# INVESTIGATION OF THE ROLE OF TCR/CD3 ζ SUBUNIT IN SIGNAL TRANSDUCTION AND THE IMMUNE RECOGNITION IN CASE OF INFLUENZA VIRUS HAEMAGGLUTININ USING SYNTHETIC PEPTIDES

**PhD Thesis**

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## PUBLICATIONS RELATED TO THE THESIS

I. Hollósi, M., Ismail, A. A., Mantsch, H. H., Penke, B., Váradi, G., (1992) *Eur. J. Biochem.* **206**, 421-425

II Tóth, G. K., Váradi, G., Nagy, Z., Monostori, É., Penke, B., Hegedűs, Z., Andó, I., Fazekas, G., Kurucz, I., Mák, M., Rajnavölgyi, É. (1993) *Peptide Research* **6**, 272-280

III. Holly, S., Majer, Z., Tóth, G. K., Váradi, G., Rajnavölgyi, É., Laczkó, I., Hollósi, M. (1993) *BBRC* **193**, 1247-1254

IV. Váradi, G., Tóth, G. K., Penke, B. (1994) *Int. J. of Peptide and Protein Res.* **43**, 29-30

V. Nagy, Z., Rajnavölgyi, É., Hollósi, M., Tóth, G. K., Váradi, G., Penke, B., Tóth, I., Horváth, A., Gergely, J., Kurucz, I. (1994) *Scand. J. Immunol.* **40**, 281-291

VI. Rajnavölgyi, É., Nagy, Z., Kurucz, I., Gogolák, P., Tóth, G. K., Váradi, G., Penke, B., Tigyi, Z., Hollósi, M., Gergely, J. (1994) *Mol. Immunol.* **31**, 1403-1414

VII. Hegedűs, Z., Andó, I., Tóth, G. K., Váradi, G., Monostori, É. (1995) *BioTechniques* **18**, 3-4

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## CONTENTS

# INTRODUCTION

There are three major cell types involved in acquired immunity: T cells, B cells and macrophages. The first two are called as lymphocytes. They come from a common precursor cell but differentiate along different developmental lines. T and B cells have different functions but both of them have cell surface receptors for antigens, so they represent specificity of the immune response toward antigens. Macrophages constitute the third cell type. They are phagocytic cells whose function is to ingest immunogens, to process them, and to present them to the T cells. Macrophages lack the capacity of specific antigen recognition.

The immune response has two arms: humoral and cellular immunity. Humoral immunity is mediated by antibodies which are secreted by the B cells. Antibodies are a heterogeneous mixture of serum globulins, all of which have the ability to bind to specific antigens. Cell-mediated immunity consists of T lymphocytes. The response to any particular antigen involves a complex interaction between humoral and cell-mediated immunity.

The definitive T cell marker is the T cell antigen receptor (TCR). It has two types. Most of blood cells express TCR-2 that has a heterodimer of the disulphide-linked $\alpha$ and $\beta$ polypeptide chains. TCR-1 has similar structure but consists of $\gamma$ and $\delta$ polypeptide chains. Both TCR-1 and TCR-2 are associated with a complex involving other polypeptide chains making up the CD3 complex [65]. These chains are the $\gamma$, $\delta$, $\varepsilon$, $\zeta$, $\eta$ and $\omega$ chains. Each of them is a transmembrane peptide and expect $\varepsilon$ and $\omega$ chains active phosphorylation sites can be found in their cytoplasmic regions containing either serine ($\gamma$ and $\delta$ chains) or tyrosine ($\zeta$ and $\eta$ chains) residues. It shows that CD3 complex mediates signal transduction when T cells are activated by antigen binding to the TCR .

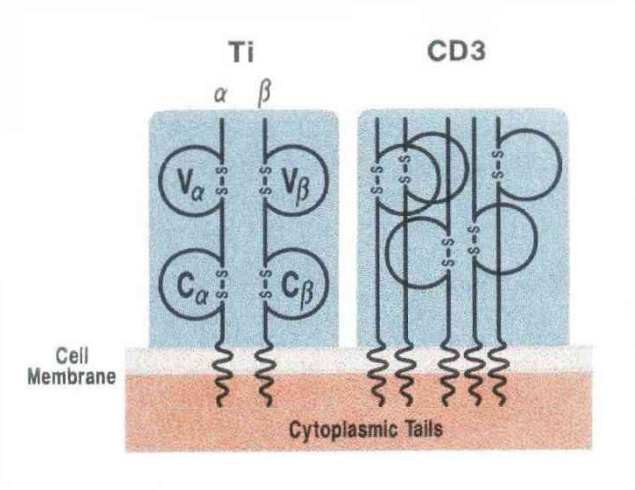



*Figure 1.* The TCR polypeptides showing the two-chain antigen-recognizing molecule (Ti) and the five-chain CD3.

A widespread disease of our age is the influenza. The influenza virus has two major surface antigens: haemagglutinin and neuraminidase. Both of them, as the viral antigens in generally, can be potential targets of immune response. The haemagglutinin is an envelope glycoprotein that is involved in attachment to cells. Antibodies against it are protective. (Antibodies against the other surface protein, neuraminidase, are much less effective.) The haemagglutinin has multiple biological functions and it is one of the major targets of immune recognition. The amino acid sequence of the haemagglutinin molecule is different in diverse influenza virus serotypes and as a consequence of mutations it shows antigenic variations of the neutralizing epitopes of haemagglutinin.

Investigation of the role of TCR/CD3 ζ subunit in signal transduction and the immune recognition in case of influenza virus haemagglutinin can make a success of understanding some small details of the functioning of the immune response.

# AIMS

I. Investigation of the role of TCR/CD3 complex ζ subunit in signal transduction

Our first aim was to investigate the role of TCR/CD3 ζ chain in signal transduction. For this purpose it was necessary to produce antibodies against this subunit. Because the classical way - using of epitope-carrier molecule conjugates - failed, we wanted to try out the multiple antigenic peptide (MAP) method in order to increase the immunogenicity of the antigenic determinants of the ζ chain.

We intended to investigate which tyrosine residues of this subunit are phosphorylated in the process of T cell activation, which kinases take part in this process and which phosphorylated chain sequence serves as substrate for the tyrosine phosphatase CD45.

On the other hand the conformational consequences of the tyrosine phosphorylation planned to be examined using synthetic peptides. For these studies we wanted to develop a suitable method for the chemical synthesis of phosphorylated peptides and a new, efficient, reproducible method for the synthesis of the acid-labile 4-(2',4'-dimethoxyphenylhydroxymethyl)-phenoxymethylpolystyrene support.

II. Investigation of the immune response in case of influenza virus haemagglutinin

Our aim was to investigate the effect of post-translational enzymatic cleavage on antibody recognition of the intersubunit region of the influenza virus haemagglutinin. We wanted to examine whether the conformation of this region of HA plays role in T and B cell recognition.

We planned to use multiple antigenic peptides in order to increase immunogenicity of the small synthetic peptides. We wanted to compare the conformation of haemagglutinin fragments in MAP systems to that outside of these constructions.

Our aim was to investigate the intersubunit region also in H2 and H3 human influenza A virus serotypes.

# THEORETICAL BACKGROUND

## I. Investigation of the role of TCR/CD3 ζ subunit in signal transduction

Antibodies, or immunoglobulins, are involved in the acquired immunity provides protection against infectious organisms and their toxic products. They are glycoproteins present in the serum and tissue fluids. Monoclonal antibodies are homogeneous populations of antibody molecules, derived from a single antibody-producing cell. They are powerful tools of studying cell-surface receptors and the function of their subunits.

In order to produce monoclonal antibodies first an immunogenic agent must be prepared that is capable of inducing an immune response. This agent must contain one or more antigenic determinants (epitopes) and must have a certain minimum molecular weight. Previously conjugates of antigenic determinants and high molecular weight carriers (e.g. proteins) were used for this purpose. But these conjugates have some disadvantages, such as the unknown number of epitopes bonded to the carrier molecule, the laborious purification and isolation of the prepared antigen, the carrier-mediated epitope competition.

The multiple antigenic peptide (MAP) doesn't possess these drawbacks. It was successfully used for several peptide fragments of different proteins [1, 2, 3, 4]. Even these kind of branched polypeptides were synthesized using the major immunogenic regions of the foot-and mouth disease (FMDV) [5], the circumsporozite antigen of malaria [6, 7, 8], hepatitis [8, 9] and Schistosoma mansoni [10]. The multiple antigenic peptide system has well-known and reproducible structure, the purification of it can be realizable and there is no carrier-mediated epitope competition.

The effect of copy number was systematically studied with FMDV-epitopes [5] and tetramers proved to be as effective as octamers. In the MAP method it is possible to use different built-in adjuvants, such as lipopeptides [11, 12, 13]. An other advantage of the method is that both B and T epitopes can be incorporated to the same molecule and the immunogenicity can be improved if B epitopes are linked to T epitopes in the appropriate position [14, 15].

After the T cell activation proliferation of these cells is coming and the different T cells perform their effector functions. In the first part of signal transduction there's change in the concentration of some ions, cyclic nucleotides and other compounds. First of all hydrolysis of lipids is increasing catalyzed by guanosinetriphosphate binding proteins (GTP). During this process phosphatidylinositol-4,5-diphosphate decomposes to diacylglycerol (DAG) and inositol-1,4,5-triphosphate. The latter bonds to intracellular receptors and mobilizes $Ca^{2+}$ ions inside of the cell, so the intracellular calcium ion concentration increases. DAG and calcium ion induce activation and translocation of the protein kinase C enzyme. Even other protein kinases are activated on the effect of complex formation of calcium ions and calmoduline. Kinases that take part in this process are serine, threonine or tyrosine kinases, such as $p56^{lck}$ and $p59^{fyn}$ of the src gene family [16, 17, 18, 19]. Tyrosine phosphatase CD45 not only phosphorylates but also dephosphorylates some intracellular substrates. One of the possible substrates of it is the tyrosyne-phosphorylated ζ chain of the TCR/CD3 complex [20].

