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A. Freese
N. Zavazava

Determination of HLA-B7 T-cell epitopes

A. Freese (✉) · N. Zavazava
Institute of Immunology,
University of Kiel,
Brunswiker Strasse 4, D-24105 Kiel,
Germany
Tel. + 49 (0)431/5973364;
Fax + 49 (0)431/5973335

Abstract A series of peptides derived from the α1 and α2 regions of the HLA-B7 and HLA-A2 molecules was synthesized with an automatic peptide synthesizer using the FMOC (9-fluorenylmethoxycarbonyl) technique. Peptides were analyzed and purified by reversed-phase high pressure liquid chromatography (HPLC). Peptide purity was > 96 %. The effect of B7 peptides on mixed lymphocyte cultures was tested in vivo. The alloresponse in the cultures with B7 peptides was strongly enhanced. The peptides that were most effective inhibited cytotoxic T-lymphocyte (CTL) cytolysis. The data show that the peptides are immunogenic and that they are recognised by both the direct and indirect pathways. Further, the mechanism of peptide recognition was studied. We coupled peptide B7 (residues 62–70 in HLA-B7) and peptide A2 (residues 62–70 in HLA-B2) covalently to fluorescein isothiocyanate (FITC). B7-specific CTLs were incubated with these peptides at 37 °C for 90 min and cell fluorescence measured by flow cytometry. The B7 peptide was bound by 60 % of the CTLs whereas the A2 peptide, as a negative control, bound only 10 %. The molecular size of the ligands to which the peptides bind are being characterized by immunoprecipitation.

Key words HLA-B7 · HLA-A2 · T-lymphocytes · Synthetic peptides · T-cell recognition

Introduction

Acute rejection of transplanted allogeneic organs is strongly dependent on T-cell activity against foreign human lymphocyte antigen (HLA) molecules. There are three possible explanations for the alloresponse. In one model, T-cell recognition requires the foreign HLA molecule together with a presented self peptide. Another model states that foreign HLA molecules are presented by antigen-presenting cells as processed peptides together with self HLA class I molecules on the cell surface. In the third model, the intact allogeneic HLA molecule is recognized independently of bound peptide [1]. The results of our inhibition assays showed that purified class I molecules [9] and papain-digested class I molecules [3] inhibit alloreactive cytotoxic T-lymphocytes (CTLs) specifically and so support the model of direct HLA recognition. CTLs show marked specificity for HLA class I molecules. These studies were continued using HLA-derived synthetic peptides. The major objective of the current studies is to perform CTL epitope mapping to determine those regions of the HLA molecule that are responsible for T-cell recognition. Defined overlapping peptides of the polymorphic region of the HLA-A2 and HLA-B7 molecules were utilized. Assuming that the differences between HLA specificities are anchored in this polymorphic region, the mismatched residues should be responsible for specific allo-recognition. To investigate the ligand bound on T-cells, peptides were coupled to a fluorescein isothiocyanate (FITC) molecule and tested in binding assays with allospecific T-cells. Furthermore, we investigated which regions of

the HLA molecule are preferentially presented. For this reason we incubated HLA-B7-derived peptides with naive HLA-B7-negative peripheral blood lymphocytes.

Materials and methods

Peptide synthesis

We synthesized peptides derived from the polymorphic regions of the HLA-A2 and HLA-B7 molecule. The HLA-B7-derived peptides 10 (91-105: GSHTLQSMYGCDVGP), 11 (62-70: RNTQIYKAQ), 12 (106–120: DGRLLRGHDQYAYDG), and the HLA-A2-derived peptides 4 (75–89: RVDLGTLRGYYNQSE) and 21 (62–70: GETRKVKAH) were synthesized by the standard FMOC (9-fluorenylmethoxycarbonyl) technique with an automatic peptide synthesizer (Millipore, USA). Peptide chains are generally assembled from the carboxyl-terminus to the amino-terminus. The first amino acid is covalently attached to an insoluble polystyrene support (PEG-PS). The remaining amino acids are added serially in cycles consisting of deblocking steps (i. e., removal of the alpha-amino-protecting FMOC group), dimethylformamide (DMF, Millipore, USA) washes, and binding of the next amino acid. The deblocking step is monitored by measuring the release of the UV-absorbing FMOC group.

Following synthesis, the product is cleaved from the resin and all side chain-protecting groups are removed. For this reaction we used a solution containing trifluoroaceticacid (Millipore, USA), phenol (Riedel-de Haen, Germany), and triisopropylsilane (Aldrich, UK). Cleavage was completed after 8 h. The peptide was finally precipitated and washed 3 times in cooled ether (Merck, Germany).

The peptide was analyzed and purified using reversed phase (Pep-S, C_2C_{18}, Pharmacia high pressure liquid chromatography (HPLC).

