Posters: topic C14

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Peptidomimetics

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NEW PROCTOLIN ANALOGUES: SYNTHESIS AND BIOLOGICAL EVALUATION IN INSECTS

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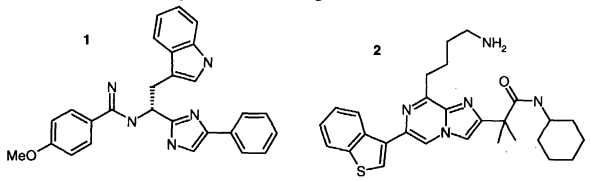
To explain the role of Tyr^2 and Thr^5 residues of proctolin (Arg-Tyr-Leu-Pro-Thr) chain in the myotropic activity of this insect neuropeptide we synthesized three grouop of its analogues: 1/ Arg-X^2-Leu-Pro-Thr where X^2 = Phg (I), D-Phg (II), Phg(p-NO_2) (III), D-Phg(p-NO_2) (IV), Phg(p-NH_2) (V), D-Phg(p-NH_2) (VI), Phg(p-OH) (VII), D-Phg(p-OH) (VIII), Phg (p-N,N-di-Me) (IX), D- Phg (p-N,N-di-Me) (X) 2/ ArgTyr-Leu-Pro-X^5, where X^5 = Val (XI), D-Val (XII), Ile (XIII), D-Ile (XIV), Ala (XV), D-Ala(XVI), Ser(XVII), Asn (XVIII), Gln (XIX), Asp(XX), Glu(XXI), Arg (XXII), D-Arg (XXIII), Lys (XXIV) and Gly(XXV) and 3/ Arg-Tyr-Leu-Pro-NH(R'), where R' = isobutyl (XXVI), S-1-methy-1-phenylethyl-(XXVII), R-1-methyl-1-phenylethyl (XXVIII), R-2-hydroxyethyl (XXIX), S-2-hydroxyethyl (XXX), R-2-hydroxypropyl (XXXI), S-2-hydroxypropyl (XXXII), 3-hydroxy-propyl (XXXIII). Synthesis all peptides were carried out by solid-phase method. All peptides were bioassayed *in vitro* on the semi-isolated hearts of *Tenebrio molitor* using a cardioexcitatory test and on the foregut of locust *(Schistocerca gregaria)*. Among the analogues I - XXXIII studies for cardiotropic activity 7 peptides (XI, XIII, XV, XVIII, XXI, XXII and XXIV) incerased heartbeat frequency in *T. molitor*. These peptides shoved 20-50% activity of proctolin at the 10^{-9} – 10^{-7} molar range of concentration. In myotropic test performed in foregut of *S. gregaria*, peptides XI, XIII and XIX retained 50-70% proctolin activity.
Basing on these data, the structure function relationship of proctolin in insect was discussed.

THE DISCOVERY OF NON PEPTIDE SOMATOSTATIN AGONISTS FROM FOCUSED LIBRARIES BASED ON ELABORATION OF 2-AMINOMETHYL IMIDAZOLES

Christophe Thurieau[1], Marie-Odile Contour-Galcéra[1], Lydie Poitout[1], Christophe Moinet[1], Barry Morgan[2], Tom Gordon[2], and Pierre Roubert[1]; [1]Institut Henri Beaufour, Les Ulis France, and [2]Biomeasure Inc., Milford MA USA.

Somatostatin (SRIF) is a widely distributed peptide that exhibits multiple biological functions such as inhibition of the release of growth hormone, insulin, glucagon and gastrin, presumably through interaction with five G-protein coupled somatostatin receptors ($hSSTR_{1-5}$). Peptide analogs of SRIF, such as BIM 23014 (Lanreotide), have been synthesized and developed for clinical use. Considerable efforts have been devoted to the development of peptidomimetics of SRIF, which would have the potential for oral bioavailability. We wish to report the design, synthesis and biological evaluation of new potent ligands for the sst_3 and the sst_5 receptor.
These ligands were discovered from focused libraries derived from imidazole derivatives which we hypothesize might mimic the topography of critical structural features of potent peptide analogs of somatostatin. The libraries were constructed by a solution-phase parallel-synthesis strategy using resin scavenger methodologies. These methods allowed rapid synthesis of thousands of discrete molecular entities in good to excellent purity.
The synthesis and structure activity relationships of several series will be described, including an imidazole-amidines series (e.g. **1**) of selective ligands at $hSSTR_3$,and an imidazopyrazine series (e.g. **2**) of selective agonists at $hSSTR_5$.



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α-KETOAMIDE SYNTHESIS AND REACTIVITY FOR A NEW PSEUDOPEPTIDE FAMILY

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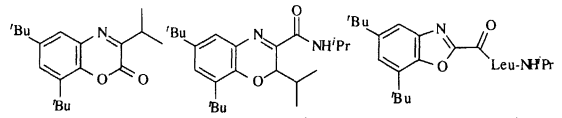
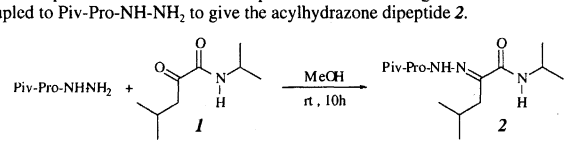
When coupled to α-aminohydrazides, α-ketoamides give rise to a new pseudopeptide family in which the amide bond is replaced by the CO-NH-N=C moiety. In a first step, we tried to synthesize α-ketoamides by Corey's reaction using 3,5-di-*tert*-butyl-1,2-benzoquinone as an oxidizing agent for amines, but we obtained several cyclic derivatives when applied to α-amino esters or amides, or to the Gly residue.



from H-Leu-OMe from H-Leu-NH^{i}Pr from H-Gly-Leu-NH^{i}Pr

In order to circumvent these drawbacks, we have used successfully 2,6-di-*tert*-butyl-1,4-benzoquinone and obtained the expected Leu keto-analogue ***1*** which was further coupled to Piv-Pro-NH-NH_2 to give the acylhydrazone dipeptide ***2***.



The structural properties induced by the acylhydrazone fragment have been studied in solution by ^{1}H-NMR and IR spectroscopy, and in the solid state by X-ray diffraction. It assumes a rigid planar conformation in which the NH interacts with the imine nitrogen to close a 5-membered cycle. The synthesis and structural analysis of various acylhydrazone pseudopeptides are in progress.

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SELECTIVITY OF AZAPEPTIDES AS CYSTEINE PROTEASES INHIBITORS

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In last years in our laboratory we were interested in the syntheses of selective inhibitors of cysteine proteases. Among them were azapeptides in which the α-CH group of an appropriate amino acid residue in the peptide chain was replaced by a nitrogen atom. Azapeptides fulfil all requirements for inhibition of proteases. Presumably, the azapeptide acylates the enzyme, but this intermediate is more resistant to hydrolysis for electronic and steric reasons, than the normal acyl – enzyme intermediate. The carbonyl group of the azapeptide is deactivated toward nucleophilic attack by the adjacent aza group, and a leaving group can be incorporated which interacts with the leaving group binding site.
Therefore we have synthesized some potential inhibitors of cysteine proteases that contain the azaglycine, azaphenylalanine and azatyrosine residues (Agly, Aphe, Atyr, respectively) instead of the appropriate amino acid residues. The sequences of some of the azapeptides were based on the structure of the N-terminal binding segment of cystatins – natural inhibitors of cysteine proteases. They had the azaglycine residue in a place of the expected hydrolysis. The azapeptide bond was formed by means of the carbonyldiimidazole.
For these substances the inactivation constants towards papain and cathepsins B and K have been determined. Azamodification of one of these proteases substrates: Z-Arg-Leu-Val-Gly-Ile-Val-OMe, proved to be very selective inhibitor for cathepsin B, whereas the other azapeptides were more selective either to cathepsin K or papain. In our communication we are going to present some synthetic aspects of azaamino acids and azapeptides, as well as to discuss structure – activity relationships of azainhibitors.

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TARGETING *CIS/TRANS* CONFORMATIONAL CHANGES IN PROTEINS USING PSEUDOPROLINE-CONTAINING PROTEIN MIMETICS

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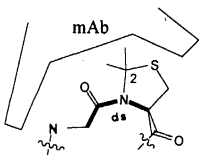
Mimicking bioactive conformations by synthetic surrogates is a helpful approach for understanding conformational effects during biological processes. C2-dimethylated thiazolidine and oxazolidine derivatives (pseudoprolines) are presented as new *cis*-proline mimics [1,2]. By introduction of 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid (Cys($\Psi^{Me,Me}$pro)) into a sequence derived from the V3 loop of the HIV-1 gp120 surface protein, the recently proposed infection-active loop tip conformation involving a Xaa-Pro *cis*-peptide bond is induced. The *cis*-constrained V3 loop analogue is used as immunogen for the preparation of a monoclonal antibody (mAb) (Figure). Experimental data obtained with the cyclic and linear, *cis*-constrained and natural V3-sequences show a correlation between the *cis*-content determined by one- and two-dimensional NMR spectroscopy and the recognition by the mAb, demonstrating that the mAb is selectively distinguishing between the *cis* and the *trans* conformation of Xaa-Pro imide bonds. The potential use of the *cis*-directed mAb for the detection of conformational changes during the HIV-1 infection process as well as a diagnistic tool for targeting *cis*-amide bonds in bioactive peptides and proteins will be discussed.



