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T cell epitope analysis with peptides simultaneously synthesized on cellulose membranes: fine mapping of two DQ dependent epitopes

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Abstract Several MHC class II restricted, CD4$^+$ human T cell clones from three donors were induced with soluble matrix protein of influenza virus. The epitopes recognized by these clones were mapped using a complete set of overlapping 15-mer peptides synthesized with the newly developed SPOT method which allows the simple simultaneous synthesis of numerous peptides on cellulose membranes. Fine analysis of two clones by truncation of the stimulatory peptide by single subsequent amino acids from either the NH_2- or the COOH-terminus revealed the minimal stimulatory determinants of two DQ dependent T cell epitopes.

Key words: T cell; Epitope; MHC class II; Peptide synthesis

1. Introduction

T cells only recognize antigens in a processed form as short peptides bound to molecules of the MHC [1,2]. For characterisation of a particular T cell response it is therefore necessary to map the individual epitopes recognized by the T cell clones, usually by scanning the protein sequence with a series of overlapping synthetic peptides. This requires a preparation of a large number of peptides amongst which only a few are stimulatory. To this end it would be desirable to have a method which allows the fast, simultaneous synthesis of multiple peptides with a minimal requirement of cost and labour. Recently, the synthesis of peptides on cellulose membranes – SPOT synthesis – has been introduced by one of the authors which fulfills all of the criteria mentioned above. This represents an easy and flexible method for the rapid and economic preparation of large numbers of peptides on a small scale [3]. To date, this method has primarily been applied to B-cell epitope mapping – the determination of antibody binding specificity to arrays of membrane bound peptides. Here we demonstrate that SPOT synthesis is equally convenient to prepare solution phase peptides and that the quantities and qualities of peptides are suitable for such delicate assay systems as T cell stimulation. Using a series of soluble peptides prepared by the SPOT method, we studied the fine specificity of two DQ restricted CD4$^+$ T cell clones specific for the matrix protein of influenza virus.

2. Materials and methods

2.1. Preparation of Influenza M protein

Influenza A/FPV/Rostock 34 (H7N1) virus was grown in alantois fluid of day 12 chicken embryos. Matrix protein was purified from concentrated virus suspension by lysis with 2% SDS at 37°C for 2 h followed by gel filtration on AcA44 (Serva, Heidelberg, Germany). Purity was assessed by immunoblotting using polyclonal rabbit anti-total virus proteins (kindly supplied by Dr. H.D. Klenk, Marburg).

2.2. Induction of human T cell clones

Peripheral blood lymphocytes of two donors were stimulated with various dilutions of matrix protein starting with 10 μg/ml. Cloning, maintenance and proliferation assays were performed as described [4].

2.3. Peptide synthesis by the SPOT method

Assembly of the peptides shown in Figs. 2 and 3 was carried out utilizing Fmoc-chemistry essentially as described [3]. Appropriate cellulose membranes derivatized with spots of Boc-Lys-Pro-dipeptide anchors were used to allow a final cleavage of peptides in aqueous solution [5]. The solubilized peptide thus remains modified C-terminally by a Lys-Pro-diketopiperazine. Chr-1 chromatography paper (Whatman, Maidstone, UK) was derivatized first with N$^\alpha$-Fmoc-proline to give a substitution of 0.25 μmol per cm^2. After cleavage of Fmoc with 20% piperidine in DMF, 1 μl aliquots of a 0.3 M solution of N$^\alpha$-Boc, N$^\epsilon$-Fmoc-lysine HOBf ester were applied at intervals of 1 cm. Residual free proline residues were blocked by acetylation and the Fmoc group at the lysine removed with piperidine. The resulting spots had a diameter of 0.7 cm and carried about 25 nmol of dipeptide anchors as determined by amino group titration [2,3]. Peptides were assembled by spotting 1.5 μl aliquots of 0.3 M solutions of Fmoc-amino acid pentafluorophenyl esters per elongation cycle. Special side chain protection was Arg(Pmc), Asn(Trt), Gln(Trt), His(Trt), and Trp(Boc).