To complete the process of activation, the T cell must receive a second signal in the form of lymphokines or other cytokines. These lymphokines interact with and bond to the appropriate subunits of TCR. One important role of ζ chain phosphorylation is the following: phosphotyrosine containing polypeptides are able to associate with SH2 domain of lymphokines. This interaction ensures the coupling of the subsequent biochemical steps of the cell activation, so coupling of the extracellular signal (antigen binding) to the intracellular activation pathway.

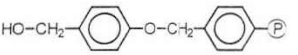
Zeta chain is phosphorylated on multiple tyrosine residues. There are seven tyrosine residues in the intracellular region of this subunit of which six are found in a conservative YL...YL motif. When these motifs are mutated the activation of the T cells is impaired. Dephosphorylation of this protein is necessary for the down-regulation of T cell activation [21].

MKWKALFTAAILQAQLPITEAQSFGLLDTKLCYLLDGILFIYGVILTALFL
RVKFSRSAE***PPAYQQG***Q***NQLYNEL***NLGR***REEYDVL***DKRRGRDPEMGGK
PRRKNP***QEGLYNEL***QKDKM***AEAYSEIG***MKGERRRGKGH***DGLYQGLS***TA
T***KDTYDAL***HMQALPPR

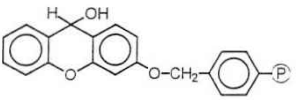
*Figure 2.* The primary structure of the TCR/CD3 ζ subunit.

Phosphorylation of synthetic peptides is widely used for the in vitro assay of protein kinases. Two methods can be used for this purpose: the synthon approach and the global method [22, 23, 24, 25, 26]. In the earlier approach phosphorylated amino acids (phosphotyrosine, phosphoserine or phosphothreonine) are built in the peptide chain. The use of commercially available Fmoc-Tyr($PO_3Me_2$)-OH or derivatives of the two other amino acids above mentioned is a safe but expensive method and the deprotection of the phosphate moiety can lead to partially deprotected by-products. In the global method the peptide chain is phosphorylated after the synthesis. Phosphorylation of the tyrosine moiety of the ready peptide with dibenzyl-phosphochloridate was unsuccessful both in solid and in liquid phase. In the case of tyrosine only the phenolate seemed to be sufficiently nucleophilic, but its preparation from the protected peptide resin was not feasible. Phosphitylation with di-*tert*-butyl-N,N-diethylphosphoramidite and subsequent oxidation seemed to be the most effective and useable method.

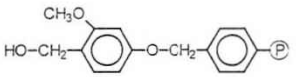
The global method combining with solid phase peptide synthesis can be carried out with Fmoc chemistry. For this technique an acid-labile carrier is needed. The ideal polymer used for this purpose remains stable during cleavage, and the peptide can be removed by mild acidic treatment after the synthesis. Simultaneously, the side-chain protectig groups are also cleaved. Such polymers are the 4-alkoxybenzylalcohol resin of Wang [27], the 3-xanthydrol-oxymethylpolystyrene of Sieber [28], the 2-methoxy-4-alkoxybenzylalcohol resin of Mergler [29] and the 4-(2',4'-dimethoxyphenylhydroxymethyl)-phenoxymethylpolystyrene of Rink [30].



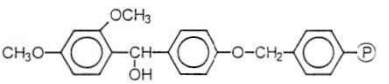

4-alkoxybenzylalcohol resin (Wang)




3-xanthydrol-(9)-oxymethylpolystyrene (Sieber)




2-methoxy-4-alkoxybenzylalcohol resin (Mergler)




4-(2',4'-dimethoxyphenylhydroxymethyl)-phenoxymethylpolystyrene
(Rink)

*Figure 3.* The structure of some acid-labile carriers.

The most commonly used procedure for the preparation of such carriers is the synthesis of the "keto-resin" and the subsequent reduction. Among others it was used for the preparation of Rink's polymer.

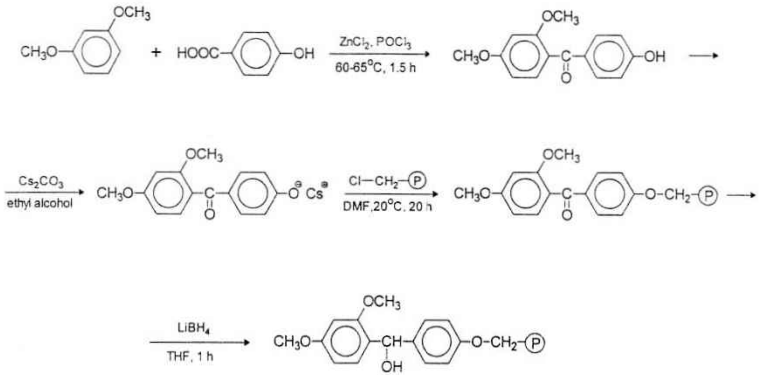



*Figure 4.* The synthesis of 4-(2',4'-dimethoxyphenylhydroxymethyl)-phenoxymethylpolystyrene.

But following this procedure some difficulties came up. Therefore it was necessary to find a new, reproducible method instead of the way written above.

## II. Investigation of the immune recognition in case of influenza virus haemagglutinin

The influenza virus haemagglutinin has an important role in the infectious cycle of the virus and it is one of the major targets of antibody recognition [31, 32, 33]. The haemagglutinin forms envelope spikes on virions or is expressed in the membrane of infected host cells [34]. Spike or integrated proteins of enveloped viruses are degraded in antigen-presenting cells via receptor mediated endocytosis. MHC class II molecules present viral peptides for virus-specific helper T cells [35, 36]. If infective viruses enter a host cell, virus replication occurs and it generates newly

synthesized viral proteins. In this process primarily MHC class I molecules are involved [37, 38, 39, 40]. MHC class I and class II molecules have a chance to select from different sets of viral peptides, but some fragments of viral proteins can be bonded to both of them [41, 42].

The influenza virus haemagglutinin (HA) has multiple biological functions: it is responsible for receptor binding and it mediates fusion of the viral and the host cell endosomal membranes [34, 43, 44]. The HA molecule is synthesized in the infected host cell as a precursor polypeptide chain with an intact intersubunit region (HA0) and upon maturation it is cleaved to HA1 and HA2 subunits [45, 46]. The enzymatic cleavage at $Arg^{329}$ is mediated by host cell proteases [47].

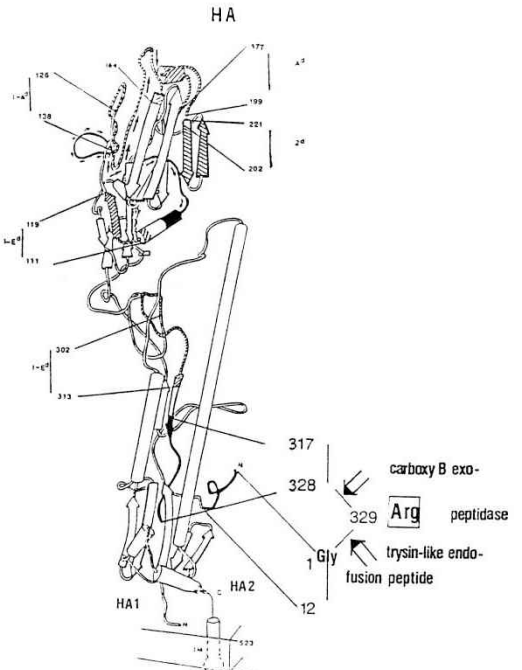



*Figure 5.* The polypeptide chains of the influenza virus haemagglutinin.

This event is a basic requirement for the infectivity of the virus and ensures a pH-dependent conformational change of the HA molecule [46, 48]. The consequences of it are the reorganization of the overall structure of the HA trimers and the exposure of the so called fusion peptide comprising the N-terminal 1-14 amino acids of the HA2 subunit. It is the peptide that enables the fusion of the viral envelope with the endosomal membrane of the host cell resulted in driving the virus core into the cytoplasm to initiate infection and facilitate uncoating processes essential for viral propagation [49, 50, 51].

Influenza A viruses are subdivided into 14 serotypes based on antigenic differences [34]. The amino acid sequences of the haemagglutinin subtypes are different in diverse influenza virus serotypes [52, 53]. In addition, the sequence changes continuously resulting in antigenic variations of the epitopes of HA [54]. The epidemological success of influenza A viruses is largely due to this variability of their surface glycoproteins. In contradistinction to these variations the amino acid sequence of the fusion peptide is identical in all influenza virus subtypes [51]. Also the amino acid sequence of the C-terminal part of HA1 subunit is relatively conserved and comprises an enzyme cleavage motif determining enzyme susceptibility and thus influencing tropism of the virus [55, 56, 57]. The enzyme involved in cleavage of HA0 molecule is a trypsin-like serine protease [58]. Antigen-presenting cells such as B cells, macrophages and dendritic cells lack this enzyme and thus upon infection generate immature, uncleaved molecules, giving rise to non-infective viruses [36, 58].

