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Rational design of biologically active peptides: inhibition of T cell activation through interference with CD_4 function

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Unfortunately, in the article by Pozzetto et al., the figures were omitted. We apologize for the oversight and the corrected version of the article is reprinted below:

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Abstract In our laboratory we generated one synthetic cyclic peptide (Pep4) and tested it in human mitogen stimulation assays (MSA) and mixed lymphocytes reactions (MLR) generating dose-response curves showing a dose-dependent inhibition of MSA up to 80% and MLR up to 98%. MSA and MLR were repeated after pre incubation of the Pep4 with each separate responder cell subset and subsequent reconstitution: these experiments showed inhibition only when the peptide was present in culture. Pep4 showed species specificity since it was ineffective in inhibiting rat MLR. Combination effect analysis with Pep4 and cyclosporine showed a combination index > 1. This rationally designed peptide (Pep4) shows powerful inhibition of human T cell activation and, although the exact mechanism is still undefined, it seems to exert its major action on the T cell surface, interfering with co receptor interaction and disrupting the same activation signal pathway inhibited by cyclosporine A.

Key words Immunosuppression · CD_4 · Synthetic peptides · Rational drug design

Introduction

Even if the development of new immunosuppressive strategies had allowed the defeat of acute rejection, a lifetime of pharmacological therapy is still required with its complications of increased incidence of viral and parasitic infections, tumors, growth deficits in children and diabetes. The achievement of antigen specific tolerance, i.e., organ engraftment without the need for generalized immune suppression, has been attempted with various strategies during the past three decades but always through strong combined induction therapies not always suitable for application in clinical practice. Having to deal with immunosuppressive drugs, an ideal immunosuppressant should be **specific,** targeting only mechanisms unique in the allo/xeno recognition, **selective,** acting on early events in T cell activation which, once inhibited, would eventually set suppressive mechanisms, **safe,** with low toxicity and possibly a regional area of action, and **suitable,** with pharmacokinetic and pharmacodynamic characteristics which would render its action synergistic with other agents with similar profiles. A potential specific and selective immunosuppressant would electively disrupt the antigen presentation properties of dendritic cells, monocyte/macrophage [antigen presenting cells (APCs)] without affecting their chemotactic, phagocytic and oxidative properties, which are important for the destruction of microbial pathogens. Advances in understanding the mechanism of interaction among APCs and T helper lymphocytes and in particular the importance of the costimulatory signals in addition to the T cell receptor (TCR) interaction, have suggested new strategies which have been successful in several experimental models, achieving permanent graft survival with agents given only at the time of grafting. In fact, exposure of the host to the foreign donor antigen in a fashion different from the way they are presented during organ transplantation may alter the host response from destructive to tolerogenic: agents which bind and inactivate surface coreceptors

could prevent specific alloreactive lymphocyte activation; lymphocytes which do not commit to activation may go to programmed cell death, or apoptosis, which is a physiological process that regulates the immune response by triggering a suicide mechanism in cells, ultimately abolishing allograft rejection. The immunosuppressive drugs available today generally act on intracellular activation pathways which tend to be, but are not completely, cell specific and selective receptor ligands as monoclonal antibodies (mAb) have the disadvantage of the need for intravenous administration and the induction of neutralizing antibodies against themselves.

Major histocompatibility complex (MHC) alloantigens can be recognized by two distinct pathways. The direct presentation involves T cell recognition of intact foreign MHC molecules on the surface of allogeneic cells. Dendritic cells are highly efficient APCs while endothelial cells, B lymphocytes and mononuclear phagocytes are less powerful but also efficient stimulators. Indirect presentation is less efficient than direct presentation of antigens. It involves MHC alloantigens being handled like conventional protein antigens, being processed and presented as peptides by responder antigen-presenting cells; this pathway is believed to contribute to a later, more chronic form of graft rejection. In T cell/APC contact, **CD_4** and CD8 promote adhesion by binding non-polymorphic sites of MHC class II and I molecules, respectively. The binding is relatively weak compared to other adhesion molecules, such as CD2 and LFA-1, but the interactions between CD_4 and CD8 and MHC molecules are more important in mediating activation signals to the T cells since they also participate in the delivery of proper stimulatory signals by the TCR. In the absence of correct Ag-MHC ligand, non-specific interactions dissociate after a short period of time allowing cells to part. Dynamic events also take place on the cell surface in order to optimize the signal delivery; the threshold number of receptors that must be occupied in order to elicit activation has been estimated to be between 20 and 200 per cell, which could explain the need for receptors to be brought into close proximity with one another. TCR engagement of cognate MHC assembles $TCR:CD_3:CD_4$ complexes, perhaps excluding larger molecules, resulting in the induction of phosphorylation of a number of proteins. CD3 cytoplasmic domains contain a motif called the antigen recognition activation motif which associates directly with two classes of cytoplasmic protein tyrosine kinases (PTK) of the SRC family, $p56^{lck}$ and $p59^{fynfynf}$; $p59^{fynfynf}$ has been shown to interact directly with TCR. CD_4 molecules associate with the TCR during T cell activation and the association is stabilized and prolonged when T cells are triggered by Ag-MHC on APCs. CD_4 (and CD8) cytoplasmic tail is associated (non-covalently) with $p56^{lck}$ and association of CD_4 brings the PTK into close proximity with $CD_3\xi$ thus allowing more effective phosphorylation to occur [1]. Antibodies to CD_4 can block the T cells activation of both Ag-MHC and non-specific triggers as mitogens or anti-receptor antibodies. The interest in the CD_4 interactive sites relies also on the fact that deficiency of expression or functional blockade of costimulatory molecule on donor DC and/or on their counter receptors on T helper lymphocytes predisposes to allograft survival and may also play a role in tolerance induction [2–5].