[1] Dumy, P.; Keller, M.; Ryan, D. E.; Rohwedder, B.; Wöhr, T.; Mutter, M. *J. Am. Chem. Soc.* **1997**, *119*, 918-925.

[2] Wittelsberger, A; Keller, M; Scarpellino, L; Patiny, L; Acha-Orbea, H; Mutter, M. *Angew.Chem. Int. Ed. Engl.* **2000**, 39, 6, *in press*.

CYCLOLINOPEPTIDE A ANALOGUES CONTAINING N-BENZYLGLICYNE AS A PEPTOID ELEMENT.

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Cyclolinopeptide A [cyclo(Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-), CLA], natural cyclic nonapeptide possesses a strong immunosuppresive activity comparable with that of Cyclosporin A. It has been postulated (*Angew. Chem. Int. Ed. Eng.*,**1986**, 25, 997) that the Pro-Pro-Phe-Phe segment of CLA is important for its biological activity. In this communication we will report our further results related to the modification of this particular fragment of CLA molecule. The synthesis of six linear and cyclic CLA analogues containing N-benzylglicyne (BzlGly) in position 8 and 9 has been achieved using standard SPPS technique.

1. Leu-Ile-Ile-Leu-Val-Pro-Pro-BzlGly-Phe
2. Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-BzlGly
3. Leu-Ile-Ile-Leu-Val-Pro-Pro-BzlGly-BzlGly
4. cyclo(Leu-Ile-Ile-Leu-Val-Pro-Pro-BzlGly-Phe-)
5. cyclo(Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-BzlGly-)
6. cyclo(Leu-Ile-Ile-Leu-Val-Pro-Pro-BzlGly-BzlGly-)

The cyclization of linear precursors has been made in solution using TBTU and/or HATU as a coupling reagents. The structure of all peptides has been confirmed by FAB-MS. The immunomodulating activity of all synthesised peptide as well as conformational search using molecular mechanics will be evaluated.

This work was supported by Polish State Committee for Scientific Research (KBN), grant 4 P05F 004 13.

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[AZPRO^3]-TRH: IMPACT OF AZAPROLINE ON *CIS-TRANS* ISOMERISM.

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The β-turn is a well-studied motif in both proteins and peptides. Four residues, making almost a complete 180° turn in the direction of the peptide chain defines the β-turn. Several types of the β-turn are defined according to the Φ and Ψ backbone torsional angles for residue i+1 and i+2. The type VI β-turn, usually contains a proline with a *cis* amide bond at residue i+2. In an aza-amino acid, the alpha carbon is changed to a nitrogen. Aza-peptides have been shown to prefer the type VI β-turn in crystals and by NMR in organic solvents. MC/MD simulations using the GB/SA solvation model for water were used to explore the conformational preferences of aza-proline(azPro)- containing peptides. An increase in the conformational preference for the *cis*-amide of azPro was clearly seen, but the increased stability was relatively minor compared to previous suggestions from the experimental data. In order to test the validity of the calculations in view of the experimental data from crystal structures and NMR in organic solvents, [azPro^3]TRH and [Phe^2, azPro^3]TRH were synthesized by novel synthetic routes due to the relative lack of reactivity of azaproline. The conformational preference of these TRH analogs was examined by NMR in water and methanol. In methanol at –10°, the signals from the *cis*- and *trans*-conformers were resolved and could be clearly assigned. Their relative percentages were determined to be nearly equal (38.5% *cis* to 51.5% *trans*). Thus, while the exchange of the carbon-α by a nitrogen enhances the propensity for the *cis*-conformer, the conformational preference of azPro appears to be environmentally sensitive. The strength of this constraint is, therefore, not sufficient to hold the amide in the *cis*-conformer. Other constraints such as the 1,5-tetrazole for amide bond or bicyclic analogs may be more appropriate to probe recognition of the *cis*-amide bond conformer by the receptor. The affinity of [azPro^3]TRH for the TRH receptor was reduced 35-fold compared with TRH itself, consistent with the lack of activity seen in general for analogs with *cis*-amide constraints (Supported in part by NIH grant GM53630).

DETERMINATION OF T AND B CELL EPITOPES OF A MAJOR ALLERGEN OF *ASPERGILLUS FUMIGATUS* FUNGUS *ASP F2* IMPLICATED IN ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS (ABPA).

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ABPA is a severe form of allergy to *Aspergillus fumigatus* proteins including *Asp f1, f2, f3, f5, f6, f12* and some others. The major allergen *Asp f2* is involved in ABPA and not in less severe forms of hypersensitivity. The induction of a protective immune response or the state of tolerance to *Asp f2* could be a way to treat ABPA. Having in mind this remote purpose, we have studied T and B cell epitopes of *Asp f2*. BALB/c (H-2^d) mice were immunized twice with *Asp f2* with a month interval between the immunizations. IgG1 and $IgG2_a$ B cell epitopes were tested by ELISA using 14 individual peptides spanning 66% of the *Asp f2* molecule as a coating agent. To determine T cell epitopes T-cell hybrids were produced by fusion of *in vitro* restimulated lymph nodes (LN) cells with TCR^- $CD3^-$ BW5147 partner cells. T hybrids were selected by high level of T-cell receptor (TCR) expression and tested for antigen specificity. The later was estimated by IL-2 release when T hybrids were stimulated with *Asp f2* in the presence of antigen presenting cells. To characterize the type of T helper immune response the production of IL-2, IL-4 and INF-γ by immune splenocytes was analyzed by reverse-transcription polymerase chain reaction (RT-PCR). We have found that this route of immunization induced Th2 immune response with a regulatory subset of Th1, which was concluded from both the results of RT-PCR and $IgG1/IgG2_a$ analysis. RT-PCR demonstrated the presence of high level of IL-4 mRNA and a moderate levels of IL-2 and INF-γ. We have found both *Asp f2* specific IgG1 and $IgG2_a$ antibodies. To our surprise the only dominant epitope of *Asp f2* recognized by IgG1 was the C terminus peptide spanning the 296-310 amino acid sequence. Peptides spanning the 155-169 and 182-196 sequences recognized the $IgG2_a$ antibodies. Out of 107 T hybrids 23 % recognized the 277-291 peptide and 13% recognized peptides 89-100 and 19-33.

SYNTHETIC LONG PEPTIDE USED IN THE ELABORATION OF A VACCINE AGAINST MALARIA

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We compared the immunogenicity of two preparations of a peptide derived from the *Plasmodium berghei* circumsporozoite protein which is expressed on the surface of the preerythrocytic stage of the malaria parasite (*Pb*CS242-310). The peptide was synthesized using a Fmoc/tBu solid phase strategy with a HOBT/DIPC activation, cleaved with the Barany's emulsion, precipitated in ether, purified by RP-HPLC and folded. Two different preparations were used to immunize mice in combination with the soluble adjuvant QS21. Preparation A contained the purified folded peptide which was soluble in PBS/QS21 ; B contained the crude peptide filtrated on a size exclusion column and folded without further purification. This peptide formed insoluble aggregates which were dispersed in the adjuvant. Immunization with preparation B induced high titers of antibodies (Ab) against the peptides A and B and specific cytotoxic T-lymphocytes (CTL). However, the preparation A was poorly immunogenic. The challenge experiment performed with alive sporozoites confirmed these results : the suspension of peptide B gave higher protection than the soluble preparation. Consequently, the presence of aggregates appears to be necessary for the induction of a protective immune response. Therefore, the immunogenicity of the synthetic peptide may be improved by its ligation on particules or incorporation into microspheres.

COMPARATIVE STUDY OF PEPTIDE ANTIGENS AND POLYMER SURFACE INTERACTIONS. THE INFLUENCE ON SENSITIVITY AND SPECIFICITY IN SERODIAGNOSIS OF HCV AND HIV

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Synthetic peptides, representing the specific antigenic determinants possess the certain advantages as compared to natural antigen in the course of the detection of viral infections. However, the interaction with polymer surface may have appreciable influence on the efficiency of their application in ELISA test-systems.