# RESULTS

## I. Investigation of the role of TCR/CD3 complex ζ subunit in signal transduction

For investigation of TCR/CD3 ζ subunit in signal transduction it was necessary to produce antibodies against this protein chain. The first step was determining epitopes in the extracellular region of it. The WISCONSIN program was used for computer modelling. This program calculated an antigenic index of the amino acids considering hydrophilicity, flexibility of the protein chain and formation of different types of the secondary structure (α-helix, β-sheet, β-turn). The largest value of this index, the most promising the epitope.

In the beginning epitope-protein conjugates were prepared. Three peptides were synthesized from the extracellular region of the ζ chain using solid-phase peptide synthesis and Boc-chemistry: $CD3\zeta_{22-32}$ (QSFGLLDPKLC), $CD3\zeta_{17-33}$ (PITEAQSFGLLDPKLCY) and $CD3\zeta_{21-37}$ (AQSFGLLDPKLCYLLDG) . The completed peptide resins were treated with liquid hydrogen fluoride. The resulting free peptides were solubilized in 10% aqueous acetic acid and lyophilized. The crude peptides were purified by reverse-phase HPLC. Amino acid analysis and analytical RP-HPLC were used for characterization. In addition, dimers were formed from them by air-oxidation, because the TCR/CD3 ζ subunit can be found in dimer form in the receptor.

The peptides were bonded to bovine serum albumin (BSA). The conjugates were used for immunizing rats. The formed antisera recognized the appropriate peptides. Specificity of the immune reaction was proved by ELISA. But none of the antisera recognized the native ζ chain presents in T cells.

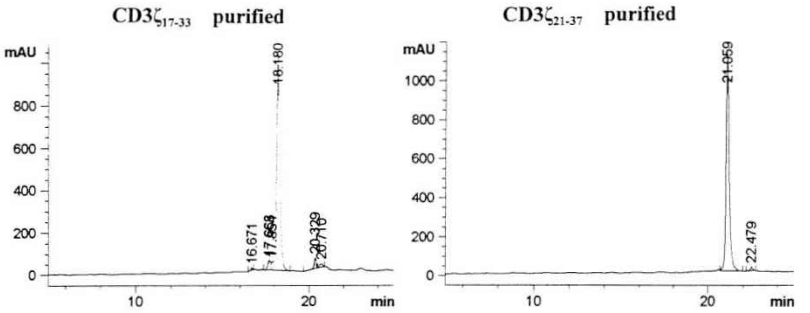



*Figure 6.* RP-HPLC profiles of two ζ chain fragments.
HPLC conditions: Nucleosil 5C-18 column (4 X 250 mm); detection at 220 nm; eluent system, (A) 0.1% TFA in water and (B) 0.1% TFA and 80 acetonitrile in water; linear gradient from 20% B to 60% B in 25 min; flow rate 0.9 ml/min

Because of this difficulty the MAP method was used to increase the immunogenicity of the previously mentioned $CD3\zeta_{21-37}$ peptide fragment. First an octameric MAP was synthesized (MAP-1). We used solid-phase peptide synthesis with Boc-chemistry. After removing the protecting groups, the polypeptide was cleaved from the resin with liquid hydrogen fluoride. Then it was solubilized and purified by reverse-phase HPLC without any difficulties. The FAB MS couldn't be used for the characterization of this branched polypeptide, but the matrix-assisted laser desorption ionization mass spectrometry proved to be applicable for determining the molecular ion. Later ESI MS was also successfully used for this purpose. Antibodies, raised against this MAP, didn't recognize the native ζ chain. It could be explained by the closeness of the epitopes in this construction resulted in a change of the native conformation.

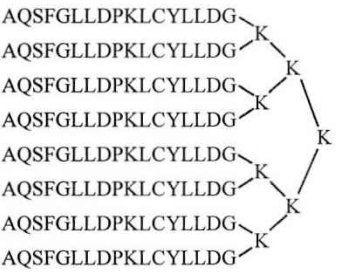



*Figure 7.* The structure of MAP-1.

To avoid difficulties a tetrameric MAP of the same peptide was synthesized (MAP-2). In addition, the influenza-derived FP3 fusion peptide was built into the construction as a T cell epitope and a hydrophobic region that can stabilize the conformation of the N-terminal peptide.

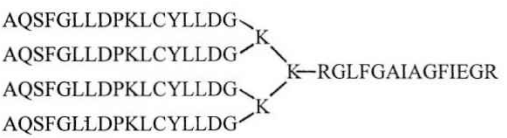



*Figure 8.* The structure of MAP-2.

Monoclonal antibodies were raised against it, that were highly reactive with the corresponding ζ-chain fragment. Higher than 50% inhibition was achieved when ζ-peptide was used as inhibitor. The experiment showed specificity of the antiserum against ζ-peptide but the antibodies didn't recognize the native ζ-chain.

The immunologically efficient MAP method was used for the investigation of different tyrosine kinases. The influenza-derived FP3 was grafted by four peptides represent the $p56^{lck}$ and $p59^{fyn}$, belong to the src oncogene family (MAP-3 and MAP-4) and the syk family member ZAP 70 (MAP-5).

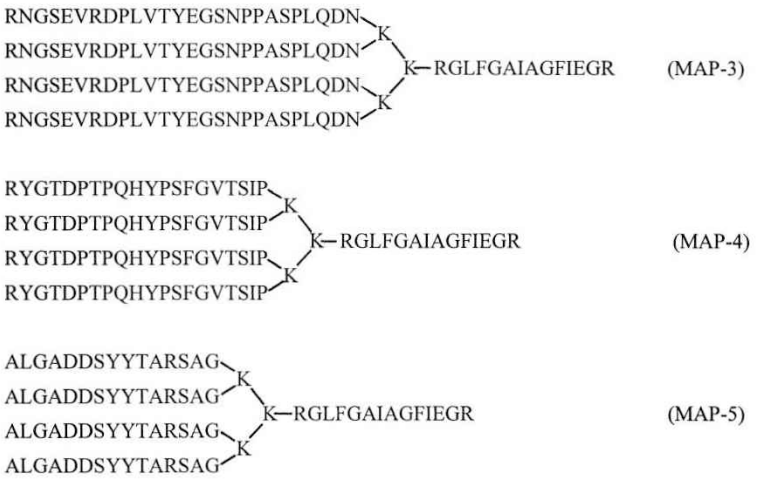



*Figure 9.* The structure of MAP-3, MAP-4 and MAP-5.

Rats were immunized with these constructions. The antisera raised against MAP-3 and MAP-5 were highly reactive with the corresponding multiple antigenic peptide constructions and both of them recognized the native protein chains. Monoclonal antibodies, raised against MAP-4, recognized the immunizing MAP but recognition of the native protein chain couldn't be proved in this case.

Seven short peptides and the phosphorylated derivatives were synthesized for the investigation of different tyrosine residues of TCR/CD3 ζ subunit in T cell activation. (The sequences are shown in Table 1.)

The nonphosphorylated peptides were synthesized in the solid phase using Boc-chemistry. After cleaving the peptides from the resin by liquid hydrogen fluoride they were solubilized and purified. Amino acid analysis and analytical RP-HPLC were used for characterizing them.

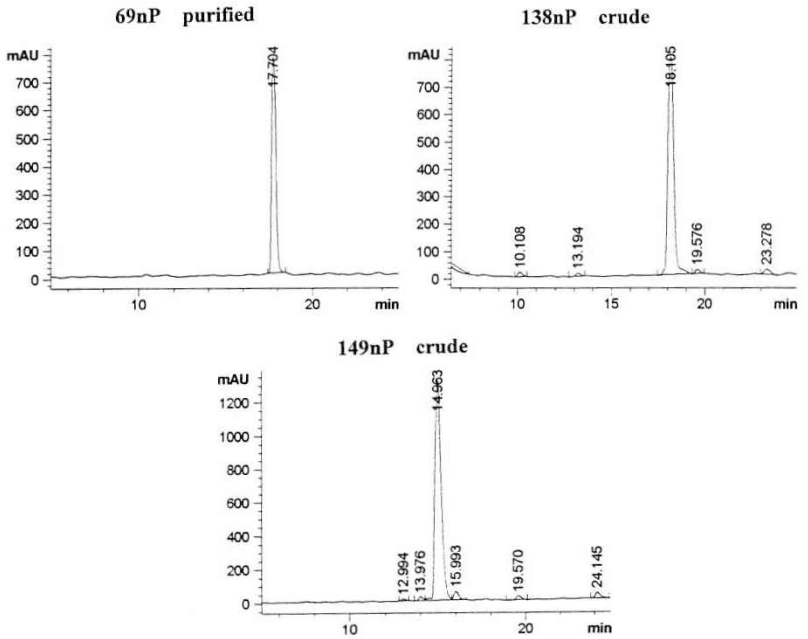



*Figure 10.* RP-HPLC profiles of several TCR/CD3 ζ subunit fragments. HPLC conditions: Lichrosorb-100 RP-18, 10 μm column (4 X 250 mm); detection at 220 nm; eluent system, (A) 0.1% TFA in water, (B) 0.1% TFA and 80% acetonitrile in water; linear gradient from 5% B to 45% B in 25 min; flow rate 1.0 ml/min.

**Table 1.** Amino acid sequences of the TCR/CD3 ζ subunit fragments.

| Code | Peptide sequences |
|---|---|
| 61nP | PPAYQQG |
| 69nP | NQLYNEL |
| 80nP | REEYDVL |
| 106nP | QEGLYNEL |
| 119nP | AEAYSEIG |
| 138nP | DGLYQGL |
| 149nP | KDTYDAL |

For the synthesis of phosphorylated fragments we tried out several methods. First polyphosphoric acid was examined, but because of a technical problem we couldn't use it for the phosphorylation of peptides. Polyphosphoric acid can phosphorylate the hydroxy group of tyrosine. The excess of it, used for the phosphorylation, can't be more than 10-15 equivalent. Because of its large viscosity it was impossible to stir so small amount of this consistent substance. We tried to use the appropriate acylchloride, phosphorus oxychloride, but we didn't observe the formation of any phosphorylated product. Phosphorylation with di-*tert*-butyl-N,N-diethylphosphoramidite could be used successfully both in liquid and in solid phase. The preparation of this reagent could be carried out with a good yield [59].