After completion of chain assembly, peptides were N-terminally acetylated and side chain protecting groups removed by treatment with 50% TFA, 3% triisobutylsilane in DCM for 2 h. The membranes were then washed successively with DCM (4 times for 10 min), methanol/water (1:1, v/v) containing 0.1% hydrochloric acid (3 times for 20 min) and 1 M acetic acid in water (3 times for 20 min), and then vacuum dried overnight. Individual spots were punched out with a *DotPunch* from Inotech Biosystems (purchased from Bibby Dunn Labortechnik GmbH, Asbach, Germany) and placed into 2 ml Eppendorf tubes. Peptides were eluted by overnight incubation at 37°C with 0.5 ml of 0.1 M triethylammonium acetate (pH 7.5) containing 20% ethanol. These solutions were transferred to a second tube and the paper spots eluted again with 0.5 ml of the same buffer. The combined eluates were dried in a vacuum centrifuge, dissolved in 0.5 ml PBS and stored at −20°C.

HPLC-separations were carried out on an analytical reversed-phase silica gel column (Nucleosil 300–7C18, 4x250 mm, Macherey-Nagel, Düren, Germany) with gradients of acetonitrile/0.1% TFA in water/

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Abbreviations: MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; EBV, Epstein-Barr virus.

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0.1% TFA at a flow rate of 1 ml/min. Mass spectra were recorded on a Kratos MS 50RF instrument with a high field magnet (mass range ca. 10,000 at 8 kV) and a Kratos FAB source using a beam of neutral xenon atoms at 8–9 kV and thioglycerol as matrix. Peak fractions from HPLC separations containing 5 to 10 μg peptide were evaporated to dryness, dissolved in 3 μl DMSO and aliquots thereof were injected into the thioglycerol matrix.

3. Results

3.1. Synthesis, elution and mass analysis of soluble peptides

Spot synthesis allows a flexible choice of format with respect to number and scale of individual peptide syntheses [3]. Here we set the final yield to be in the 10–50 nmol range. This resulted in an array of 10 × 7 spots arranged of 1 cm apart on a 12 × 9 cm Chr1 chromatography paper sheet, each spot carrying about 25 nmol of dipeptide anchor. The elegant concept of an orthogonal peptide-support anchor developed by Bray et al. [5] was adapted to spot synthesis. It allows the final deprotection and removal of contaminating chemicals while the peptide remains attached to the support. This is followed by the direct release of the peptide into an aqueous buffer of choice.

Spots of Boc-Lys-Pro anchor were generated on the cellulose membrane, onto which the peptides were assembled via the ε-amino function of the lysine residue. Completed peptides were released from individual punched out spots by diketopiperazine formation at neutral pH. The diketopiperazine moiety remains C-terminally bound to the peptide. Valerio et al. [6] have demonstrated that this modification does not significantly impede biological activity in T-cell stimulation assays. For peptide synthesis, conventional Fmoc chemistry was utilized and peptides up to a length of 20 amino acid residues were successfully assembled as proven by HPLC and mass spectrometry analyses (Fig. 1). This material was used in the functional T cell assays.