By using HPLC technique we have studied the adsorption characteristics on polystyrene plates (MaxiSorp, Nalge Nunc International) of 6 synthetic peptides representing the antigenic regions from core, NS3 and NS4 proteins of the hepatitis C virus (HCV). It was shown that some of them have high affinity to polymer surface whereas one of two overlapping NS4 epitopes (20 amino acids) has poor adsorption properties. These results prompted us to synthesize the consensus NS4 epitope sequence (21 amino acids) which permits to improve the sensitivity of parent HCV ELISA test-system (Medical Centre «Avicenna»). The optimized peptide composition has been tested using standard control HCV sera (Boston Biomedical Incorporated).

The similar study of peptide - polymer surface interaction for the components of HIV-1/HIV-2 test-system (Medical Centre «Avicenna») was significantly more complicated. The great difference in solubility and other physico-chemical properties of peptides, representing immunodominant antigenic regions of gp41, gp36, gp120 and p24 (comprised 16-35 amino acids) permits us only to detect the poor adsorption of p24 epitope. Based on this result, we have tried to enhance the quantity of peptide bound to polystyrene surface by using its conjugate with carrier protein or Multiple Antigen Peptide System (MAPS) peptide. Simultaneously, the synthesis and study of properties for peptides belongs to others p24 antigenic regions permit us significantly improve the sensitivity and specificity of test-system.

These results illustrate the utility of peptide - polymer surface interaction study for the choice and optimisation of peptide composition in ELISA test-systems.

MEMBRANE AFFINITY AND CELLULAR UPTAKE OF A CHIMERIC PEPTIDE INDUCING CYTOTOXIC T LYMPHOCYTE RESPONSES IN SALINE

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There is considerable interest in the development of vaccine strategies that will enhance the generation of virus-specific CTL responses. We have studied the immunogenicity of a 9-mer peptide representing a cytotoxic T-cell epitope from the nucleoprotein of measles virus (NP6: residues 52-60). The presence of two copies of a T-helper epitope (T) resulted in the induction of strong CTL responses after administration of the TT-NP6 peptide in saline, whereas the presence of only one copy of the T-cell epitope (T-NP6) revealed a weak immunogenicity[1]. In addition to the presence of more T-cell help, the TT-NP6 peptide exhibited an α-helical conformation, whereas the T-NP6 peptide adopted a random coil conformation; the α-helical conformation being known to favor the interaction of peptide with membrane.

As part of a program to investigate a possible correlation between membrane affinity, cellular uptake and immunogenicity of soluble peptide containing a CTL-epitope, we have studied the putative interaction with membrane of the T-NP6 and the TT-NP6 peptides and their subsequent internalisation into living cells. Determination of partition coefficient from aqueous phase and lipids was done by circular dichroism and fluorescence in the presence of small unilamellar vesicules as mimicry of cell membranes. In the presence of living cells, membrane localisation and cellular uptake were followed by FACS and microspectrofluorometry with the peptides labeled with either 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD) or oregon green 514. The most immunogenic peptide, the TT-NP6, was better internalized than the T-NP6 peptide. In addition, the former one exhibited the highest affinity for model and plasma membrane. As the TT-NP6 peptide is self-associated under physiological conditions[2], we wonder whether its membrane affinity is not due to a more compact conformation allowing it to pack more densely at the surface of the membrane.

[1] Partidos C.D., Delmas A., and Steward M.W. (1996) Molec. Immunol. 33, 1223.
[2] Delmotte C.,Le Guern E., Trudelle Y. and Delmas A. (1999) Eur. J. Biochem. 265, 336.

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DESIGNING PEPTIDE PROBES FOR INTRAVITAL MICROSCOPY OF TUMOR CELL ADHESION MOLECULES

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Peptides which contained the sequence Asn-Gly-Arg (NGR motif) have recently been identified as a cell adhesion motif. Moreover, the tumor homing of such peptides is rather specific (Arap et al. (1998) Science 279, 377-380). Thus they may be suitable tools in tumor targeting for diagnostic and therapeutic purposes.

1 phrase pour situer microscopie intravitale et dire son intérêt

In order to localize tumor by specific binding of circulating ligands on blood vessel receptors, we have designed a peptidic molecule with NGR motif which can bind to fluorescent microspheres used in intravital microscopy analysis. The cyclic peptide CNGRC was chosen as the binding sequence, and avidin-biotin interaction used as linkage between peptide and fluorescent bead. To circumvent the steric bulk of the cyclic sequence and the avidin molecule in one hand, and the necessity of a free N terminus in the other hand, we added as a spacer a C terminal poly-Gly extension. A Lysine residue was also incorporated for additional spacer and allowing biotin covalent coupling via the ε functional amine group.
Various synthesis strategies were developed. Linear sequence was assembled using Fmoc solid-phase chemistry. Intramolecular disulfide bond formation was performed by polymer-supported procedure using mild oxydant agents, as potassium ferricyanide and DMSO. Biotinylation was carried out on resin at the end of the peptide assembly and cyclisation, or during peptide elongation, by use of Fmoc orthogonal protection compatible with acid labile-resin.
The different synthesis strategies and the preliminary biological data will be presented and discussed.

THE EMPLOYMENT OF RANDOM PHAGE-DISPLAYED PEPTIDE LIBRARIES FOR THE IDENTIFICATION OF ANTIGENS ASSOCIATED WITH AUTOIMMUNE DISEASES

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[a]Dept. of Neurology and [b]Laboratory of Human Biology and Genetics, Democritus Univ. of Thrace, School of Medicine, Alexandroupolis, Greece, [c]Dept. of Neurology, Univ. of Pennsylvania School of Medicine,Philadelphia,PA USA, [d]Dept. of Pharmacology Univ. of Wisconsin, USA, [e]Dept. of Pediatrics, Univ.of Pennsylvania School of Medicine and the Children's Hospital of Philadelphia,Philadelphia,PA USA, [f]Dept. of Pharmacy , Univ. of Patras ,Patras, Greece, [g]Dept. of Neurology and Microbiology, Univ. of Colorado Health Sciences Center, Denver,CO. USA.

The nature of antigens recognized by the G-Immunoglobulin (IgG) antibodies of the oligoclonal bands in the cerebrospinal fluid (CSF) of patients with subacute sclerosing panencephalitis (SSPE) or multiple sclerosis (MS) is unknown. It is believed that the immune response developed against these unknown antigens may be responsible for the demyelination process that leads to disease. IgGs derived either from the brain tissue of an SSPE or MS patient or from an oligoclonal band of an MS patient, were tested against phage peptide libraries for target peptides that would bind the particular IgGs. The peptide sequences identified as specific to the brain-eluted antibody of the SSPE patient suggested a particular motif. This motif was identified as YNPxDLLG. Among those proteins that were identified to satisfy the motif was the nucleocapsid protein of measles virus. This infection agent is known to cause SSPE. It is possible we have identified the sequence portion on the nucleocapsid protein implicated in the demyelinating activity. The specificity of IgGs isolated either from brain or CSF of the MS patient were identified. The brain eluted IgG was reactive to the VKxxNxL sequence motif, while the IgG isolated from CSF was reactive to a number of peptide sequences without forming a comprehensive motif. These sequences served as a basis to search the data bank for homologous peptides and proteins. Appears that a number of proteins from Herpes Virus I include homologies with the peptides recognized by both, the brain eluted IgG and the CSF-derived IgG. These data will be compared to those derived from several patients to identify relevant with the disease peptide sequences.

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HAPTENISATION OF OVALBUMIN T-CELL EPITOPE WITH THE SKIN SENSITIZER METHYL OCTANESULFONATE

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In the course of our studies on Allergic Contact Dermatitis (ACD) to lipophilic methylating agents, we have been interested in the haptenisation of ovalbumin (OVA) with the strong skin sensitizer methyl octanesulfonate. We have previously shown that Lys, Met and His residues are preferably methylated by the alkylsulfonate. One of our objectives is to study the effect of methylation on presentation and recognition of ovalbumin T-cell epitope OVA323-339 (ISQAVHAAHAEINEAGR) in which His^{328} and His^{331} are the two potential sites of methylation.

In this report, we describe the synthesis of the modified monomer N-α-Fmoc-N-τ-methyl-L-histidine and its incorporation in the solid phase synthesis of the three possible methylated analogues of $OVA_{323-339}$ that were needed as references.

We also report the identification of the methylated histidine residue in $OVA_{323-339}$ after haptenisation of native OVA with methyl octanesulfonate. The modified epitope was isolated by gel permeation and HPLC after CNBr cleavage and trypsic digestion. The site of methylation was determined using Edman sequencing and mass spectrometry. Our data show that $OVA_{323-339}$ was selectively methylated at His^{331}.