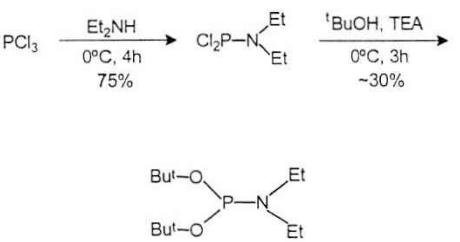



*Figure 11.* Preparation of di-*tert*-butyl-N,N-diethylphosphoramidite.

Phosphorylation with this reagent consists of two steps: phosphitylation and oxidation with *tert*-butylhydroperoxide.

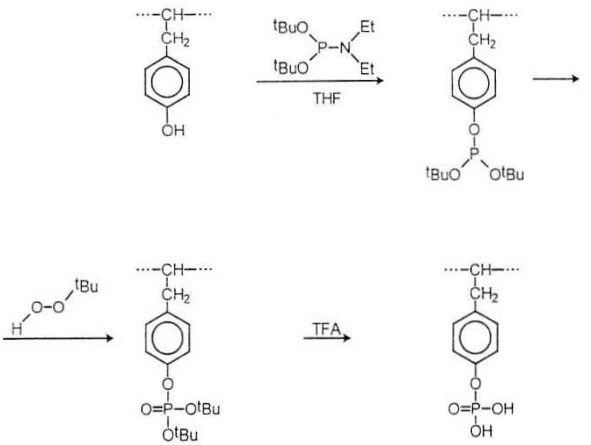



*Figure 12.* Phosphorylation with di-*tert*-butyl-N,N-diethylphosphoramidite.

In solid phase first the peptide is synthesized on an acid-labile polymer, using Fmoc-chemistry, until the tyrosine or serine residue that will be phosphorylated. The phosphitylation and the subsequent oxidation are carried out on the solid support. The solvent of these reactions is tetrahydrofurane. It's very important to use completely pure, peroxide and water free solvent. Following it the synthesis of the N-terminal part is coming. When it is ready, the peptide is cleaved from the polymer and solubilized.

In liquid phase just the peptides that have no other reactive functional groups can be phosphorylated in this way. 61nP is an example for it. First the N-terminal amino group had to be protected with Boc-group. Then the same method could be used as in the solid phase.

Using di-*tert*-butyl-N,N-diethylphosphoramidite we synthesized the phosphorylated analogs of the seven short ζ-chain fragments, summarized in Table 2.

**Table 2.** Amino acid sequences, calculated and observed masses of the phosphorylated peptides

| Code | Peptide sequences | Mass spectra | |
|---|---|---|---|
| | | Calculated | Found |
| 61P | PPAY(*P*)QQG | 838.81 | 839.2 |
| 69P | NQLY(*P*)NEL | 971.96 | 972.7 |
| 80P | REEY(*P*)DVL | 1001.99 | 1002.0 |
| 106P | QEGLY(*P*)NEL | 1044.02 | 1044.6 |
| 119P | AEAY(*P*)SEIG | 917.86 | 918.4 |
| 138P | DGLY(*P*)QGL | 843.83 | 845.1 |
| 149P | KDTY(*P*)DAL | 903.88 | 904.1 |

The crude peptides were purified by a preparative Knauer RP-HPLC system. Purity was checked by RP-HPLC.

Amino acid analysis and mass spectrometry (FAB MS) were used for characterization of the peptides. Data are shown in Table 2.

The incorporation of the phosphate moiety was verified by using UV spectroscopy. The phosphorylation caused a 10 nm hypsochromic effect in the UV spectra recorded from the HPLC.

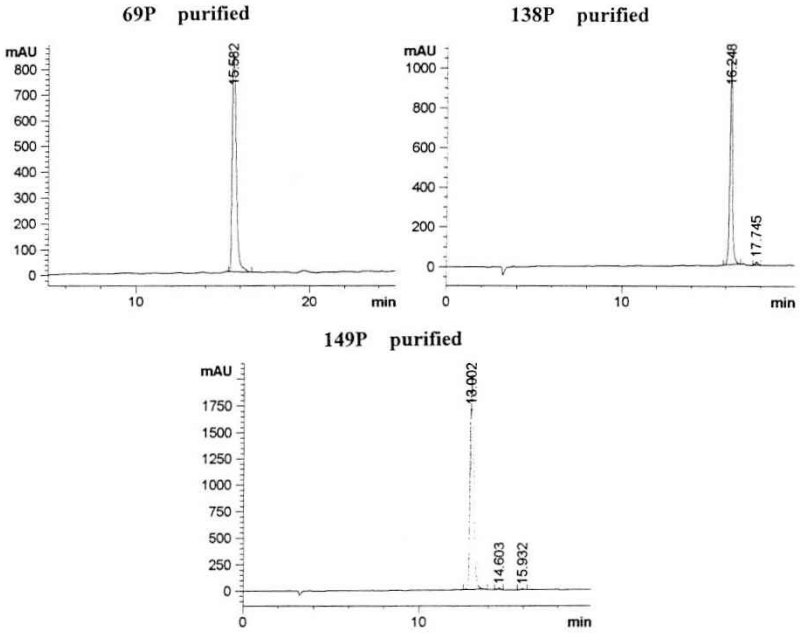



*Figure 13.* HPLC chromatograms of several phosphorylated peptide fragments of the TCR/CD3 ζ subunit.
HPLC conditions: Lichrosorb-100 RP-18, 10 μm column (4 X 250 mm); detection at 220 nm; eluent system, (A) 0.1% TFA in water, (B) 0.1% TFA and 80% acetonitrile in water; linear gradient from 5% B to 45% B in 25 min; flow rate 1.0 ml/min

The conformational consequences of the phosphorylation were investigated by CD and IR spectroscopy. The peptides were dissolved both in water and in trifluoroethanol (TFE), a structure promoting organic solvent. In water, both the phosphorylated and non-phosphorylated peptides

showed predominantly random conformation. In TFE the peptides existed as mixtures of conformers. Four groups of peptides could be distinguished on the base of the changes in the CD spectra of the phosphorylated peptides relative to the non-phosphorylated ones.

1, 61nP, 61P, 69nP, 69P

CD spectra of the non-phosphorylated peptides indicated the presence of an unordered and β-turn conformation. Introduction of the negative $PO_3H_2^-$ on the tyrosine residue alters the spectra. It indicated unordered and β-sheet conformation. The stability of the β-sheet conformation of 69P in TFE was demonstrated by adding water to the TFE solution. The β-sheet character was still preserved in 50% TFE.

The FT-IR spectra showed same kind of shifts in the conformation upon phosphorylation. The 69nP existed as a mixture of β-sheet, β-turn and unordered conformers, the phosphorylated analog of it represented mostly β-sheet structure.

2, 119nP, 119P

Similar changes could be observed for the 119 fragment. A well-expressed β-sheet character could be observed on the CD spectrum that showed the presence of a higher amount of β-sheet conformation comparing to the 61 and 69 peptides.

3, 149nP, 149P

The CD spectrum of the 149nP peptide showed a β-turn/unordered conformer mixture. This equilibrium was shifted to the unordered structure upon phosphorylation.

4, 80nP, 80P, 106nP, 106P, 138nP, 138P

The non-phosphorylated peptides contained β-turn and/or β-sheet conformation and the proportion of the ordered structure was further increased in the phosphorylated fragments.

CD and FT-IR investigations showed that the phosphorylation of the peptides changed their conformation. Upon phosphorylation an increase in the β-sheet conformation could be observed.

Several tyrosine kinases are responsible for the ζ-chain phosphorylation that follows T cell activation. Three of them were used in immunological investigations: $p56^{lck}$ and $p59^{fyn}$ of the src oncogene family and the syk family member ZAP 70. The ZAP 70 was able to phosphorylate any of the peptides. The two src kinases phosphorylated six short ζ-related peptides, those in which

the tyrosine residue could be found in a conservative motif. The $p56^{lck}$ phosphorylated the peptides with different pattern: 61nP was not phosphorylated, 69nP, 80nP, 106nP and 138nP were preferentially phosphorylated compairing to the others. When HPBALL membrane was used as a source of tyrosine kinases, 61nP was not phosphorylated, 119nP and 149nP were preferentially phosphorylated. It showed that different kinases phosphorylate different tyrosine residues. The 61nP (PPAYQQG) was not phosphorylated by any of the tyrosine kinases. This peptide lies outside the conservative motifs, so it's not a target of in vitro phosphorylation by the tyrosine kinases.

CD45, a lymphocyte surface protein, that has tyrosine phosphatase activity, dephosphorylated our phosphopeptides. It indicates that this tyrosine phosphatase can be responsible for the dephosphorylation of the ζ-chain, so it can have role in the down regulation of T cell activation.

For solid-phase synthesis using Fmoc-chemistry acid-labile polymers are needed. One of these is the 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxymethylpolystyrene. The most commonly used procedure for the preparation of such carriers is the synthesis of the "keto-resin" and the subsequent reduction on the resin. We tried to synthesize the polymer of Rink by following this method but we obtained low and unrepeatable substitution. Besides the structure of the polymer couldn't be determined accurately.