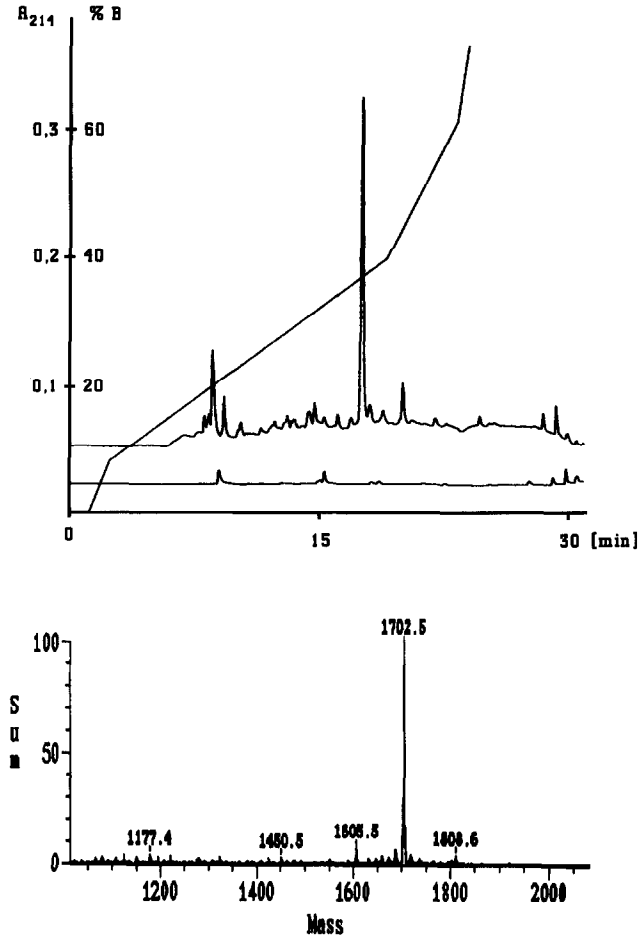


Fig. 1. Analysis of an eluted 20-mer peptide synthesized on a cellulose matrix. Upper panel: HPLC trace of crude Ac-Val-Leu-Ser-Ile-Ile-Pro-Ser-Gly-Pro-Leu-Lys-Ala-Glu-Ile-Ala-DKP (Lys-Pro) which is peptide No. 3 from Fig. 2 (% B = concentration of acetonitrile, A_{214} = absorbance at 214 nm). Lower panel: FAB mass spectrum of the prominent peak from upper panel showing the expected correct mass.

3.2. T-Cell epitope mapping by overlapping peptides

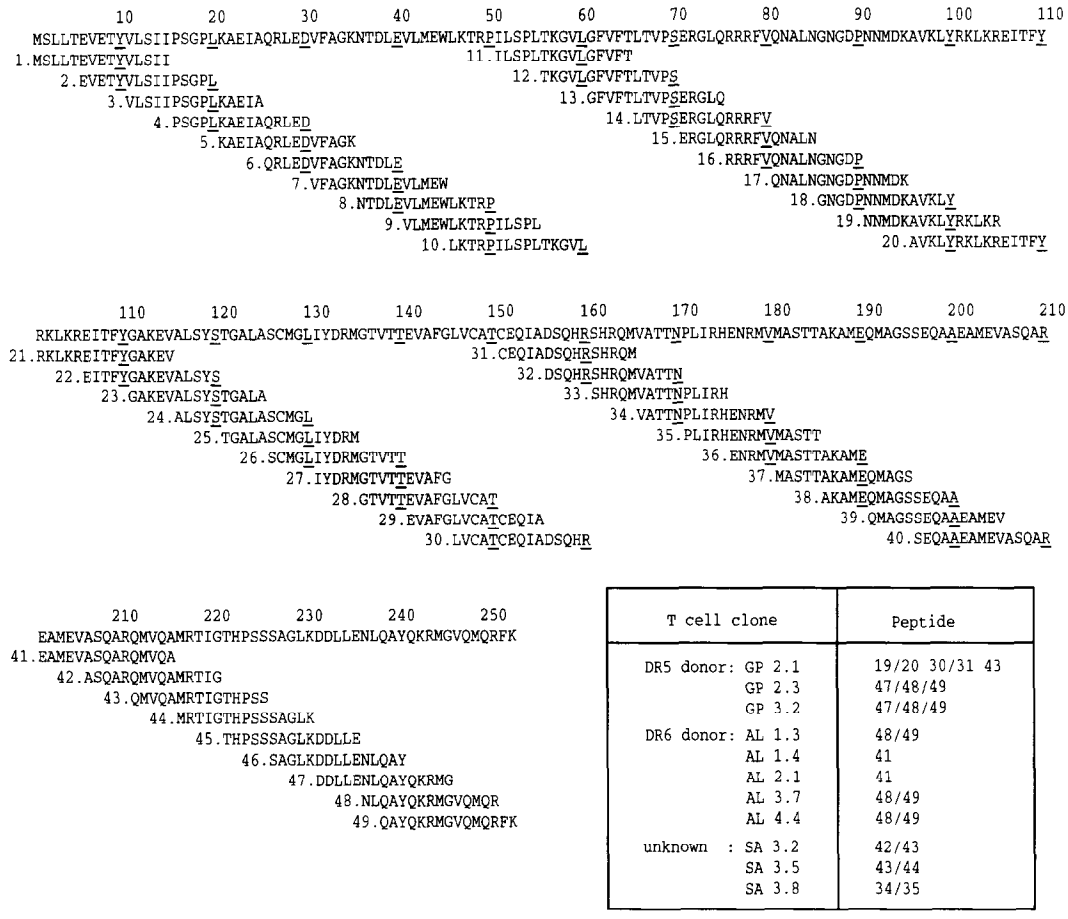
For characterization of the T cell clones which had been established by stimulating PBL with purified influenza matrix protein, a series of peptides was synthesized (Fig. 2), to reduce the chances of destruction of the antigenic determinant. A length of 15 AA was chosen. For the same reason, an overlap in sequence of 10 AA was used beween neighbouring peptides. The eluted peptides (ca. 20 nmol) were dissolved in 500 μl PBS, and 10 μl were mixed with T cell clones together with either irradiated PBLs or EBV transformed B cell lines as antigen presenting cells. For most T cell clones it was possible to assign one or two overlapping stimulatory peptides (Fig. 2). This indicates that the peptides are of suitable quality, as already suggested by the analyses using a mass spectrometer, and in addition that they are free of toxic substances.