Secondary structure of synthetic $OVA_{323-339}$ methylated at His^{331} was studied by circular dichroism and compared to the non-methylated peptide.

SYNTHESIS OF A DIFLUOROMETHYL-PHOSPHOSERINE (F_2Pab)-CONTAINING PEPTIDES FOR GENERATING *SPECIFIC ANTIBODIES TO PHOSPHORYLATION SITES*

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Phosporylation of p53 is a mechanism for responding to signaling events iniziated by DNA damage or other stresses. To study how multiple phosphorylation sites control p53 activity, polyclonal antibodies have been developed that are capable of detecting specific phosphorylation sites. However, several attempts to generate an antibody to Ser6 of human p53 using phosphoserine-containing peptides as immunogens were unsuccesfully. Recently, we used F_2Pab as a phosphoserine mimetic and incorporated it as the Fmoc-F_2Pab-OH derivative in the solid-phase synthesis of the p53 peptide Ac-1-12(6F_2Pab)Cys (Ac-MEEPQ(F_2Pab)DPSVEPC). Synthesis of Fmoc-F_2Pab-OH was achieved by an improved procedure based on Otaka's (*Tetrahedron Lett.* 1995, 36, 927) and Berkowitz's (*J. Org. Chem.* 1996, 61, 4666) methods. The peptide purified by HPLC was coupled to keyhole limpet hemocyanin (KLH) and antibodies were made by immunizing rabbits. Phosphoserine 6-specific antibodies were affinity purified using Sulfolink-conjugates of the phosphorylated and unphosphorylated peptides and were tested by ELISA and immunoblot assays. They recognized p53(1-39) peptide phosphorylated at Ser6 alone, but did not recognize the unphosphorylated peptide or the same peptide phosphorylated at Ser9, 15, 20, 33 and 37. Using these immunopurified antibodies, we showed that exposure of cells to either ionizing radiation or UV light increased phosphorylation at Ser6 2- to 12-fold over the low, constitutive background of phosphorylation seen in undamaged cells treated with a proteosome inhibitor to stabilize p53. Our data indicate that the F_2Pab-derivatized peptides are stable, effective mimics of protein phosphorylation sites that can be used to develop site specific polyclonal and potentially monoclonal antibodies.

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CARRIER EFFECT ON BIODISTRIBUTION OF COVALENTLY ATTACHED ANTIBODY EPITOPE PEPTIDE FROM MUCIN 1

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Mucin 1 glycoprotein (MUC1) contains a polypeptide core composed of variable number (30-100) repeats of a 20 amino acid sequence, APDTRPAPGSTAPPAHGVTS. Majority of MUC1 specific monoclonal antibodies react with protein core epitopes of 3-5 amino acids within the hydrophilic region APDTRPAP of the repeat unit. Recently it has been also demonstrated that peptide APDTRPAPG (AG) is capable to induce MUC1 specific CTL responses in carcinoma patients. Based on these findings the aim of this study was to design and synthesise new group of peptide antigen conjugates containing multiple copies of peptide AG attached covalently to branched chain polypeptides with different charge properties (1). First the sequence of AG was elongated by an N-terminal Cys residue and the thiol group was utilised for conjugation with SPDP modified side chains of carrier polypeptides with the general formula poly[Lys-(X_l-DL-Ala_m] where X = 0 (AK), Glu (EAK) or Leu (LAK) or with poly-lysine. Polypeptides was modified with prelabelled ^{125}I using N-succinimidyl 3-(4-hydroxy-phenyl)propionate. After gel filtration BALB/c mice were injected intra-venously via a tail vein with labelled polypeptides. Serial blood samples were taken from the tail tip at 1, 10 and 30 min and at 1, 2, 3 and 4 h after injection. Blood clearance curves were constructed. At 4 h mice were killed, and weighed samples of blood, visceral organs, and residual carcass were assayed for radioactivity (2). Results of the tissue-distribution analysis showed that the charge properties of the carrier polypeptide have a marked influence on the blood clearance profile and on the tissue accumulation of the epitope peptide conjugates.

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INVESTIGATION OF THE ANTIGENIC STRUCTURE OF TREPONEMA PALLIDUM'S MEMBRANE PROTEIN TPP17

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Usage of recombinant antigens in enzyme linked immunosorbent assays (ELISAs) for detection of anti-Treponema pallidum-antibodies often leads to false positive results because of their cross reactivity. Development of immunosorbents based on synthetic peptides that have higher antigenic specificity, allows to overcome this problem.
We have carried out an investigation of antigenic structure of membrane lipoprotein Tpp17 (156 aa), one of major antigens of Treponema pallidum. For this purpose overlapping peptides have been synthesized and their immunochemical reactivity has been studied on a panel of sera from syphilis patients.
Peptides 26-46, 102-121, 126-156, 138-156 were synthesized on PAM resins, using "fast-Boc" technology. Peptide 34-93 was accomplished by the method of convergent synthesis of full-protected fragments. Fragments 34-46, 47-51, 52-60, 61-71 were synthesized on 2-chlorotrityl resin, using Fmoc-chemistry. The peptides were cleaved from the polymers by treatment with mixture of acetic acid : threefluoroethanol : dichloromethane (AcOH:TFE:DCM = 2:2:6), purified by flash-chromatography and introduced in reaction with fragment 72-93 as corresponding 1-hydroxybenzotriazol-esters.
Study of immunoreactivity of the products demonstrated that regions 26-46 and 138-156 take part in major antigenic determinants of Tpp17 and synthetic peptides homologous to these regions can be used as antigens.

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MULTIPLE ANCHORING OF MYELOPEPTIDES ON SEQUENTIAL OLIGOPEPTIDE CARRIERS (SOC_n) : SYNTHESIS, CONFORMATION AND STUDIES IN HUMAN LEUKEMIA CELLS.

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Myelopeptides (MP_s) are a new class of small bioactive peptides of bone marrow origin with definite immunoregulatory activity that differs from one to the other. MP-6 (Val-Asp-Pro-Pro) and MP-4 (Phe-Arg-Pro-Arg-Ile-Met-Thr-Pro) display differentiating effect, inducing terminal differentiation in human leukemia cell line HL-60. These peptides were coupled to the Sequential Oligopeptide Carrier (Lys-Aib-Gly)$_n$, SOC_n , separately, in four copies, from the Lys-$N^{\varepsilon}H_2$ groups. The differentiating activity (decrease of DNA synthesis and increase of protein synthesis simultaneously) of MP-4, free and bound to the (Lys-Aib-Gly)$_4$, in human leukemia cell line HL-60 is in progress for determining the character of the dose dependences for MP-4 and (MP-4)$_4$-SOC_4 in the metabolic changes. Preliminary experiments indicated that the differentiation effect of (MP-4)$_4$-SOC_4 is increasing in accordance to the enhancement of the (MP-4)$_4$-SOC_4 concentration (up to 25 μg/ml). ^{1}HNMR studies have shown that the helicoidal structure of SOC_n (3_{10}-helix) , which contributes to the reduction of steric hindrances and conformational restrictions of the carrier, is not affected by the covalent attachment of the myelopeptides to the Lys-$N^{\varepsilon}H_2$ groups, resulting thus to a favorable disposition of the myelopeptides in the biological process. Further conformational studies will give us information on the structural requirement of MP_s for obtaining the most bioactive derivatives. The indepth study of the structure – biological activity relationships of MP-4, MP-6 and their SOC_n analogs will offer new alternatives in the leukemia therapy and the antileukemia medicines.