Because of these difficulties we decided to work out an entirely different strategy: preparing the ready linker and coupling it to polystyrene. Investigation of the reduction of 2,4-dimethoxy-4'-hydroxybenzhydrol was confirmed by IR and NMR spectroscopy. Then the linker was coupled to chloromethylated polystyrene via its cesium salt. This process resulted in an acceptable degree of substitution. Later we used the potassium salt of the phenolic hydroxy group instead of the expensive cesium salt. The degree of substitution was proved by coupling of an Fmoc amino acid to the polymer. We achieved a convenient level of substitution (0.35-0.47 mmol/g), suitable for the synthesis of any peptide.

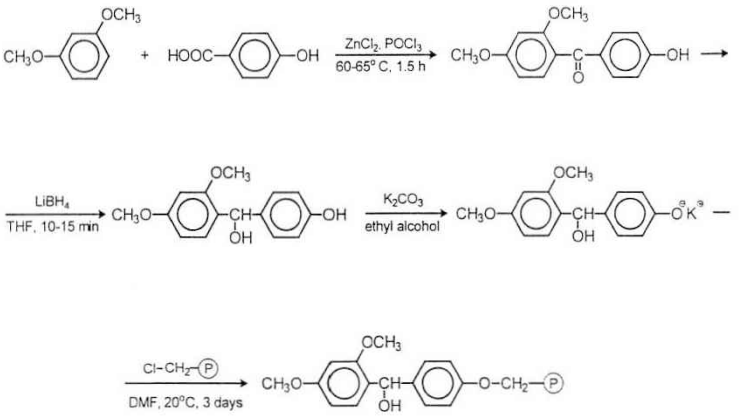



*Figure 14.* The new synthesis of 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxymethyl-polystyrene.

II. Investigation of the immune response in case of influenza virus haemagglutinin

Peptides were synthesized from the intersubunit region of the H1 serotype of influenza A virus. H1IP is a 25-mer peptide comprises the intact intersubunit region of the HA0 molecule. Both this peptide and its D-Arg$^{329}$ analog at the position of coupling the fusion peptide to the C-terminal part of HA1 were synthesized. The fusion peptide was synthesized with and without arginine residue at the N- and C-terminuses. H1 peptides represent the C-terminal region of the HA1 molecule after the enzymatic cleavage at the position 329.

The peptides were synthesized in solid phase by Boc-chemistry. The cleavage from the polymer was performed by liquid hydrogen fluoride. After solubilization they were purified by preparative Knauer reverse phase HPLC system and characterized by analytical RP-HPLC, amino acid analysis and mass spectrometry. Data are shown in Table 3.

**Table 3.** Amino acid sequences, calculated and observed masses of the synthesized peptides

| Code | Peptide sequences | Mass spectra | |
|---|---|---|---|
| | | Calculated | Found |
| H1IP | VTGLRNIPSIQSRGLFGAIAGFIEG | 2574.0 | 2574.3 |
| (D-Arg)[13]H1IP | VTGLRNIPSIQS*R*GLFGAIAGFIEG | 2574.0 | 2573.6 |
| FP1 | GLFGAIAFIEG | 1151.3 | 1151.5 |
| FP2 | RGLFGAIAFIEG | 1307.5 | 1307.8 |
| FP3 | RGLFGAIAFIEGR | 1463.7 | 1463.2 |
| $H1_{317-329}$ | VTGLRNIPSIQSR | 1438.8 | 1439.9 |

The peptides were used for immunological and conformational investigations.

An IgM type monoclonal antibody was isolated from an influenza virus infected mouse. This antibody recognized the uncleaved intersubunit region of the HA both in the uncleaved HA0 molecule and in a synthetic peptide. It demonstrates that this monoclonal antibody was generated in the adaptive immune response against the virus. The reactivity pattern of this antibody was studied with synthetic peptides. It showed that a B cell epitope was located in the C-terminal $H1_{317-329}$ region of the HA1 subunit. H1IP and the D-Arg analog of it were able to induce antibody production against this region without the introduction of any carrier. In contrast, $H1_{317-329}$ or FP3 proved to be not immunogenic. Antibodies were able to react with H1IP and (D-Arg)[329]H1IP, but were unable to recognize $H1_{317-329}$. These results show the presence of T cell epitopes in this region of the HA molecule and demonstrate that the covalently linked fusion peptide, present H1IP and (D-Arg)[329]H1IP is necessary for antibody recognition of the $H1_{317-329}$.

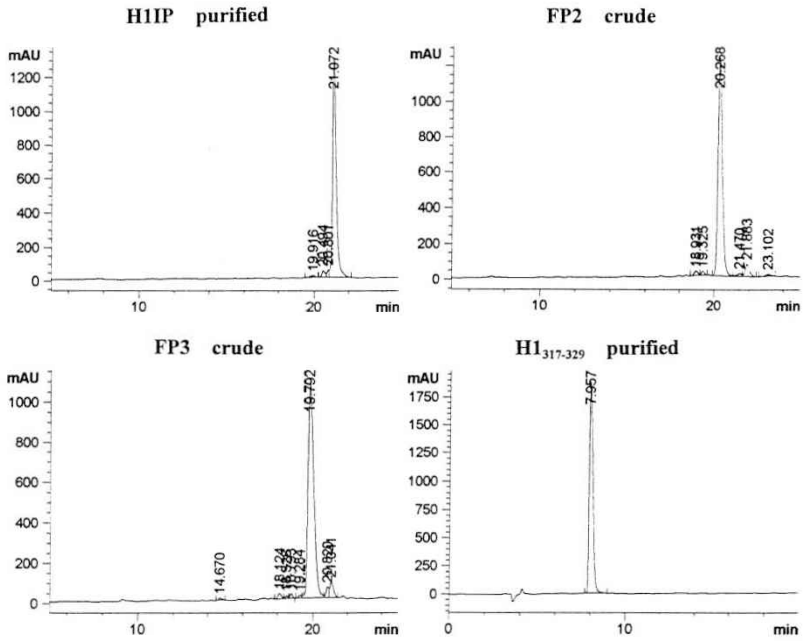



*Figure 15.* Analytical HPLC profiles of some peptides from the haemagglutinin of the H1 human influenza A virus serotype.
HPLC conditions: Nucleosil 5 C18 column (4 X 250 mm); detection at 220 nm; eluent system, (A) 0.1% TFA in water, (B) 0.1% TFA and 80% acetonitrile in water; linear gradient from 20% B to 65% B in 25 min; flow rate 0.9 ml/min.

Conformational characterization of these peptides was of particular interest. The reason of it is that B cell recognition is highly dependent on the conformation [60, 61] and also T cell epitopes are characterized by restricted conformational requirements [62]. Circular dihroism (CD) and

Fourier-transform infrared (FT-IR) spectroscopy were used for determining the secondary structure of the synthetic peptides in solution. The peptides were dissolved both in water and in trifluoroethanol (TFE), a structure-promoting organic solvent. Both CD and FT-IR spectra showed a significant α-helix content for H1IP in TFE and α-to-β conversion in mixtures containing increasing amount of water. Both of these spectra indicated evidence of β-sheet conformation in H1IP, FP1 and FP3 in aqueous solution. Also X-ray analysis of the cleaved haemagglutinin was done that supported the presence of β-turn in the glycine-rich N-terminal HA2 chain (containing FP1 and FP3) [45]. These results demonstrate that both HA1 and HA2 parts of the cleaved haemagglutinin molecule are capable of adopting multiple conformations. They can be present in different conformations in aqueous solution or in the vicinity of cell membranes supporting the possibility for both B and T cell recognition. It is therefore very likely that the interaction with MHC molecules plays an important role in stabilizing peptide conformations suitable for T cell recognition.

**Table 4.** Amino acid sequences and analysis of the peptide fragments by mass spectrometry.

| Code | Peptide sequences | Mass spectra | |
|---|---|---|---|
| | | Calculated | Found |
| $H1_{320-329}$ | LRNIPSIQSR | 1182.39 | 1183.3 |
| $H1_{319-329}$ | GLRNIPSIQSR | 1239.39 | 1239.8 |
| $H1_{320-328}$ | LRNIPSIQS | 1026.20 | 1026.9 |
| $H1_{319-328}$ | GLRNIPSIQS | 1083.25 | 1083.8 |
| $H1_{317-328}$ | VTGLRNIPSIQS | 1283.5 | 1283.9 |
| $H1_{317-327}$ | VTGLRNIPSIQ | 1196.5 | 1196.9 |

It was of particular interest to investigate whether the C-terminal arginine of the HA1 subunit has any effect on T cell recognition. (This arginine is eliminated under natural conditions.) To answer this question the $H1_{317-328}$ was synthesized, that doesn't have C-terminal arginine residue. There was no difference between the T cell recognition of the originally used peptide and its shorter analog. This result is in good correlation with the fact that the C-terminal arginine residue

is eliminated under physiological conditions by a virus associated host cell derived carboxypeptidase enzyme.

In order to localize the core region of the T cell epitope on the C-terminal part of the HA1 subunit N- and C-terminally truncated analogs of the $H1_{317-328}$ were synthesized. None of the other peptides were able to activate T cells. So the core region of T cell epitope is the 317-328 region of the HA1 subunit.