Generation of CTL lines

Peripheral blood lymphocytes (PBLs) were isolated from HLA-typed B7-negative volunteers. An HLA-B7-specific cell line, HN.B7, was generated by coculturing PBLs (HLA-A26,28, -B60,-C3, -DR3,6) with irradiated (50 Gy) stimulator Epstein-Barr virus (EBV)-transformed B cells, prostaglandin F (HLA-A3, -B7) at a 10 : 1 ratio in RPMI 1640 medium (Gibco, USA) supplemented with 15 % heat inactivated human AB serum, 125 µg/ml penicillin, and 250 µg/ml streptomycin. T-cells were restimulated every 10 days with stimulator cells. At the third and subsequent restimulations, interleukin-2 was added at a concentration of 30 U/ml. After the second restimulation, CTLs were used in functional assays.

Stimulation of PBLs with allogeneic peptides

PBLs were isolated from HLA-typed B7-negative volunteers. These PBLs were stimulated at day 0 with 10 µg/ml of a mixture of HLA-B7-derived peptides (peptides 10, 11, and 12). After 10 days, the cells were restimulated with autologous PBL blasts at a 10 : 1 ratio [6]. The blasts were preincubated with a 100-µg/ml peptide mixture for 90 min at 37 °C. After irradiation (25 Gy) the blasts together with the peptide mixture were added to the cells. At 5–6 days after the first restimulation, cells were tested in functional assays.

Proliferation assay

Primed PBLs were cocultivated with 100 µg/ml of the HLA-B7-derived peptides 10, 11, and 12. After 5 days the cells were pulsed with [^{3}H]thymidine and harvested after 16 h. The uptake was measured in a β-counter.

FITC coupling of synthetic peptides

We modified the method described by Kropshofer et al [4] by coupling peptides before cleavage. Peptides attached to the solid support were dissolved in 50 mM borate buffer and mixed with FITC in a molar ratio of 1 : 3. The solution was shaken gently for 6 h. The peptides were washed 3 times with borate buffer and then cleaved. The purity of the coupled peptides was analyzed by reversed-phase HPLC.

Binding assays with FITC-coupled peptide

An HLA-B7-specific cell line was incubated with 500 µg/ml of the HLA-B7-derived peptide 11 (residues 62–70) and the HLA-A2-derived peptide 21 (residues 62–70) as a negative control. We incubated the cells at 37 °C for 90 min. Subsequently, the cells were washed 3 times with phosphate-buffered saline (PBS) and the fluorescence intensity was measured in a FACScan.

Results

The efficiency of amino acid coupling was reflected in the HPLC profiles. After synthesis, the peptide was purified by means of preparative HPLC. A purity of more than 96 % was achieved. These highly purified peptides were used in the assays to study the regions of the HLA molecule responsible for T-cell recognition.

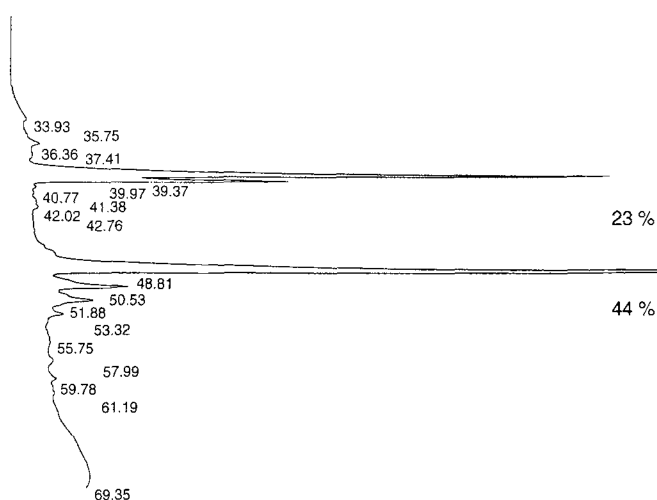

Fig. 1 The high pressure liquid chromatography (HPLC) profile of fluorescein isothiocyanate (FITC) coupled peptide 11 at 214 nm. The first peak, marked 23 %, represents the uncoupled peptide and that marked 44 % the FITC coupled peptide. This was the highest yield obtained

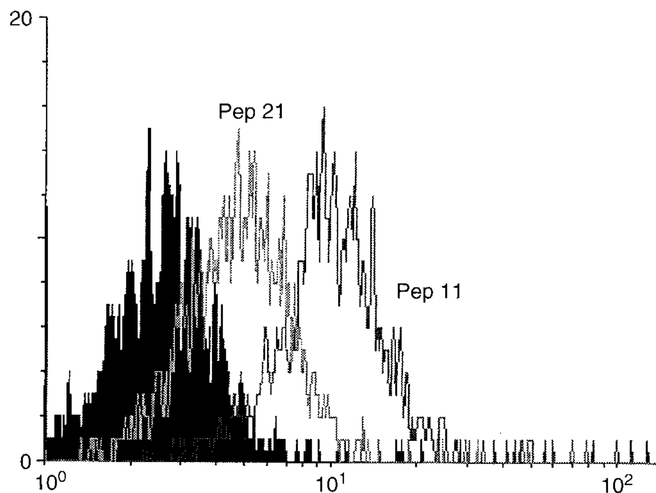

Fig. 2 Results of a binding assay with FITC-coupled peptides. The HLA-B7-derived peptide 11 *(Pep 11)* shows a greater affinity to the HLA-B7 cell line, HNB7, than does the HLA-B2-derived peptide 21 *(Pep 21)*. This result suggest that the binding is stable