PEPTIDE SCANNING IN ANTIGENIC AND FUNCTIONAL MAPPING OF MONOOXYGENASE SYSTEM PROTEINS

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Parallel synthesis and testing of peptides with the help of pin technology approach (PEPSCAN, Chiron Technology, Australia) allowed us to reveal linear antigenic determinants of several cytochromes P450 and their redox partners cytochrome b5 and putidaredoxin and to estimate linear sites responsible for protein-protein interactions. For this purpose overlapping oligopeptides covering the whole sequences of the proteins under study were synthesised on polyethylene pins and tested for antibody binding without peptide cleavage from the pins. Several antigenic sites were covered with peptides of smaller length and containing amino acid substitutions, to achieve a more precise mapping of protein linear B-epitopes. Amino acid and immunochemical analysis of peptides upon their repeated testings (about 100 times) showed the preservation of their structure and antibody binding properties.
Antigenic mapping of proteins by PEPSCAN approach with the use of antibody preparations from different animal species allowed to reveal immunodominant (immunogenic for the majority of species and individual animals) B-epitopes which were treated as an "antigenic core" of a protein. Based on cytochrome P450 101and cytochrome b5 cytosolic fragment (proteins with known tertiary structures) antigenic mapping results, the immunodominant B-epitopes were shown to have a statistically greater content of water-accessible amino acid residues than the protein molecule in a whole. Individual as well as group-specific linear B-epitopes were found in cytochromes P450, and the ability for immune cross-reactions was mentioned upon antibody interactions with amino acid-substituted peptides.
Mapping of linear protein fragments responsible for protein-protein interactions in monooxygenase system proteins with the use of pin-bound peptides required the elaboration of a special ELISA-based procedure. Results obtained by PEPSCAN solely were confirmed with the help of optical biosensor technique by evaluating protein-protein interaction inhibition by peptides. Peptides for these experiments were synthesised on pins of a special type designed for cleavable peptide synthesis.
Thus, PEPSCAN approach to monooxygenase protein studies allowed to receive an important information on their functionally active sites.
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FURTHER INVESTIGATION OF IMMUNOMODULATORY PROPERTIES OF GAMMA-GLUTAMYL CONTAINING PEPTIDES

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Earlier we described gamma-glutamyl and beta-aspartyl containing peptides that manifest immunomodulatory properties [A. Kolobov et al., *Proc. 25th EPS, 1998, p. 526*]. On the basis of structure-activity relationship studies the most promising analog, gamma-D-glutamyl-L-tryptophan (SCV-07), has been further investigated.
In this study we evaluated the immunomodulatory potential of SCV-07 by determining its influence on the specific immune response of mice to a specific antigen, ovalbumin (OVA), and on the expression of cell surface antigens on human lymphocytes *in vitro*. Mice were immunized with OVA solution in incomplete Freund's adjuvant; SCV-07 was administered i.p. in physiological saline. SCV-07 treatment resulted in stimulation of OVA-induced IL-2 and γIFN production by spleen cells, a significant dose-dependent enhancement of OVA-induced spleen cell proliferation, and an increase in titers of total IgG and IgG_1 anti-OVA antibodies in sera of mice after either primary or secondary immunization. For *in vitro* experiments mononuclear cells were separated from peripheral blood of healthy volunteers by Ficoll-Paque gradient centrifugation and cultured with various concentrations of SCV-07. Cells were analyzed by flow cytofluorimetry using monoclonal antibodies. It was found that SCV-07 treatment increased the quantity of cells possessing the CD25 antigen and changed the CD4+/CD8+ ratio.
Our results suggest that SCV-07 stimulates the immune response through preferential activation of the Th1 lymphocyte subset.

ADJUVANT-CONJUGATED-SEQUENTIAL OLIGOPEPTIDE CARRIERS (SOC_n) FOR VACCINE APPLICATIONS.

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A novel class of oligopeptide carriers termed Sequential Oligopeptide Carriers (SOC_n) is successfully applied in our laboratory. SOC_n is formed by the $(Lys\text{-}Aib\text{-}Gly)_n$ sequential motif, where n=2-7, and adopts a predetermined secondary structure of 3_{10}-helix. This helicoidal structure contributes to the reduction of steric hindrances and conformational restrictions of the carrier, and thus allows the antigens anchored to the Lys-$N^{\varepsilon}H_2$ groups to retain their original structure, as confirmed by ^{1}HNMR and molecular modeling studies, and induce favorable molecular recognitions. A new generation of adjuvant -conjugated-SOC_n is now presented. With the aim to improve the SOC_n carrier for human usage and avoid the use of complete and incomplete Freund's adjuvant, the IL-1β sequence (163-171) is covalently coupled to the N-terminal part of SOC_n. A "promiscuous" T-cell epitope, derived from the Tetanus Toxoid (TT), is also attached to the C-terminal part of the carrier, to stimulate a broad spectrum of isotypic and allotypic forms of human MHC. The immune responces in mice, immunized with three different antigents (La/SSB, Sm and AChR epitopes) anchored to the carrier, without using Freund's adjuvant, as well as the conformational properties of the adjuvant-conjugated SOC_n, by ^{1}HNMR spectroscopy,are presented.

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PEPTIDE CONSTRUCTS INCLUDING IMMUNOACTIVE FRAGMENTS OF THE FOOT-AND-MOUTH DISEASE VIRUS VP_1 PROTEIN.

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Earlier the VP_1 protein of the foot-and-mouth-disease virus (FMDV) was described as inducing the protective antiviral immunity and its immunoactive fragments carrying B- and T-epitopes have been localized. Here we report a study of longer peptide constructs containing various immunoactive regions of the FMDV A_{22} protein.
We have synthesized three constructs representing linear peptides 44-53 amino acid residues long. Construct **I** represents a dimer of the protein's main immunogenic region 135-159. Construct **II** combines the main immunogenic region 135-159 and the minor immunogenic region 197-213. Construct **III** includes the main immunogenic region 135-159 and the virus specific T cell epitope 170-190. In the process of studying the immunogenic activity of the constructs in mice of three strains, we have found that construct *III* induced the highest level of antipeptide antibodies. Construct **III** was the most efficient in the test of protection of guinea pigs against experimental FMDV infection. Single immunization by construct III in 10 μg dose protected 100% of the inoculated animals. Protective activity of the two other constructs was lower however exceeding the activity of their shorter precursors. The antibodies induced by all constructs were directed to the immunodominant region 135-159. Anti-**III** antibodies possessed considerably higher virus-neutralizing activity than the antibodies against other constructs. We also tested the ability of construct **III** to protect pigs against FMDV infection. In this experiment, the animals were immunized twice (days 1 and 42) with the peptide and inoculated with the virus (day 56). All immunized animals survived from the disease. Thus, the combination of 135-159 and 170-190 VP_1 fragments in a single linear molecule is the most effective artificial construct for protection of animals against FMDV ever created.

SELECTION FROM SEVERAL CONSTRAINED AND UNCONSTRAINED PHAGE LIBRARIES OF PEPTIDES MIMICKING OF A DISCONTINUOUS EPITOPE RECOGNIZED BY AN ANTI-THYROGLOBULIN ANTIBODY: PASSAGE FROM PHAGE TO PEPTIDE

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Phage-displayed peptide libraries have been used for several years to identify peptide-ligands for antibodies, and other binding molecules. A high success rate was obtained with antibodies recognizing continuous epitopes. Finding peptides able to mimic discontinous epitopes has proven more difficult.
Anti-human thyroglobulin (hTg) autoantibodies in patients with thyroid disorders exhibit a restricted epitopic specificity towards a particular region of hTg (region II). Therefore it is of interest to select mimotopes recognized by these autoantibodies to improve diagnosis of autoimmune thyroiditis. To try to select functional mimetics of region II, we used Tg10, a monoclonal antibody which specifically recognizes region II, to screen a constrained 9 aa library and 3 unconstrained libraries (9, 15 and 28 aa).
Three rounds of biopanning were performed with each library. Several clones which showed a high reactivity with Tg10 were isolated and sequenced. The most reactive phage displayed a sequence (sequence1) with no homology with region II. Sequences from three reactive 28 aa-phages were synthesized by the Spot method in the form of overlapping 16-mer peptides. They did bind to hTg whereas peptides derived from the region II of hTg were not reactive. When the same set of 16-mer peptides was tested with normal and patient's sera, some peptides from sequence 1 show a specific reactivity with a serum having a high-titer of anti-hTg autoantibodies. Finally, synthetic peptides corresponding either to the complete sequence 1 (28 mer) or to 16 mer peptides derived from sequence 1 were synthesized by conventional SPPS and used in BIAcore analysis. They exhibit K_D values for Tg10, in the 10^{-6}—10^{-7} M range. A 102-aa recombinant protein corresponding to the whole region II had only a 50 times better affinity. So, thanks to the combination of phage-displayed libraries and chemical peptide synthesis, we have selected small ligands of a monoclonal antibody that recognizes a discontinuous epitope on an autoantigenic region of hTg. These peptides have potential diagnostic applications.