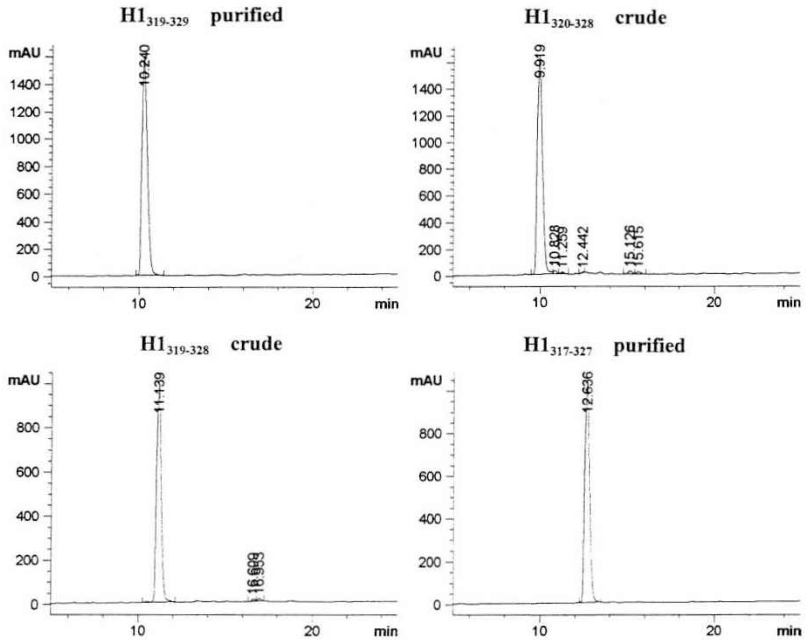



*Figure 16.* HPLC profiles of several shorter peptides from the haemagglutinin of the H1 human influenza A virus serotype.
HPLC conditions: Nucleosil 5 C18 column (4 X 250 mm); detection at 220 nm; solvent system, (A) 0.1% TFA in water, (B) 0.1% TFA and 80% acetonitrile in water; linear gradient from 15% B to 60% B in 25 min; flow rate 0.9 ml/min.

To clarify the antigenic and immunogenic properties of the HA-derived peptides, multiple antigenic peptides comprising repeated copies of the $H1_{317-329}$ sequence were also synthesized. In MAP-7 this peptide was combined with the FP3 fusion peptide, that was efficient in inducing a proliferative T cell response and characterized as a highly hydrophobic sequence.

Solid-phase synthesis and Boc-chemistry was used for the preparation of these branched peptide antigens. The syntheses were carried out manually. The products were cleaved from the resin with liquid HF. The resulting peptides were solubilized in 10% aqueous acetic acid and lyophilized. The crude peptides were purified by reverse-phase HPLC, using an Astec 300 C4 5 μm column (8 X 250 mm). Purity was checked by C4 RP-HPLC. As in the case of the earlier described MAPs the MALDI MS could be used for determining the molecular weight of the branched polypeptides.

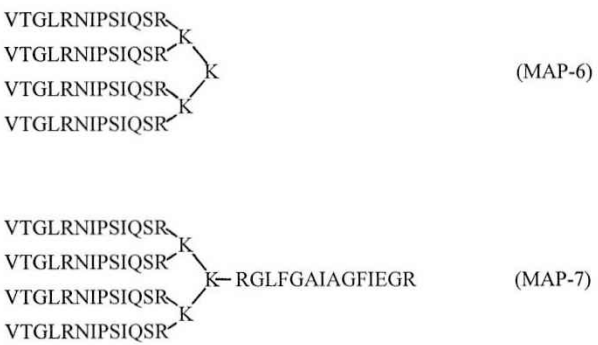



*Figure 17.* The structure of MAP-6 and MAP-7.

The IgM type monoclonal antibody, isolated from an influenza virus infected mouse and mentioned earlier, showed increased reactivity with MAP-6 compared to $H1_{317-329}$. Specificity of polyclonal serum antibodies induced by H1IP, $(D\text{-}Arg)^{329}$H1IP and MAP-6 were investigated. The

results demonstrated similar specificity of these serum antibodies to that of the mentioned monoclonal antibody.

It was of particular interest to investigate whether synthetic peptides comprising the intact form of the intersubunit region of haemagglutinin can confer protection against a lethal dose of influenza virus infection. Pre-immunization with H1IP and with the MAP-6 resulted in prolonged survival and complete protection of a given number of the pre-treated animals. The mortality was 80% in the case of H1IP and only 60% when MAP-6 was used for pre-immunization. Despite of the fact that the molar mass of the branched polypeptide is much higher than that of H1IP, 100 μg of MAP-6 had similar efficiency as 200 μg of H1IP.

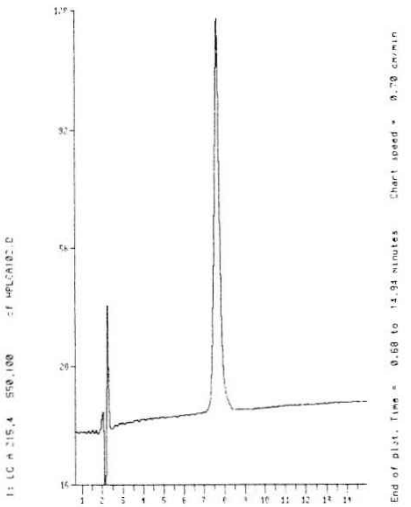


*Figure 18.* Analytical RP-HPLC profile of purified MAP-7.
HPLC conditions: Vydac C4 column (4 X 250 mm); detection at 220 nm; solvent system, (A) 0.1% TFA in water, (B) 0.1% TFA and 80% acetonitrile in water; linear gradient from 0% B to 90% B in 20 min; flow rate 0.8 ml/min

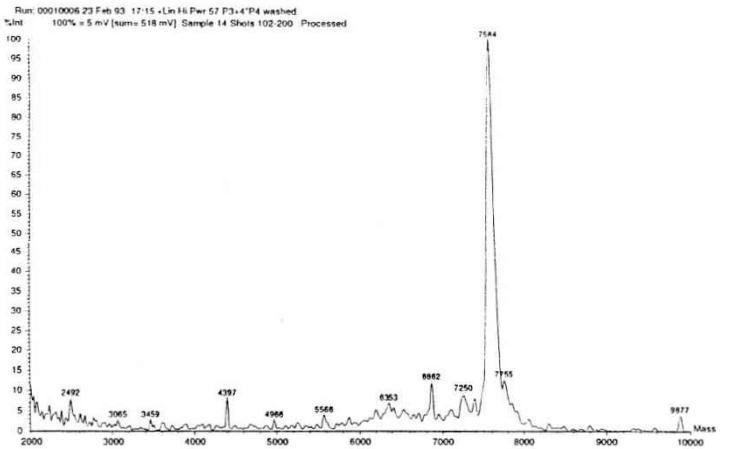



*Figure 19.* Matrix-assisted laser desorption ionization spectra of MAP-7.

MAP-7 was used to elicit immune response and to investigate protection against influenza virus. This branched polypeptide proved to be immunogenic and was able to induce IgG-type, MAP-7-specific antibodies cross-reactive with the acid pre-treated virus. This type of IgG antibody could be detected after repeated MAP-7 treatment and at an elevated level after viral challenge in all survivals. In contrast, the $H1_{317\text{-}329}$ and FP3 monomers were unable to elicit an antibody response directed to the corresponding peptides, MAP-7 or to any form of the pre-treated mice, compared to the $H1_{317\text{-}329}$-pre-immunized mice.

In protection experiments 60% of MAP-7-pre-immunized BALB/C mice acquired complete protection against a lethal dose of influenza virus. This number was 40% in the case of $H1_{317\text{-}329}$. The level of virus-specific antibodies detected in MAP-7- and $H1_{317\text{-}329}$-pre-treated mice was significantly higher than that in the control group of mice.

The conformation of MAP-6 and MAP-7 was investigated by CD and FT-IR spectroscopies. In trifluoroethanol both of them showed very low α-helix content (20% was calculated for MAP-7 and 18% for MAP-6). Interestingly, MAP-6 has a higher helix content than $H1_{317\text{-}329}$ peptide from

which it is composed. The FT-IR spectra showed the presence of weakly H-bonded helical structure.

According to the CD and FT-IR studies both of the branched peptides may also adopt β-turn-like 10-membered H-bonded conformations. Up to 3 different types of 10-membered H-bonded structures may be formed with the participation of the lysine α- and ε-amide groups playing the role of either the acceptor or the donor amides of H-bonds.

Two analogs of the $H1_{317-329}$, representing the C-terminal region of the HA1 molecule, and some other analogs of the nona- and decapaptides were synthesized from the H2 and H3 human influenza A virus serotypes. The peptides were synthesized in solid-phase using Boc-chemistry. The syntheses were carried out manually. The peptides were cleaved from the resin with liquid HF, then were solubilized in 10% aqueous acetic acid and lyophilized. The crude peptides were purified by RP-HPLC using a C-18 column. The purity was checked by analytical RP-HPLC and FAB MS was used for characterization. (Data are shown in Table 5.)

