was used. The cells were incubated for 4 days and pulsed with $H^3$-thymidine for 18 h before harvesting. In the proliferation assays, the responder cells were co-cultivated with peptide concentrations of 100, 50, 25 and 12.5 μg/ml, pulsed after 4 days and harvested after another 18 h.

### Phenotyping

OX12, Ox41 and antibodies against CD4, CD8, CD5, CD25 and MHC class II molecules were used to study the phenotype of the Lew cells by flow cytometry. Antibodies were purchased from Serotec, Germany. Antibodies were diluted 1:100 and incubated with $5 \times 10^5$ splenocytes for 30 min at 4 °C. Cells were washed three times and further incubated with a fluorescein-conjugated goat anti-mouse serum for another 30 min. The cells were subsequently washed and cell fluorescence measured by flow cytometry.

## Results

Rats received two injections of the DA peptides on days 0 and 14. The peptides were not supplemented with adjuvant in order to be able to study the effect of peptides alone as one would expect in a transplantation set-up. On day 16, the rats were sacrificed and the splenocytes were used in mixed lymphocyte cultures. The splenocytes were responders both to irradiated DA stimulator cells and third-party CAP splenocytes. Peptide 56–81 (RT1) induced a concentration-dependent immunosuppression as can be seen on Fig. 1. The lowest response was measured at 400 μg/rat. This result was rather unexpected. Interestingly third-party stimulator cells, CAP, failed to abolish this suppression, suggesting that the effect was not allospecific.

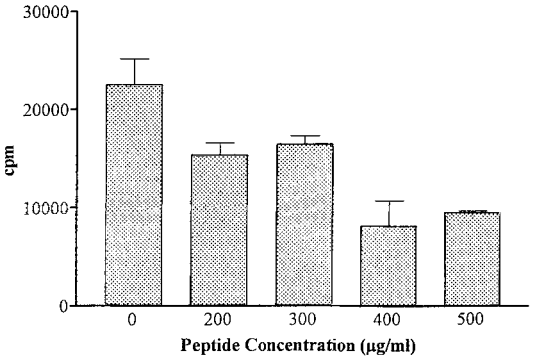


**Fig. 1** Lew responder rats treated with peptide RT1 showed a concentration-dependent suppression of the mixed lymphocyte reaction to DA stimulator cells. The lowest response was measured in the animals treated with 400 mg

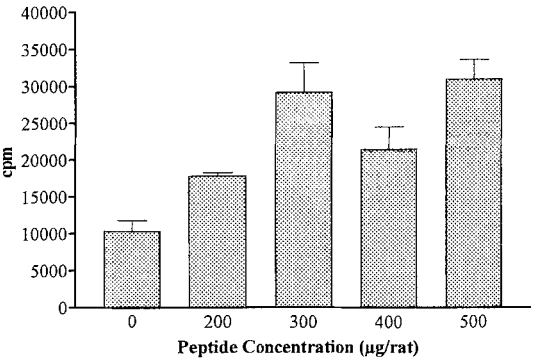


**Fig. 2** Lew responder rats were treated by peptide RT4. A significant enhancement of the response was measured in all animals that had received the peptide. This result shows that the peptide sensitized and that this region is apparently a relevant region of the native DA class I molecule

The second sequence studied, 96–120 (RT4), had contrary effects. A concentration-dependent augmentation of the alloresponse was observed in the mixed lymphocyte response (Fig. 2). This result suggested that this peptide represented a region of the class I molecule that is immunogeneic in vivo and, thus, sensitizes. To study the mode of peptide recognition, we performed proliferation studies in which the splenocytes of the immunized animals were used as responders to peptides added in culture. Only peptide RT1 was clearly presented and led to T-cell proliferation, as shown in Fig. 3. RT4 and a control human peptide 19 (HLA-A3: 56–69) failed to induce proliferation. Further, antigen-presenting cells were enriched by adherence and pulsed by these peptides before-hand. The cells were subsequently irradiated and then used as stimulator cells. Only antigen-presenting cells pulsed with RT1 could induce T-cell proliferation (data not shown). This experiment showed that recognition was through the indirect presentation. On phenotyping, animals treated with either RT1 or with RT4 did not

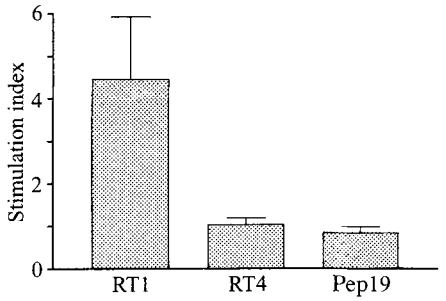


**Fig. 3** Indirect recognition of RT1. Splenocytes of responder Lew rats previously treated by 200 μg of each peptide were cultivated in 50 μg of each of these for 4 days. After pulsing for 18 h, the cells were harvested. The highest proliferation was measured in the splenocytes of the rat treated by RT1. RT4 and the control human peptide 19 were not presented. RT1 appears to contain anchor residues for the Lew MHC class II molecule

show any significant differences to control untreated animals for all cell markers studied.

DTH has been clearly shown to be one of the mechanisms by which allografts are damaged and subsequently rejected during rejection episodes. We immunized rats by subcutaneous injection of 400 μg (RT1) or 200 μg (RT4) peptide and the thickness of the footpads was measured after reimmunization 14 days later. None of these peptides was able to induce DTH. However, when the peptides were supplemented with Freud's complete adjuvant, peptide RT1 strongly induced foot thickness and inflammation. When both peptides were mixed and used for immunization, DTH was as severe as that observed by injecting RT1 alone (data not shown).

## Discussion

Although the two peptides described here have been described by others [3], few in vitro studies have been performed. Our data showed two different reaction patterns. RT1 was well presented in vitro, as shown by the proliferation assays. We assume that the peptide was presented by class II molecules of the Lew antigen-presenting cells, since enrichment of the class II expressing antigen-presenting cells resulted in augmentation of the immune response. Surprisingly, however, after in vivo treatment of Lew responder animals, RT1 decreased the T-cell response. Two possibilities are likely explanations for this phenomenon. First, it is quite likely that since this peptide is presented, there could be induction of T-cell suppressor cells or regulatory Th2 cells that downregulate the immune response [7]. Second, a much easier hypothesis could be that the peptide binds to some cell receptor required for T-cell proliferation and thus blocks cell response. This effect was not allospecific since response to third-party CAP stimulator cells was also reduced. Nisco et al. [4] and Cuturi et al. [2] have described a human decamer B7 peptide that induces prolongation of graft survival. Although our peptide RT1 is a 25mer, the B7 decamer and this peptide share the same residues at seven positions. It remains unclear whether we are describing a similar phenomenon or not.

The second peptide, RT4, sensitized recipient animals so that they reacted strongly in the mixed lymphocyte reaction. This peptide remained ineffective in proliferation assays, suggesting that it may require further processing in vivo before presentation. It was also interesting to observe that this peptide did not induce DTH, unlike RT1. In preliminary transplantation studies, recipient Lew responder rats were pre-treated with either peptide and received DA allogeneic cardiac allografts. The grafts were rejected in the normal fashion in both groups. This indicated that without further immunosuppression, the in vitro effects observed were not sufficient either to induce accelerated rejection or prolong survival. These studies are being continued with low levels of cyclosporin A.

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