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DEVELOPMENT OF SPECIFIC ANTIBODIES AND A DIRECT ELISA ASSAY FOR DETERMINING THYMOSIN BETA-15 IN EXTRACTS OF BREAST CANCER TISSUES

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Thymosin beta-15 (TB15) is a 44 amino acid polypeptide (MW: 5,173) which has been recently detected in human prostate tumours, but not in healthy human tissues. As the homologous peptide thymosin beta-4 (TB4) or thymosin beta-10 (TB10), TB15 has been reported to inhibit actin polymerization, thus being possibly involved in cell division and motility mechanisms.
In order to develop specific antibodies against this peptide, TB15 secondary structure was theoretically studied and three major antigenic determinants, i.e. residues 2-10, 15-25 and 35-43, were predicted to occur. The fragments 1-16, 31-44 and 38-44 were selected as haptens for antibody development, and then synthesized, due to their putative antigenic properties and minimal structural similarity with the TB15 homologues TB4 and TB10, which occur in human tissues. In addition, these fragments end at a Lys residue in the C- or N- terminus, respectively, which is ideal for their coupling to the KLH carrier protein. Antisera were raised in rabbits against the above synthetic peptides as well as against intact synthetic TB15, after they had been coupled to KLH. All antipeptide antisera were able to recognize TB15 as shown in an ELISA system except the one developed against the fragment 38-44. The antisera raised against the fragment 31-44 or the intact synthetic TB15 were evaluated for cross reactivity with TB4 and TB10 by displacement experiments. For both antisera, excellent specificities were obtained, which may indicate that the C-terminus is the predominant immunogenic epitope of TB15. A TB15 ELISA assay was developed using the antiserum raised against intact synthetic TB15 as well as synthetic TB15 as well-coating and standard material. Due to its appropriate sensitivity, the assay developed was capable of directly determing TB15 in extracts of human breast tumours. According to preliminary data, TB15 levels were found to be significantly higher in malignant tissue than in the adjacent normal tissue areas in five tumour samples out of seven till now examined.

A SUPER-AGONIST OF THE T CELL RESPONSE GENERATED BY INTRODUCING A NEW PEPTIDOMIMETIC MOTIF IN A CD8 T CELL EPITOPE

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With the aim of designing major histocompatibilty complex (MHC) ligands able to enhance cytotoxic T cell activity, we have synthesized a new pseudopeptide analogue of the immunodominant H-2D^b-restricted GP33 epitope of the lymphocytic choriomeningitis virus. A carba bond $\psi(CH_2\text{-}CH_2)$ was introduced between positions 6 (Phe) and 7 (Ala) of the peptide and the C^α of the seventh residue was replaced by a nitrogen (N^α), thus leading to a unique carba/aza hybrid. The biological activity of the resulting peptide presenting this new motif -C^α (R_n)-CH_2-CH_2-N^α (R_{n+1})-, named carbaza peptide, was analyzed in vitro and in vivo, and compared to that of the GP33 parent peptide and to the control carba and aza analogues, respectively. The four peptides bound equally well to H-2D^b MHC molecules. However, in a proliferation assay using GP33-specific transgenic T cells, the activity of the carbaza peptide was significantly superior to that of the parent peptide and the aza or carba analogues. Moreover, the carbaza analogue induced more efficiently the major functions of CD8 T cells (i.e. lysis of target cells and interferon γ production) than the parent peptide. X-ray analyses of the carbaza dipeptide and molecular modeling of MHC/pseudopeptide complexes suggest that the carbaza modification induce a more stable orientation of the side chain of the major T cell receptor contact residue.

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DELAYED AND LONG-TERM PRESENTATION OF MHC CLASS I-RESTRICTED LIPOPEPTIDES

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The efficiency of vaccines against many intracellular infectious agents requires the stimulation of an immune response resulting in the induction of cytotoxic T cells (CTLs) that recognize specific peptide epitopes in the context of MHC class I molecules. Cytotoxic epitopes are usually 8-11 residues long, and are mostly produced by the cytoplasmic endogeneous processing pathway for protein degradation and presentation.

Intervention strategies aimed at inducing CTLs have been elaborated in two opposite directions. The first one is based upon the use of the 'optimal' ligands which are presumably able to associate directly with MHC molecules on the cell surface without needing further intracellular processing. An alternate approach is based upon the expression or introduction of peptide or protein antigens into the cytoplasm of antigen presenting cells (APCs), providing access to the MHC class-I pathway.

Among different vehicles that have been designed for antigen delivery into the cytoplasm, palmitoyl-modified peptides (lipopeptides) were able to induce CTL responses *in vivo* in mice, primates and humans. We have hypothesized that antigenic lipopeptides could favor internalization and cytoplasm delivery in APCs, thereby functioning as immediate precursors of the epitope.

As a model peptide, we have selected the HLA-A*0201 restricted HIV-1 polymerase (pol) 476-484 conserved epitope. Several analogs of this epitope were obtained by modifying P0, P1 or P10 positions by a single N^ε-palmitoyl-lysine residue. The use of fluorescent derivatives confirmed the cell-permeating activities, and suggested that a P0- and a P1-modified lipopeptide possessing ionizable extremities fulfill the structural requirements for MHC loading. Both lipopeptides induced a considerably prolongated expression of conformationally correct HLA-A*0201-peptide complexes at the surface of TAP-deficient T2 cells. This expression was dependent upon the integrity of the exocytosis pathway, suggesting a dynamic mechanism of formation or reloading of the complexes from an intracellular pool. The agonistic activities of the different HLA-peptide complexes were evaluated using peptide-specific CD8+ T-cell lines from HIV-infected donors in IFN-γ ELISPOT assays. The P0 modified lipopeptide was able to increase the life-span of functional presentation to class I-restricted T cells specific for the parent peptide.

ANTIPEPTIDE ANTIBODIES FOR THE L6 AND L13 RIBOSOMAL PROTEINS OF *Escherichia coli*

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Synthetic peptides are used extensively for the induction of antibodies specific for the corresponding primary amino acid sequences in the intact proteins. Thus, antibodies for relevant surface-exposed sequences of ribosomal proteins from *E.coli* are valuable probes to study the fine structure at the surface of the ribosome and its functions.

In the present study the C-terminal hexapeptide Lys-Glu-Ala-Lys-Lys-Lys of ribosomal protein L6 and the C-terminal hexapeptide Pro-Gln-Val-Leu-Asp-Ile of L13 were synthesized by SPPS, using 2-chlorotrityl chloride resin as solid support and 9-fluorenylmethoxycarbonyl- (Fmoc-) as the N-terminal protecting group of the amino acids used. The peptides were purified by HPLC on C18 column with acetonitrile/water as eluent and identified by N-terminal sequencing and nanoelectrospray tandem mass spectrometry. They were further coupled to ovalbumin and the conjugates were used for the immunization of rabbits and the induction of the antipeptide antisera.

The reactivity of the antipeptide antibodies against these synthetic peptides was determined by enzyme-linked immunosorbent assay (ELISA) and against the ribosomal proteins of *E.coli* by immunoprecipitation and immunoblotting. It was found that the antipeptide antisera recognize the corresponding and other ribosomal proteins of *E.coli*.

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ANTIGENIC MAPPING OF CYTOCHROME P450CAM BY PEPTIDE SCANNING

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Protein linear antigenic determinants can be revealed using short synthetic peptides that overlap a known protein sequence (Pepscan method). Since the whole protein surface is considered to possess antigenic properties, a question arises if a set of linear B-epitopes determined by Pepscan correlates with a protein spatial structure. We have chosen the P450cam (CYP 101), which is one of the well-investigated P450s with known 3D structure, as a template.
Sera of 3 rabbits and antibody egg yolk preparations from 2 chicken were produced against the holo-form of P450cam, and sera of another 3 rabbits were produced against the apo-form. These antibodies were analysed separately in ELISA with 409 overlapping P450cam hexapeptides. Differences between antigenic maps were estimated by X^2 criterium.
The whole set of linear antigenic sites (total antigenic map) revealed by Pepscan with antibody preparation against holo-P450cam covered about 60% of P450cam sequence. However, immunodominant sites (those revealed with more than 3 antibody preparation), the so called 'antigenic core', represent only 17% of the protein sequence. While the content of water-accessible residues in the total antigenic map (42%) was close to that in the whole native P450cam molecule (38%), the content of water-accessible residues in the antigenic core was significantly higher (54%). In addition, immunodominant sites are shown to be devoid of α-helical structures.
The maps received against holo- and apo-forms of protein differ only slightly. Only one of immunodominant regions of holo-P450cam, 311-318 (QLKKGDQI), was not revealed by any of anti-apoprotein antibody preparations.
Functionally important residues of P450cam Gln108, Arg109 and Arg112 were located in an immunodominant site, thus making this region perspective in producing the functionally directed antipeptide antibodies.
The work was supported in part by grants from RFBR (No. 98-04-48684).

COMPLEMENTARY PEPTIDES TO B AND T-CELL EPITOPES OF THE La/SSB AUTOANTIGEN FOR IMMUNOREGULATION IN SJOGREN'S SYNDROME (pSS).