**Table 5.** Amino acid sequences, calculated and observed masses of the peptides.

| Code | Peptide sequences | Mass spectra | |
|---|---|---|---|
| | | Calculated | Found |
| $H2_{317-329}$ | ATGLRNVPQIESR | 1439.63 | 1440.2 |
| $H2_{320-328}$ | LRNVPQIES | 1054.21 | 1054.9 |
| $H2_{320-329}$ | LRNVPQIESR | 1210.40 | 1211.0 |
| $H2_{319-328}$ | GLRNVPQIES | 1111.26 | 1111.9 |
| $H3_{316-329}$ | LATGMRNVPEKQTR | 1599.87 | 1599.7 |
| $H3_{320-328}$ | MRNVPEKQT | 1101.29 | 1101.6 |
| $H3_{320-329}$ | MRNVPEKQTR | 1257.47 | 1258.0 |
| $H3_{319-328}$ | GMRNVPEKQT | 1158.34 | 1158.8 |

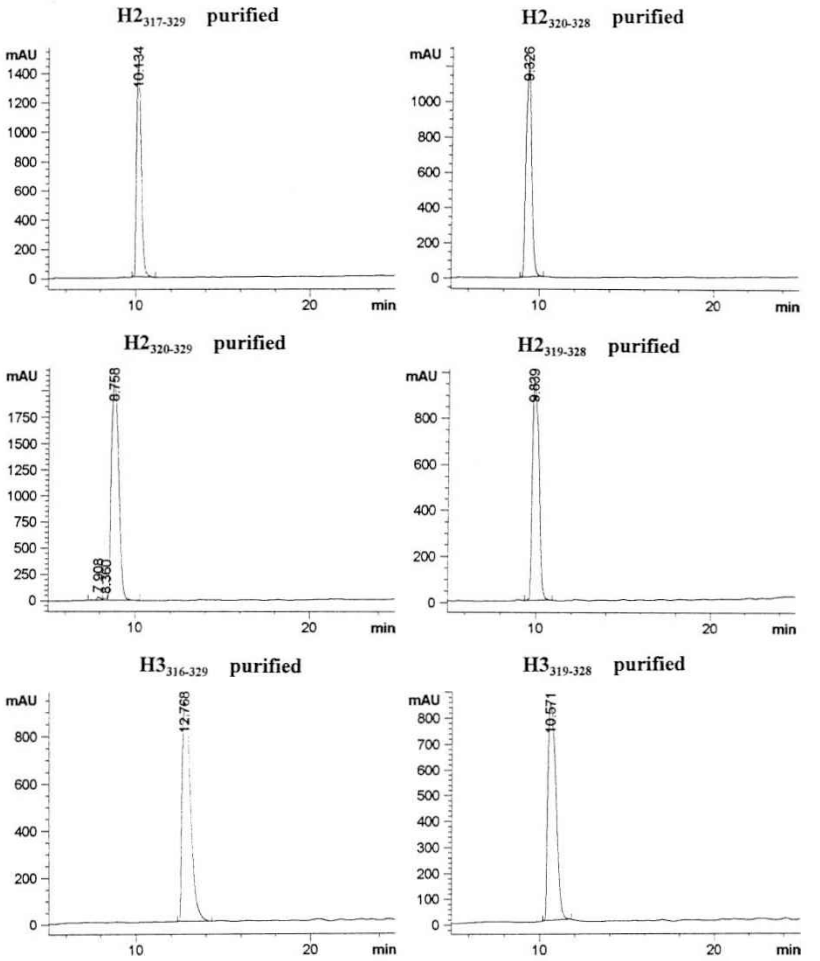



*Figure 20.* Analytical HPLC profiles of several peptide fragments from the haemagglutinin of H2 and H3 human influenza A virus serotypes.

HPLC conditions: Nucleosil 5 C18 column (4 X 250 mm); detection at 220 nm; eluent system, (A) 0.1% TFA in water, (B) 0.1% TFA and 80% acetonitrile in water; linear gradient from 5% B to 50% B in 25 min; flow rate 0.9 ml/min.

CD spectra showed that $H2_{317\text{-}329}$ and $H3_{316\text{-}329}$ peptides existed predominantly in α-helical conformation in TFE. This conformation is broken at $Pro^{324}$. Also a small amount of β-sheet and unordered conformation could be observed. In contrary, $H1_{317\text{-}329}$ occurs as a mixture of different conformers even in TFE as written earlier.

FT-IR spectroscopic data indicated the presence of β-turn in all three peptides.

Comparing these data to the results of the immunological investigations, it can be concluded that the $H2_{317\text{-}329}$ peptide, having the highest α-helix content on the basis of both CD and FT-IR spectra, was proved to be less potent to induce T cell response. The reason of it can be the following: in a water rich environment α-helicity doesn't play a central role in the recognition of T cell epitopic peptides representing the intersubunit region of the influenza virus haemagglutinin.

Conformational and immunological investigations of the other peptides are in progress.

# MATERIALS AND METHODS

## Chemicals

Dichloromethane (DCM), dimethylformamide (DMF), diisopropylethylamine (DIEA), methanol (MeOH), trifluoroacetic acid (TFA) and HPLC-grade acetonitrile (ACN) were purchased from E. Merck (Darmstadt, Germany) and were used without further purification. Fluka (Bucks, Switzerland) supplied puriss-grade dicyclohexylcarbodiimide (DCC). Protected amino acid derivatives were from Bachem Inc. (Torrance, CA, USA). Aldrich (Steinheim, Germany) supplied 1-hydroxybenzotriazole (HOBt), piperidine, p-cresol and dimethyl sulphide. Hydrogen fluoride was obtained from UCAR (Olen, Belgium).

## Solid-phase peptide synthesis using Boc-chemistry

The peptides were synthesized on *p*-methylbenzhydrylamine (MBHA) resin. Reactive side chains of the amino acids were protected. The δ-guanidine function of Arg was masked by the tosyl group. Cyclohexyl side protection was used for the β and γ carboxyl groups of Asp and Glu, benzyl for the hydroxyl groups of Ser and Thr. The sulphydryl group of Cys was protected by the *p*-methylbenzyl, the ε-amino group of Lys by the 2-chlorobenzyloxycarbonyl and the phenolic hydroxyl group of Tyr by the 2-bromobenzyloxycarbonyl groups.

The syntheses were carried out manually. The reaction vessel used for manual synthesis was fitted at its end with a sintered glass, protected by a screw-cap and mounted on a shaker. Couplings were performed with DCC, expect Asn, Gln and Arg, which were introduced *via* preformed HOBt esters.

Amino acid incorporation was monitored by qualitative ninhydrin test [63]. In case of positive test, so incomplete coupling , this acylation step was repeated.

The completed peptide resins were treated with liquid hydrogen fluoride/ dimethyl sulphide/ anisole/ *p*-cresol/ *p*-thiocresol (100:8:2:2:2, vol/vol), at 0°C for 45 minutes. HF was removed and the free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized.

**Solid-phase peptide synthesis using Fmoc-chemistry**

The peptides were synthesized on *p*-alkoxybenzylalcohol resin. The side-chain protecting groups were the following: *tert*-butyl ester for the ω carboxyl group of aspartic and glutamic acid, trityl for the sulphydryl group of cysteine, *tert*-butyloxycarbonyl for the ε amino group of lysine, and *tert*-butyl ether for the hydroxy groups of serine and threonine.

The syntheses were carried out manually. The amino acids were incorporated *via* preformed HOBt esters, with DCC condensation. Coupling of the C-terminal amino acid was repeated once. Following the second coupling step the substitution level of the resin was determined by the Gisin test [64]. The solvent was DMF in all cases. Amino acid incorporation was monitored with the ninhydrine test. If the coupling was not complete, this step was repeated.

The completed peptide resins were treated with 95% trifluoroacetic acid for 2.5 hours. In case of cysteine containing peptides 2.5% dithiothreitol was also added to it in order to prevent oxidation of the free sulphydryl groups. TFA was removed in vacuum and the free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized.

**Synthesis of phospho-tyrosine containing peptides**

The peptides were synthesized with Fmoc-chemistry. The peptide chains were elongated just until the tyrosine residue. This amino acid was incorporated without side-chain protection. A large excess (10 to 20 equivalent) of di-*tert*-butyl-N,N-diethylphosphoramidite and 1H-tetrazole were used for phosphitylation in abs. tetrahydrofurane. Following it the phosphite was oxidized with 14% aqueous solution of *tert*-butylhydroperoxide. Then the N-terminal part of the peptide was synthesized. The N-terminal Fmoc-group was removed with 20% piperidine/DMF. The peptide was cleaved from the resin, solubilized and lyophilized as it was written in the case of the Fmoc-synthesis.

**Synthesis of multiple antigenic peptides**

*Synthesis of MAP-1*

It was synthesized on MBHA resin, using Boc-chemistry. In the first three coupling steps

Boc-Lys-OH was incorporated to the peptide. After removing the *tert*-butyloxycarbonyl group in the third step, 8 free amino groups were available on the polymer. The synthesis of $CD3\zeta_{17-33}$ was carried out on this resin.

*Synthesis of the other MAP-s*

These MAP-s were also synthesized on MBHA resin, using Boc-chemistry. First FP3 was synthesized on the polymer. In the next two steps Boc-Lys-OH was incorporated to the peptide. Removing the Boc-group at the end of the second step, 4 free amino groups were available on the polymer. The synthesis of the appropriate peptide was carried out on this resin.

**Purification and characterization of the peptides**

The crude peptides were purified by semi-preparative reverse-phase High Performance Liquid Chromatography (RP-HPLC). The HPLC apparatus was produced by Knauer (Berlin, Germany). The column was a Lichrosorb RP-18 10μm column (16 X 250 mm). The solvent system was the following: A: 0.1% TFA in water; B: 80% ACN, 0.1% TFA in water. The gradient was as follows: 0-60% B in 120 minutes, the flow rate was 3 ml/min. UV detection was used at 220 nm. The appropriate fractions were collected and lyophilized. Purity was checked by analytical RP-HPLC on a Vydac $C_{18}$ (4 X 250 mm; Hesperia, CA, USA), a Nucleosil RP-18 5μm (4 X 250mm) or a Lichrosorb-100 RP-18 (4 X 250 mm) column. Flow rate was either 0.8 ml/min or 1 ml/min.