In terms of safety and suitability, small synthetic peptides offer the advantage of inducing a selective and functional impairment of receptor interaction, being therefore otherwise inactive and rapidly degradable if left unbound. If they could be directed to the receptors on APCs interacting with their coreceptors on the T helper cells, responsible for the generation of costimulatory signals, they could render the APC inefficient in antigen presentation, deviating the host response toward tolerance, eventually by the mechanism of apoptosis of the T helper reactive clones. We investigate if a rationally designed peptide derived from the CD_4 molecule may function as a selective agent in blunting the TCR-mediated activation.

Materials and methods

Rational peptide design

Interaction sites have been largely investigated by constructing CD_4 mutants as well as CD_4- and MHC-derived peptides [6–8]. CD_4 regions interacting with MHC class II were mapped at the N-terminal region, i.e., within the CD_4 D1 domain (partially overlapping the GP120 binding sites) and D2 domain [9–1]. On the other hand, the CD_4 interaction sites with TCR-CD_3 complex have been mapped within the D_3 and D_4 domains, i.e., the two membrane proximal domains of CD_4 [8, 12]. Finally, CDR_3-like region in domain 1 of CD_4 has been implicated in CD_4 dimerization [13]. According to the studies indicating the role D_{1-4} domains play in CD_4 interactions and the T cell activation process, we identified a region from the extracellular portion of the CD_4 molecule and constructed a cyclic peptide designed to mimic the template CD_4 region and to interfere in the CD_4 interaction process. This peptide has been named Pep4.

Synthesis of peptides

Peptide synthesis has been carried out as previously described [14]. Briefly, peptides, here referred to as Pep4 as well as the corresponding scrambled version used as control, were synthesized with a solid-phase automatic synthesizer (Applied Biosystem 430A), with t-BOC technology according to standard protocols. Optimization of coupling times as well as deprotection times was required. Purity up to 90–95% was achieved by HPLC reverse-phase purification on a C-18 column, and mass spectrometry analyses were performed to check the purity and the molecular mass of the synthesized molecules. Cyclization was achieved by inducing a disulfide bridge linking between the N- and C-terminal cys residues. The disulfide link formation was carried out by dissolving

the peptide at 0.1 mg/ml concentration in 0.15 M NaCl, at room temperature for 18 h [15].

Scrambled sequence peptide and same sequence non-cyclic peptide were produced as controls.

Immunosuppressive reagents

Pep4 and other peptides and cyclosporine (CsA; Sandoz, Basel, Switzerland) were diluted with RPMI 1640 medium (Labtek, Eurobio, France) at various concentrations as indicated.

Preparation of cell suspensions

Mononuclear cells from peripheral blood (PBMC) or buffy coat (BFMC) were isolated by density gradient centrifugation over MSL (Labtek). After three washings in HBSS (Labtek) the cells were resuspended in culture medium RPMI 1640 supplemented with 2 mM L-glutamine, 100 UI/ml penicillin/streptomycin, and 5% FCS (Gibco Grand Island, N. Y., USA).