3.3. Fine specificity of two DQ restricted T cell clones

Clones from two donors of different haplotypes were found which displayed reactivity to the peptides of the carboxy terminus of the influenza M protein. The exact epitope for two such clones was established by a second set of peptides. Based on a 20-mer consisting of the complete sequence of peptides 47 and 48, variants were synthesized which displayed successive single AA truncations from either the NH_2- or COOH-terminus. As shown in Fig. 3, the two T cell clones studied recognized two similar but not identical peptides. The restriction element of both clones was determined by blocking the proliferation with MHC class II specific antibodies. Monoclonal antibodies against either DR, DP or DQ (L243, B7.21 or SPVL3, respectively) were added to the proliferation assays using the 20-mer peptide as antigen. DQ could be clearly identified as the restriction element of both clones (data not shown). One donor (DR6 homozygous) had been typed positive for DQw1, the second donor (DR5 homozygous) is most likely DQw3 due to genetic disequilibrium of the DR5 haplotype in the caucasian population [7]. The exact alleles of the DQ molecules remain to be determined.

4. Discussion

Many class II dependent antigens have been investigated in the human system. Of the antigenic determinants characterized, most have been found to be presented by DR molecules and only a few determinants have been described which depend on DQ molecules [8–10]. The finding that T cell clones isolated from different donors for reactivity against the M protein of influenza virus were mainly restricted to DQ molecules (clones GP 3.2, ALF 3.7 and data not shown) was therefore surprising.



T cell clone		Peptide
DR5 donor:	GP 2.1	19/20 30/31 43
	GP 2.3	47/48/49
	GP 3.2	47/48/49
DR6 donor:	AL 1.3	48/49
	AL 1.4	41
	AL 2.1	41
	AL 3.7	48/49
	AL 4.4	48/49
unknown :	SA 3.2	42/43
	SA 3.5	43/44
	SA 3.8	34/35

Fig. 2. Display of the simultaneously synthesized peptides used to scan the matrix protein of influenza virus for T cell epitopes. The insert shows the reactivity of various T cell clones from three donors of whom two had been typed for DR.

During an influenza infection T cells most likely encounter antigen in the respiratory tract. The high frequency of DQ restricted T cell clones might indicate that antigen presenting cells at this site display a quantitatively different composition of class II molecule isotypes than antigen-presenting cells in the blood stream. A higher expression of DP and DQ on antigen presenting cells in gut-associated tissue has been observed (Thorsby, personal communication).

The minimal stimulatory peptides of T cell clones derived from the two donors of different haplotypes are overlapping in sequence – AYQKRMGVQMQR for DQw1 and LENLQA-YQKR for DQw3 – and both require basic AA (Arg) at the COOH terminus. They differ, however, in the required length (12 AA and 10 AA, respectively). This might reflect a better fit of the shorter peptide to its particular binding site on the class II molecule, but also could reflect differences in the recognition by the T cell receptor. Since data on other peptides which bind to identical DQ alleles are not yet available, it is difficult to speculate as to which residues are essential for binding to the DQ molecules.