Amino acid analysis or mass spectrometry was used for the characterization of the peptides. The mass spectra were recorded on a VG ZAB SE FAB mass spectrometer or on a Finnigan TSQ 7000 tandem quadrupole mass spectrometer equipped with an electrospray ion source.

**Conformational investigations**

CD measurements were carried out on a Jasco J720 dichrograph at room temperature. Double-distilled water and NMR-grade trifluoroethanol (TFE) were used as solvents. IR spectra were recorded on a Bruker IFS55 instrument. Aqueous peptide solutions were prepared in $D_2O$ and in trifluoroethanol. Solid-state spectra were recorded from KBr discs.

# EXPERIMENTAL SESSION

**Synthesis of the peptides from the TCR/CD3 ζ subunit and the influenza virus haemagglutinin**

The peptides were synthesized on MBHA resin (0,5 mmol), using Boc-chemistry. The syntheses were carried out manually. Before the synthesis the polymer was neutralized with 10% TEA in DCM, then it was washed 3 times with 10 mL of DCM. Three- or fourfold molar excess of DCC and protected amino acids were used for coupling. In case of Arg, Asn and Gln also 3 or 4 eqv. of HOBt was added to the mixture. The solvent was either DCM or DMF depending on the solubility of the protected amino acids. Amino acid incorporation was checked by qualitative ninhydrin test. If the test showed complete coupling, the Boc-group was cleaved by 50% TFA in DCM (5 and 25 min). The completed resins were treated with liquid HF. (The scavengers were written in the previous session.) The resulting free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized.

**Synthesis of the phospho-peptides**

The peptides were synthesized on *p*-alkoxybenzylalcohol resin, using Fmoc-chemistry. The syntheses were carried out manually. Coupling of the C-terminal amino acid was repeated twice. Fourfold molar excess of DCC, HOBt and protected amino acid was used in the presence of 0.2 equivalent of a tertiary base (N,N-dimethylaminopyridine). After removing the Fmoc-group the substitution level of the polymer was determined by the Gisin test. The peptides were synthesized on 0.5 mmol-s of these pre-loaded resins. Three- or fourfold molar excess of DCC, HOBt and protected amino acids were used for coupling in DMF. Amino acid incorporation was checked by the ninhydrin test. In case of complete coupling the Fmoc-group was cleaved by 20% piperidine in DMF (5 and 15 min.). Then the resin was washed. The syntheses were continued until the tyrosine residue. The polymers were washed with abs. THF. A large excess (16-20 equivalent) of di-*tert*-butyl-N,N-diethylphosphoramidite and 1H-tetrazole was used for the

phosphitylation. In 16 hours the resin was washed with abs. THF and then filtered. The phosphite group was oxidized with 14% *tert*-butylhydroperoxide for 2.5 hours. Following it the N-terminal part of the peptide was synthesized. After removing the N-terminal Fmoc-group the completed polymers were treated with 95% TFA in water. In case of cysteine-containing peptides also 2.5% of DTT was added to the mixture. The free peptides were solubilized in 10% acetic acid, filtered and lyophilized.

**Dimerization of CD3$\zeta_{22\text{-}32}$ and CD3$\zeta_{17\text{-}33}$**

The peptides were dissolved in pH 7.5 phosphate buffer (0.15M). The solutions were stirred at room temperature for 8 hours, then dialyzed against distilled water at 0°C overnight. The dimerized peptides were lyophilized.

**Phosphorylation in liquid phase**

*Preparation of N-α-Boc protected 61nP*

61nP was synthesized by solid-phase procedure, using Boc-chemistry. Crude 61nP (0.1 g; 0.13 mmol) was dissolved in a mixture of 3 mL of dioxane and 3 mL of water. Then 0.033 mL (0.14 mmol; 1.1 equiv.) of di-*tert*-butyl dicarbonate was added to it and adjusted to pH 8-9 by 2M sodium hydroxide solution. The mixture was stirred at room temperature for 6 hours, then it was diluted with distilled water and lyophilized.

*Phosphorylation of N-α-Boc protected 61nP*

Crude N-α-Boc protected 61nP (0.1 g; 0.12 mmol) was dissolved in 8 mL abs. tetrahydrofurane. Then 0.5 mL (1.8 mmol; 15 eqv.) of di-*tert*-butyl-N,N-diethylphosphoramidite and 0.126g (1.8 mmol; 15 equiv.) of 1H-tetrazole were added to it. The reaction mixture was stirred at room temperature for 18 hours. Then the solvent was removed in vacuum, the crude phosphopeptide was solubilized and purified by semi-preparative RP-HPLC.

solid, which was dried at 45-50°C and reduced pressure.

IR (KBr): $\nu_{max}$ 1240, 3240 $cm^{-1}$.

*4-(2',4'-Dimethoxyphenylhydroxymethyl)phenoxymethyl-polystyrene*

Equivalent amount of the prepared potassium salt in DMF was added to a suspension of chloromethylpolystyrene in DMF. The suspension was stirred at room temperature for 72 h. The resin was then washed in turn with DMF, MeOH, $H_2O$, MeOH, $CH_2Cl_2$ and MeOH., and dried.

*Determination of the degree of substitution of the resin*

A mixture of an Fmoc-amino acid (1.0 mmol), HOBt (1.0 mmol), DCC (1.0 mmol) and 4-dimethylaminopyridine (0.2 mmol) in DMF was added to 0.41 g of the resin in DMF. The suspension was shaken at room temperature for 4 h, and the resin was washed in turn with DMF, MeOH and DMF. The coupling was repeated with half quantities of the reagents. After removal of the Fmoc group with 20% piperidine in DMF for 5 and 15 min, the degree of substitution of the resin was determined by the method of Gisin [64].

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## SUMMARY

In order to investigate the role of TCR/CD3 complex ζ subunit in signal transduction two peptides and their dimers were synthesized from its extracellular region. Antibodies, raised against their bovine serum albumin and thyreoglobuline conjugates, didn't recognize the native ζ chain. Because of this problem the multiple antigenic peptide method was tried out. Using these peptides an octameric and a tetrameric MAP were synthesized. An influenza-derived fusion peptide (FP3) was built into the tetrameric MAP as a T cell epitope and a hydrophobic region that can stabilize the conformation of the N-terminal peptide. This kind of construction was not published earlier. Monoclonal antibodies against these multiple antigenic peptides showed high reactivity with and specificity against the corresponding ζ-peptides.

Three other tetrameric multiple antigenic peptides combining with FP3 were synthesized from the $p56^{lck}$, $p59^{fyn}$ and ZAP 70 kinases. The antisera raised against them recognized the immunizing MAPs and in the case of $p56^{lck}$ and ZAP 70 also the native protein chain.

Seven short peptides and the phosphorylated derivatives were synthesized for the investigation of tyrosine residues of TCR/CD3 ζ subunit. For the preparation of phosphate esters the phosphoramidite method was successfully used. We were the first who applied this method in liquid phase. It could be successfully used for the phosphorylation of 61nP. Preparation of di-*tert*-butyl-N,N-diethylphosphoramidite could be carried out with a good yield. Both CD and FT-IR investigations showed that the phosphorylation changed the conformation of the peptides: an increase in β-sheet conformation could be observed.

Three tyrosine kinases ($p56^{lck}$, $p59^{fyn}$ and ZAP 70) were used in immunological investigations of these peptides. The two src kinases phosphorylated all the six peptides, in which the tyrosine could be found in the conservative YL....YL motif. The syk family member ZAP 70 was not able to phosphorylate any of the peptides. Dephosphorylation of the phosphopeptides by CD45 proved that this tyrosine phosphatase could be responsible for dephosphorylation of the ζ-chain, so it could have role in down-regulation of T cell activation.

A new, efficient, reproducible method was worked out for the preparation of the acid-labile 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxymethylpolystyrene. A convenient level of substitution could be achieved that is suitable for the synthesis of any peptides in solid phase, using Fmoc-chemistry.

In order to investigate the effect of post-translational enzymatic cleavage, peptides were synthesized from the intersubunit region of the influenza virus haemagglutinin. A 25mer peptide and its D-$Arg^{329}$ analog represented the intact intersubunit region of the HA. Three peptides were prepared from the N-terminal part of the HA2 and seven fragments from the C-terminal region of the HA1 subunit.

Immunological investigations showed the presence of both B and T cell epitopes in the C-terminal region of the HA1. It was demonstrated that covalently linked 25mer peptide is necessary for the antibody recognition. There was no difference between the T cell recognition of the HA1 derived peptides in the presence and in the lack of $Arg^{329}$. It is in good correlation with the fact that this arginine is eliminated under physiological conditions. Using shorter analogs the core region of the T cell epitope was localized on the 317-328 fragment of the HA1 subunit.

CD and FT-IR spectroscopies demonstrated that both HA1 and HA2 parts of the cleaved haemagglutinin are capable for adopting multiple conformations. It can be the reason of the fact that they are capable for both B and T cell recognition.

Two tetrameric MAPs were synthesized using $H1_{317\text{-}329}$ peptide. In one of these also FP3 was built into the construction. In protection experiments 40% of MAP-6 and 60% of MAP-7 pre-immunized mice acquired complete protection against a lethal dose of influenza virus. CD and FT-IR investigations showed low α-helix content and a β-turn-like conformation.

Three analogs of the HA1 derived peptides were synthesized from both the H2 and H3 human influenza A virus serotypes. CD and FT-IR spectra showed predominantly α-helical conformation. (In contrary, the H1 analog occurs as a mixture of different conformers.) The H2 peptides, that had the highest α-helix content, proved to be less potent to induce T cell response.

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