T cell and monocyte purifications

The two subsets were purified by centrifugation on different concentrations of Percoll density gradient (Pharmacia Biotech, Uppsala, Sweden). The resulting populations of T lymphocytes and B cells/monocytes (MØ) were further purified by magnetic immunoselection using anti-CD14 and anti-CD19 for T cells and anti-CD3 and anti-CD19 for MØ. The beads were applied with a bead-to-target ratio of 10 : 1. We used a negatively selected sub-population to exclude possible interference of anti-CD3 or anti-CD14 mAb with cellular activation/suppression. After depletion the T cell population contained more than 96% CD3 + cells and the MØ population about 98% CD14 + cells.

Mitogen stimulation and mixed lymphocytes reaction (MLR)

PBMC and BCMC were stimulated in triplicate (15×10^4) with anti-CD3 mAb (CLB-CD_3, Ortho Diagnostic System, Raritan, N.J., USA) at a final concentration of 1 µg/ml or phytohemoagglutinin (PHA; Difco Laboratories, Detroit, Mich., USA) at a final concentration of 10 ng/ml in 96-well round-bottomed microculture plates (Microtest III, falcon; Becton and Dickinson, Mountain View, Calif., USA). Peptides at various concentrations were added at the beginning of the culture. Cells were incubated for a period of 3 days at 37 °C with 5% CO_2, 16–18 h before harvesting 1 µCi [3H] Tdr (Amersham, Arlington Heights, Ill., USA) was added to individual wells. Cells were then harvested using a semi-automatic cell harvester (Titertek Skatron, Norway) and the degree of thymidine incorporation was measured using a 1215 Rank Beta scintillation counter (LKB, Wallac, Finland). For MLR, PBMC and BFMC were used as responders and a pool of allogeneic PBMC as stimulators. Allogeneic cells were pretreated with mitomycin C (M-0503, Sigma, St. Louis, Mo., USA) at 0.025 mg/ml for 40 min at 37 °C. Cells were then washed 2 times to remove excess mitomycin C. PBMC and BCMC (15×10^4/ml) were cultured with mitomycin C treated allogeneic cells (30×10^4/ml) in RPMI 1640 for a period of 6 days; peptides at various concentrations were added at the beginning of the culture, 1 µCi [3H] Tdr was added to the culture 16–18 h before cell harvesting and the degree of thymidine incorporation was quantified as described above. In some experiments, the responder or stimulator cells were pretreated with peptides before the beginning of the culture; the cells were incubated for 2 h at 37 °C with various concentrations of peptides, washed 2 times with HBSS to remove excess peptide, resuspended in medium, and cultured at the indicated concentration. The percent suppression of proliferation was calculated using the formula:

$$\% \text{ inhibition} = \left(\frac{\text{cpm treated cells}}{\text{cpm untreated cells}} - 1\right) \times 100$$

In some experiments the results were reported as stimulator index (SI):

$$\text{SI} = \frac{\text{cpm activated cells}}{\text{cpm inactivated cells}}$$

Combination effect of CsA and Pep4

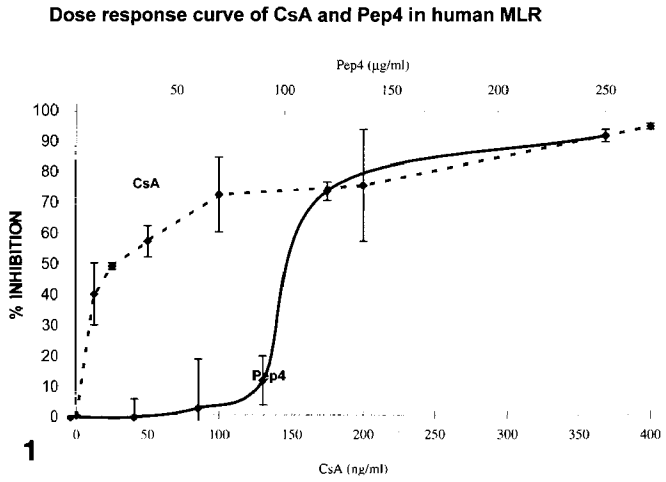
To investigate the immunosuppressive effect of CsA and Pep4 in combination, the two drugs were added at the beginning of MLR. Dose inhibition curves were obtained with two single drugs at different concentrations (CsA: 12.5–400 µg/ml; Pep4: 0.01–0.75 mg/ml). The inhibitory effects of drugs in combination were assessed by mixing concentrations of each single drug with all concentrations of the other. A quantitative analysis of the dose-effect relationship of both drugs was performed.