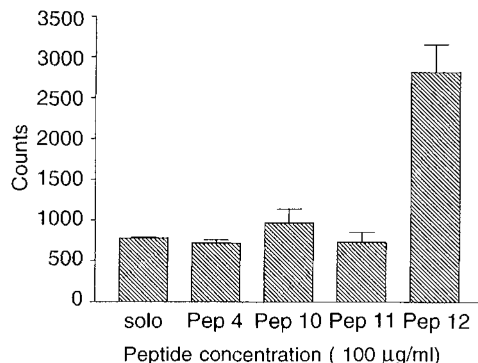

Fig. 3 Presentation of peptide 12. B7-negative peripheral blood lymphocytes (A2,–B51,60,–C3,–DR8,12) were cultured in 10 µg/ml peptides 4, 10, 11, and 12 for 10 days. Five days after restimulation, cells were cultivated in 100 µg/ml of each peptide. Cells were pulsed after 5 days and harvested. Peptide 12 *(Pep 12)* induced enhanced proliferation

The interaction of highly purified FITC-coupled peptides of the polymorphic region of the HLA molecule with allospecific cell lines was investigated in order to obtain information on the intensity, specificity, and duration of the binding of allopeptides to specific T-cells. FITC-coupling of the HLA-B7-derived peptide 11 (62–70), which was positive in several inhibition assays, was performed. The first coupling experiments were moderately successful. After numerous coupling experiments with slight modifications in the reaction time and in the molar ratio of peptide to FITC, the maximum yield of FITC-coupled peptide was raised to 44 % (Fig. 1). With this method established, it was possible to couple any peptide of interest. To have a perfect negative control for our binding experiments we synthesized an HLA-A2 peptide of the same region as peptide 11, peptide 21 (A2: 62–70). This peptide was also FITC-coupled with a similar yield.

We tested the binding specificty of allopeptides to HLA-specific T-cells. For this reason, an HLA-B7-specific cell line was incubated with 500 µg/ml of the FITC-coupled peptide 11 and the HLA-A2-derived peptide 21 as a negative control. The result obtained is shown in Fig. 2. Peptide 11 showed a greater binding affinity to the B7-specific cell line than did the negative control, peptide 21. Many different cell lines were tested. The binding intensity to peptide 11 ranged from 15 % to 90 %.

Additionally, we investigated the capability of HLA-B7-derived peptides to induce a proliferative response to PBLs. The naive PBLs were coincubated with 100 µg/ml of the B7-derived peptides 10 (91–105), 11 (62–70), and 12 (106–120). Peptide 10 caused low cell proliferation, whereas peptide 12 doubled the response (not shown). Peptide 4 is an A2 (75–89) negative control peptide. To test if the naive PBLs could be primed with peptides they were cocultivated with a 10-µg/ml peptide mixture of peptides 10, 11, and 12 for 10 days. Five days after restimulation, cells were tested in a proliferation assay with the various peptides. As shown in Fig. 3, peptide 10 slightly increased cell proliferation, whereas peptide 12 was strongly presented. In other experiments, peptide presentation was shown to be mediated by antigen-presenting cells (data not shown).

Discussion

We previously showed that purified native HLA class I molecules are capable of specifically inhibiting allogeneic cytotoxic T-lymphocytes in vitro. Continuing these studies, we established the production of synthetic peptides. The inhibition assays using synthetic peptides confirm our previous data [3, 9]. Similarly to the whole purified HLA molecule, tested peptides of the polymorphic region of the HLA molecule are able to inhibit cytotoxic T-cell reactivity specifically. These results also confirm findings by others [2, 5]. The HLA-B7-specific CTL epitopes were located at positions 62–70 and 91–105. A simple explanation for this specific inhibition of the CTLs is that the peptides bind directly to the T-cell receptor (TCR). The blocked receptor can, thus, no longer come into contact with the foreign HLA molecule of the target cell, hence no cytolysis occurs. The nature of this binding is still, however, not clear. To further investigate the interaction between the allospecific peptide and the T-cells, we coupled the peptides to FITC. Our results showed that peptide binding to the membrane ligand is much stronger than expected. In studies done with soluble TCR heterodimers, the binding of class II major his-

tocompatibility complex (MHC) molecules was reported to have a very fast off rate, with a $t_{1/2} \approx 12$ s [5]. Additionally, Seth et al. [7] reported that it was impossible to detect a class II-peptide-TCR complex in a native polyacrylamide gel because of the low affinity of these interactions. However, further characterization of this phenomenon is required. In light of these arguments, it is not clear whether our peptides directly interact with the TCR or some other cell-surface ligand. Since the interaction is allospecific, there is strong evidence for the involvement of the TCR.

The proliferation studies showed that one synthetic peptide was presented and led to T-cell proliferation. This observation is important for organ transplantation. As reported by others [8], donor soluble HLAs or their fragments could be picked up by recipient antigen-presenting cells and either lead to augmentation of the alloresponse or to T-cells that might down-regulate proliferation. These studies are helpful in elucidating alloreactivity and identification of relevant T-cell epitopes.

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