Through sequencing of peptides eluted from selected MHC class I molecules it was possible to define anchor residues which are essential for binding of a peptide to a particular class I molecule [11]. Using this knowledge it is now possible to predict potential stimulatory peptides of a given antigen with a high probability [12]. Since anchor residues for peptides binding to class II molecules seem to be more degenerate, prediction of epitopes of class II restricted T cell clones is still rather difficult, requiring scanning of whole antigens or fragments thereof by overlapping peptides. A suitable method of peptide synthesis which can be performed with minimal cost would therefore be desirable for investigators interested in T cell receptor–antigen interaction. We could show here that the SPOT method of simultaneous peptide synthesis, which has successfully been used for epitope mapping of monoclonal antibodies [3], could also be adapted to efficient screening of T cell epitopes. HPLC and mass spectroscopy revealed that even peptides up to a length of 20 AA could be synthesized with excellent yields. The peptides were stimulatory in functional T cell assays even at high concentrations, indicating that the peptides were not contaminated with toxic substances remaining from the synthesis or deprotection.

The SPOT synthesis therefore represents a convenient method to simultaneously synthesize peptides for biological assays without the requirement of expensive apparatus. In addition the synthesis has recently been semi-automated to allow an even larger number of peptides to be synthesized simultaneously. This should be of great benefit not only for epitope screening, but also for other applications such as screening for peptide antagonist which specifically interfere with T cell

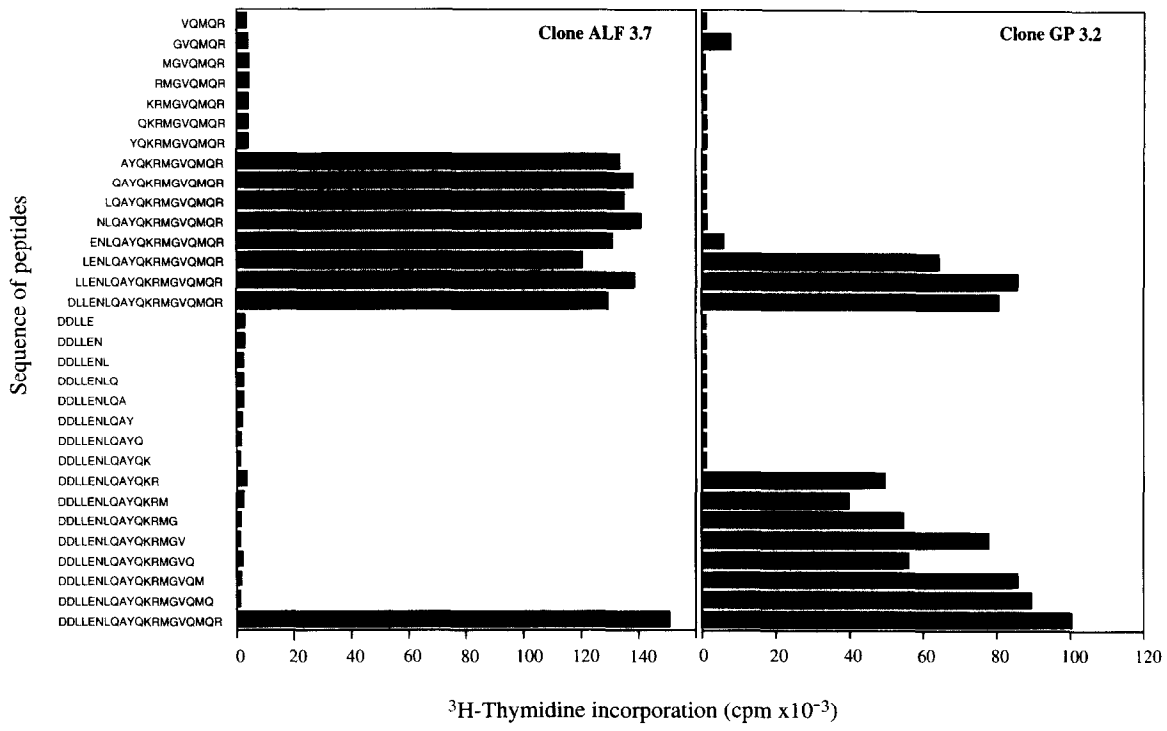


Fig. 3. Fine mapping of two DQ restricted T cell clones by proliferation assay.

responses [13], the latter for possible use in the specific treatment of immunological disorders.

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