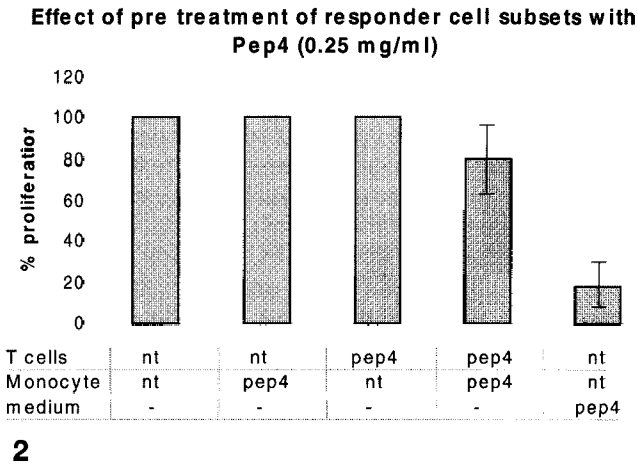
Statistics

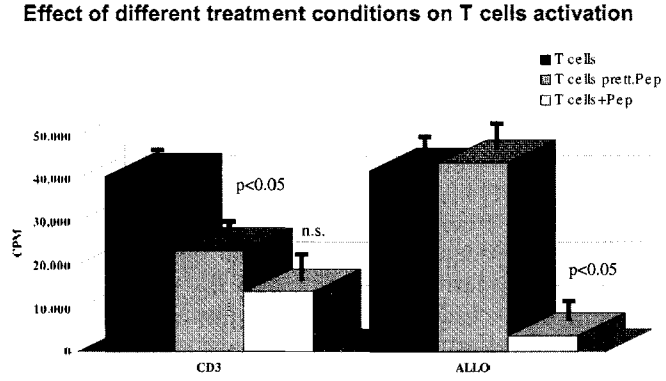
The significant differences were determined using the paired Student's *t*-test. The median effect analysis of the combination of Pep4 with CsA was performed according to the Chou-Talalay method [16].

Results

The dose-response curve in allogeneic activation (MLR) when Pep4 was added in solution showed a dose-related inhibition of T cell proliferation with a sigmoid shape, with 98% inhibition at plateau (Fig. 1). Compared to a typical CsA dose-response curve, Pep4 showed clearly a critical point at which an off/on effect could not be noticed, maybe related to the reaching of a "mass" effect and/or a full target molecule coverage. Experiments conducted with a peptide with similar sequence but different conformation (non cycled), resulted in the loss of effect (data not shown). Specificity was also proved by using the same peptide (Pep4) in full inhibitory concentration (in human MLR) in rat (Wistar/Buffalo) MLR, showing no inhibitory effect. In lymphocyte stimulation with PHA and OKT3 an inhibitory effect was also noticed, although up to lower inhibition levels (60 and 70%, respectively). When MLR experiments were done with responder cells or cell subsets alternatively preincubated with Pep4, the inhibitory effect was lost, demonstrating the absence of a stable, covalent bond with responder cells. The effect was instead restored in all combinations when peptide was added in culture (Fig. 2). When preincubation of cells was performed before OKT3 stimulation, a 50% inhibitory ef-







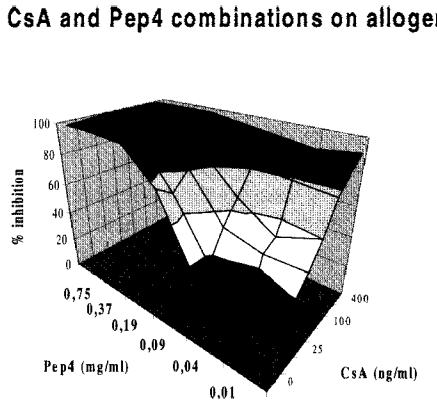


Fig. 1 The different shapes of dose-response curves for CsA and Pep4 are shown here. While Pep4 shows a sigmoidal shape, CsA shows an hyperbolic shape, confirming the relative different pharmacodinamic

Fig. 2 Responder cell subsets were separated as described (see text) and alternatively pre-incubated with Pep4. Pre-incubation showed no significative effect. Only when Pep4 was added in culture was there significant inhibition ($P < 0.05$)

Fig. 3 The effect of the peptide on T cell activation was dependent on the pretreatment conditions: in the case of allogeneic stimulation, the peptide was inactive on pretreated T cells and active when present in the culture medium. In CD3 stimulation by OKT3, the peptide was active both on pretreated cells and in the medium culture. This experiment suggests a double mechanism of action for the peptide: one specific major functional form of interference on Th/APC interaction and one minor non-specific form of interference on CD3-complex function

Fig. 4. The combination index calculated by the median-effect analysis resulted in more than 1, indicating a physiological antagonistic effect visualized in the lowest dose combination

fect was recorded, compared to a 75% inhibition when Pep4 was present in culture (Fig. 3).