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In previous studies it has been reported that vaccination with complementary peptides, encoded by complementary RNA, induces the production of anti-idiotypic and anti-clonotypic Abs, the combining sites of which are complementary to and therefore reactive with Ag receptors on disease epitope-specific B and T cells. Recently, we have demonstrated that anti-La/SSB antibodies derived from pSS patients sera are directed towards four linear epitopes spanning the regions 145-164, 289-308, 301-320 and 349-368. Predictive methods have shown that the epitope 289-308 is also a T-cell epitope. Two peptides, encoded by complementary RNA (termed cpl (289-308) La/SSB and cpl (349-368) La/SSB were used in immunization experiments with the aim to manipulate the immune network in pSS. In particular, these peptides were coupled, separately, in doublicate to the Lys-$N^{\varepsilon}H_2$ groups of the Ac-(Lys-Aib-Gly)$_4$, (SOC_4), carrier and then used in animal immunizations together with SOC_4-(289-308)$_4$ and SOC_4-(349-368)$_4$ respectively. In this study we report on: (i) the humoral and lymphoproliferative responses in mice, (ii) the presence of anti-idiotypic antibodies in pSS patients sera and (iii) the conformational properties, by ^{1}HNMR spectroscopy, of the complementary peptides. (Grants from GGSRT).

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ROLE OF THE GLYCOSYL MOIETY IN AUTO-ANTIBODY RECOGNITION IN MULTIPLE SCLEROSIS

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In the majority of human autoimmune diseases of the nervous system, and in particular in multiple sclerosis (MS), the target antigens responsible for triggering the autoreactive/pathogenic responses are not known. The essential difficulty in their identification can be related to the fact that autoreactive immune-responses involve protein carrying post-translational modifications. Post-translational modifications (such as glycosylation, acetylation and lipidation) dramatically affect the antigenic properties of protein antigens. Moreover, in the recent years, after controversial data obtained by studying the major antigens (MBP, PLP), attention has focused on minor antigens such as MOG. In previous studies [Papini, A.M., *et al.*, *Bioorg. Med. Chem. Lett.* (1999) 9, 167-172] we demonstrated that only glycosylation with a simple β-D-glucopyranosyl moiety on Asn and not on a Ser or Hyp residue, at position 31 (unique glycosylation site of MOG) of the immunodominant peptide hMOG(30-50) [Johns, T.G., *et al.*, *J. Neurochem.* (1999) 72, 1-9] carries to the first synthetic antigen able to detect autoantibodies in a high percentage of MS patients. The antibody titre correlates to the disease activity.
We synthesized different peptide sequences bearing the β-D-glucopyranosyl moiety. We chose MBP(83-99) (ENPVVHFFKNIVTPRTP) and a scramble MOG sequence (TPRVERNGHSVFLAPYGWMVK), in which the aa are randomly reordered. The glycopeptides [Asn^{31}(Glc)]hMOG(30-50), [Asn^{84}(Glc)]hMBP(83-99), and [Asn^{36}(Glc)]scramble-hMOG(30-50) were used in direct and indirect competitive ELISA. We observed that the conjugation of a glycosyl moiety to an Asn (N-glycosydic linkage), in all the different peptide sequences, allowed the detection of antibodies specific for MS in comparison with the non glycosylated ones. These data demonstrate for the first time the relevance of the presence of a sugar moiety in antibody recognition in MS. ELISA were performed for out-coming antibody response in sera and cerebrospinal fluid of a large set of patients affected by MS.

MIXOTOPE OR COMBINATORIAL PEPTIDE LIBRARY APPROACHES TO ELICIT IMMUNE RESPONSE AGAINST FOOT-AND-MOUTH DISEASE VIRUS.

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The high variability of RNA viruses is the main problem for the design of effective vaccines for virus diseases. Foot-and-mouth disease virus (FMDV) is the economically most important animal pathogen and has been used as a relevant representative model. The major antigenic site A (hypervariable G-H loop of the capsid protein VP1) has been incorporated into synthetic vaccine formulations against FMD. However, full protection has not been achieved and no synthetic vaccine is available, probably due to point mutations that give rise to escape variants, among other reasons.
Mixotope or combinatorial peptide library approaches has been suggested as a strategy that offers substantial variability of antigens to the immune system. This would induce a broader immune repertoire capable of recognizing the highest possible number of escape mutants. In this way, we have designed and synthesized mixotopes related to site A (FMDV serotype C), with 3.2×10^5 and 3.4×10^3 peptide sequences each. The variability was derived from sequences isolated in the field or in cell cultures. The sera of guinea-pig immunized with these peptide libraries were tested by ELISA. Such animals elicited broadly specific antibodies that recognized both mixotopes and peptides related to site A. Although high ELISA titers were observed when peptides were coated in plate, no recognition was observed with the virus coated in plate. This suggests that the complementary determining regions (CDRs) of the elicited antibodies have three-dimensional structures that are not suitable for interacting with the native virus. Recently, Verdaguer *et al.* determined the three-dimensional structure of neutralizing antibodies complexed with a site A peptide, showing a compact *quasi*-circular conformation for the peptide. In view of this and results observed in our group using cyclic peptides as antigens, we have designed cyclic peptide mixotopes related to site A. The results of FMDV recognition by the anti-cyclic-mixotope antibodies will be presented.

IDENTIFICATION OF NATURALLY OCCURING SELF PEPTIDES WHICH PARTICIPATE TO ANTIVIRAL MEMORY IMMUNITY.

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The trimolecular system made of the T cell receptor (TCR), the antigenic peptide and the major histocompatibility complex (MHC) glycoprotein plays a pivotal role in the interaction between target cells and effector cells of the immune system. The pharmacological principles of TCR-ligand interaction have recently been defined. It is now well established that TCR ligands may act as agonist, partial agonist or antagonist (Hudrisier and Gairin (1998) Curr. Top. Microbiol. 232, 75-97). Based on the flexibility of TCR/antigen/MHC recognition, it has been proposed that self peptides could participate in pathophysiological processes involving T cells. TCR antagonists do not trigger T cell activation and their role in T cell survival has been suggested (Bachmann and Ohashi (1999) Immunol. Today 20, 568-575). To address this issue, we used as a model the H-2D^b-restricted LCMV immunodominant epitope NP396-404, for which the T cell response is well characterized and a transgenic model of virus-induced autoimmune diabete (the RIP-NP LCMV tg mice) developed. Based on the knowledge of NP396-404 MHC anchors and TCR contact residues, we screened the protein data bases for peptide sequences sharing structural and functional homologies with the viral antigen. Among the sequences found, a peptide was identified in the TNF receptor Type I (TNFR302-310). We found that TNFR302-310 was not a TCR agonist (no induction of lysis, proliferation nor cytokine secretion) but behave as an antagonist. TNFR302-310 maintained the antiviral cytotoxic function of NP396-404-specific memory CTLs both in vitro against LCMV-infected cells and in vivo once adoptively transferred into RIP-NP LCMV tg mice as shown by the development of auto-immune diabete (IDDM). Furthermore, using on line HPLC-MS, we showed that TNFR302-310 was naturally presented at the cell surface, thus supporting its biological relevance. These data support strongly that self peptides may participate to pathological processes leading to virus-induced autoimmune disease.

NEW PEPSCAN METHODS TO DETECT DISCONTINUOUS EPITOPES OF FOOT-AND-MOUTH DISEASE VIRUS

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Scanning of protein sequences with small peptides in ELISA against antibodies is a powerful tool to determine epitopes. This technique accurately revealed many antibody-protein interaction sites, including Foot-and-Mouth disease virus (FMDV) surface proteins. Although many monoclonal antibodies were shown to be reactive in ELISA when tested against sets of hexa- up to dodecapeptides, several neutralising monoclonal antibodies did not show interactions in these scans. In new scanning techniques, testing peptides up to a lenght of 30 amino acids several of the non-reacting monoclonal antibodies were found to react. The much longer peptides used in this new scan technique are supposed to cover larger areas of the protein surface to be tested, and thus are able to reveal discontinuous epitopes. Using our set of FMDV type O1BFS monoclonal antibodies, new reactive sites were found that cover the so-called D-site discontinuous epitope located on the VP2 and VP3 surface proteins. These new sites could be used as additional epitopes in a vaccine providing long-lasting protection in cattle.