In order to explain this effect on CD3-mediated stimulation, FITC-conjugated peptides were used to trace peptide stable interaction with cell subsets, documenting a weak deposition only on T cells and no binding with other cells. Therefore we could speculate that Pep4 shows a double effect, one prevalent and acting functionally on Tcell/APC interaction and the second aspecific, directed on T cells, and interfering with CD3-mediated activation. Combination analysis with CsA (Fig. 4), evaluated by the median effect analysis, showed an antagonistic interaction (combination index > 1); results expected by the share of the same targeted activation pathway by the two compounds.

Discussion

According to the current views, antigen presentation by non-immunogenic cells or "less efficient APC" tends to induce specific unresponsiveness and the prolonged residence of an acceptal allograft can induce a state of

profound, donor-specific tolerance. Immunotoxic or immunomodulatory drugs tend to suppress the allorecognition either depleting the alloreactive cells or inhibiting their activation pathways. T cell engagement of specific MHC-peptide complexes can have a variety of consequences, partial or full activation or in alternative apoptosis, anergy and ignorance. Much of the regulation of these outcomes lies in the context in which engagement occurs; therefore inefficient APC function tends to induce T cell unresponsiveness. In our experiments we confirmed this thesis observing that Pep4 elicited a dose-related inhibition of T cell proliferation in response to allostimulation; therefore small synthetic peptides, eventually administered for a short time, or transiently, on donor cells engrafted, could be extremely specific and inactive on systems other than the ones for which they are directed. Being formed generally by few residues (up to 15) they are usually soon complexed with their target molecule, differently from other drugs that need complex catabolic processes and are eliminated often in active or less-active forms. Our results confirm this feature of synthetic peptides acting by simply interfering with molecular interactions. Their action is "functional", as they are non-covalently bound by the target molecules, and specific, because their specificity is set by their sequence and by their tridimensional conformation. In our experience, Pep4, in fact, was active selectively on allostimulation, "functionally", only when present in the medium. It showed not to engage stable interaction with target cells, no toxicity, and species specificity. Pep4 showed instead less specificity, since a small effect was evident also on mitogen stimulation and demonstrated by a weak bond, after preincubation, with T cells. While the latter observation could be explained by the fact that Pep4 derives from the D1 domain of the CD4 molecule, involved in MHC class II contact and CD4 dimerization processes, it is still unclear how it could interfere with PHA and OKT3 stimulation.

Peptides derived from polymorphic regions of the MHC may induce antigenic specific unresponsiveness when administered via certain routes. Non-polymorphic MHC peptides have been shown to be immunoregulatory in a non-allele specific manner [17]. Class II MHC peptides have also demonstrated allele non-specific inhibition of the alloimmune response, with inhibition of the MLR, cytotoxicity, and cytokine production. The identification of critical sites in alloimmune responses has been used in the rational structure-based design of peptide analogs/antagonists. Immune reactions could be modulated by specifically interfering with CD4-MHC class II interactions, in various in vitro and in vivo assays, with peptides corresponding to a region of MHC class II molecules. Depending on the chemical nature and concentration of these peptides, they modulated Ag-specific responses of CD4 + T cells. At high concentrations, these peptides inhibited T cell responses in vitro [18]. Peptides mimicking important functional regions of the CD4 molecule have been developed and shown to prolong allograft survival and also prevent GVHD in murine models [19, 20].

A peptide analog to the human CD4-CDR3-like region, was synthesized and showed inhibitory action on human MLR tests [21]. Our synthetic peptide (Pep4), selected upon four different ones, rationally designed, is derived by a different portion of the D1 domain of the human CD4 region, and similarly showed powerful inhibition of T cell alloactivation, validating the use of synthetic peptides based on rational design. Although further investigations would be necessary for the development of a clinical-oriented compound, overcoming the difficult pharmacokinetics of such small molecules, synthetic peptides, acting by conformational properties, offer an interesting opportunity for the development of a safe and specific drug. Moreover, having once identified an active site through a synthetic peptide, either more active mutants or synthesis of a specific mAb are potential future developments. Theoretically, such peptides or derived compounds, directed to donor cells, could be utilized "ex vivo": organ transplantation, like no other field, offers such an opportunity for the application of such a strategy, since organs are harvested, cold perfused, and transplanted in an other individual. Hence, peptide administration could be carried out during the organ harvesting or during the bench cold perfusion step.

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