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PEPTIDE-BASED ANALYSIS OF FINE SPECIFICITY OF B CELL IMMUNE RESPONSE TO ALLERGEN *ASP F2* MEDIATED BY T HELPER 1 AND T HELPER 2

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It is well known that T-helper cells (Th) are activated by antigen presenting cells (APC) displaying protein peptides on their surface in the association with mouse I-A or I-E molecules of major histocompatibility complex (MHC) class II. Activated CD4$^+$ T cells that drive the allergic response are polarized towards the Th2 phenotype, producing IL-4, a major IgE switch factor. Earlier we have hypothesized that the activation of Th2 or Th1 was associated with the presentation of the antigen in the context of either I-A or I-E molecules, respectively. In the present study we have synthesized 14 peptides from the major allergen *Asp f2* to study the fine specificity of B cell immune response driven by Th1 and Th2. One group of BALB/c (H-2^d) mice was immunized only with *Asp f2*, while the other one received additionally 10 injections of anti-I-A^d antibodies to block I-A molecule participation in the immune response. Antibodies were injected near the lymph node (LN) draining the site of immunization. IgG1 (Th2 associated) or IgG2$_a$ (Th1) B cell epitopes were determined by peptide-specific ELISA. Our data demonstrated that in the control group IgG1 recognized C-terminal peptide spanning the 296-310 amino acid sequence, while IgG2$_a$ recognized the 155-169 and 182-196 peptides. In the experimental group where mice were additionally treated with anti-I-A^d antibodies only in two out of four mice *Asp f2*-specific IgG1 were found. In other two mice a high level of Asp f2-specific IgG2$_a$ was detected. Their fine specificity differed from the specificity of the regulatory IgG2$_a$ found in the control group. They recognized to the same extent 5 epitopes of *Asp f2* molecule corresponding to the sites 20-33, 73-84, 134-148, 155-169 and 296-310. Thus, specific antibodies mediated by Th1 and Th2 recognize different epitopes of the antigen.

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AFFINITY OF THE HIV-NEUTRALIZING MONOCLONAL ANTIBODY 2F5 FOR CONFORMATIONALLY CONSTRAINED GP41 ELDKWA PEPTIDES

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The human monoclonal antibody 2F5 (Mab 2F5) binds to a linear B-cell peptide epitope from the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein gp41 comprising residues 662-667, which corresponds to the peptide sequence ELDKWA. The ELDKWA sequence is highly conserved, and Mab 2F5 has a broad neutralizing activity when tested against primary HIV-1 isolates. We wish to test the hypothesis that conformationally constrained peptides and peptidomimetics that bind with high affinity to 2F5 will also elicit high titers of HIV-neutralizing antibodies. Such peptides may provide leads in the design of immunogens that elicit protective immunity against HIV-1 infection. Towards this goal, two series of conformationally biased peptides have been synthesized and are being tested for their affinities for 2F5. In the first series, comprising eight peptides, the ELDKWA peptide is flanked by Ala-rich sequences containing Aib residues or side-chain lactam bridges, in order to promote the α-helical conformation. This is the native conformation that has been characterized for the flanking sequences of the ELDKWA epitope in gp41, although the native conformation of the ELDKWA epitope itself is uncharacterized at present. In the second series, comprising 42 peptides, the ELDKWA sequence is incorporated into all possible locations around disulfide-bridged loop structures of sizes ranging from 10 through 16 residues. Initial characterization of the helix-stabilized series indicate that 2F5 affinities decrease as the peptide helix stability assessed by circular dichroism spectropolarimetry increases. This suggests that the 2F5-bound conformation of ELDKWA may differ significantly from the probable native conformation of this epitope. The synthesis of the disulfide-looped series is now in progress. A complete characterization of the antibody binding properties of these two peptide series and their relationship to the peptide solution conformations will be presented.

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SYNTHETIC PEPTIDES AS FUNCTIONAL MIMICS OF A DISCONTINUOUS VIRAL ANTIGENIC SITE

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Structural and functional features of continuous antigenic sites have been successfully reproduced by linear peptides. Most antigenic sites, however, are discontinuous, i.e., made up by residues distant in the sequence or even belonging to different protein chains. A certain level of functional mimicry of this kind of antigenic sites is possible if i) the 3D structure of the antigen is known, and ii) the residues involved in antigenic recognition are identified. These data can be used to build a peptide construction that displays the antigenically relevant residues and their environment in a native-like orientation.

We have reported preliminary results in this direction[1] using foot-and-mouth disease virus (FMDV) as model. Crystal structures for FMDV are known and a discontinuous antigenic site, involving five residues from three capsid proteins, has been identifie [2]. These five residues and their adjoining regions can be connected through a polyProII helix and a disulfide bridge to give a heterotrimeric structure. In the presentation we analize how the length of the polyPro module influences performance of the peptide. The problem is approached both computationally and by immunization experiments with selected peptide construcions. Antisera to the peptides have been evaluated on several criteria: i) recognition of FMDV particle, ii) competition with mAbs mapping at the discontinuous site, and iii) infectivity reduction in cell culture. Results show clear recognition of the discontinuous site and moderate neutralization levels. Extension of the approach to cattle, a natural FMDV host, has provided similar results. We have also proved that recognition-neutralization does not arise from mere admixture of the linear sequences, i.e., structural preorganization is required. This is supported by NMR studies of the heterotrimers.

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MAPPING AND CHARACTERIZATION OF PEPTIDE EPITOPES RECOGNIZED BY SEVEN MURINE INHIBITOR ANTIBODIES TO FACTOR VIII

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Factor VIII (FVIII) is a key protein in the coagulation cascade. Hemophilia A is a severe disease in which FVIII is genetically absent. To try circumventing the lack of constitutive FVIII, hemophiliacs are given recombinant FVIII. About 10-20% of these patients mount an abnormal immune response, leading to antibody-based neutralization of the effects of FVIII. The localization of the immunodominant epitopes within the FVIII sequence is an important goal to achieve, since it may yield information about the mechanism of neutralization of FVIII activity by antibodies. Due to the large size of FVIII (2332 aa), methods of multiple peptide synthesis, allowing the efficient preparation of large sets of overlapping peptides covering the complete protein sequence, appear to be ideally suited for the mapping of epitopes recognized by an antibody. In our work, the amino acid sequence of FVIII (except for the B domain) was presented in the form of an array of overlapping pentadecapeptides (2 residue overlap) synthesized on a cellulose membrane. Several murine inhibitor anti-FVIII antibodies were evaluated individually for their capacity to react with the set of immobilized FVIII peptides. Monoclonal antibodies F7B4 and F29F11 were found to map an epitope in the a_1 acidic region: DVVRF (aa 356-360); mab F14A12 required the presence of residues Glu and Met upstream, EMDVVRFD (aa 354-360); mAb F26F6 recognized the epitope EDISAYLLSK (aa 724-733) in a_2. Mabs F19C2 and F18B1 bound to two distinct sequences in a_3: DDTISVEMK and IYDED respectively. Alanine scanning of each reactive peptide allowed the identification of the amino acid side chains that contribute significantly to binding. By using a very sensitive assay format, the epitope of the monoclonal antibody ESH8 was identified, in good agreement with results obtained by others. The results obtained by peptide analysis were consistent with those found by using other methods such as immunoprecipitation of metabolically labeled fragments of FVIII and ELISA reactivity with FVIII fragments fused to GST. Our results therefore provide a detailed understanding of the recognition of FVIII by model inhibitor antibodies.

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SYNTHETIC FRAGMENTS OF *NEISSERIA MENINGITIDIS* PROTEINS POR A AND OPA B INDUCE PROTECTION AGAINST EXPERIMENTAL INFECTION

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No vaccine is yet available against disease caused by *Neisseria Meningitidis* serogroup B. Earlier some outer membrane proteins of bacteria have been described as carrying protective B-cell epitopes and helper T-cell epitopes. We deduced potential immunoactive fragments from cation-specific protein PorA and opacity protein OpaB. 14 peptides covering all exposed loops and part of calculated T-helper epitopes of PorA protein and 11 peptides covering the part of exposed loopes and all calculated T-helper epitopes of OpaB protein were synthesized.

We studied the ability of free peptides to induce the antibody formation in a Balb/c, C57/Bl and CBA/J mice upon double immunization without conjugation with a protein carrier. It was shown that 10 PorA fragments and all OpaB peptides were immonogenic at least in one mice strain. Among them 3 PorA peptides and 7 OpaB peptides were able to induce the antibody formation in all three mice strains. Only two fragments from PorA bind the antibacterial mice serum. For the protection study a CBA/J mice were single inoculated with peptides and 35 days later were challenged with the different doses of bacteria. Three peptides from PorA with sequences 32-51, 306-332 and 346-363 and six fragments from OpaB with sequences 30-51, 41-63, 64-83, 74-93, 109-130 and 131-150 induce protection against *Neisseria Meningitidis* challenge. Mechanisms of peptide induced protective immunity and the ways of its potentiation will be discussed.

THE ROLE OF FLANKING SEQUENCES IN ANTIBODY RECOGNITION OF PTGTQ EPITOPE OF MUCIN-2

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Mucin-2 (*MUC2*) gastrointestinal glycoprotein can be underglycosylated in case of colon cancer enabling us to identify malignant tissues with protein core specific monoclonal antibodies. In our earlier work monoclonal antibody 996 (MAb 996) [1] recognising the protein core was used to identify the minimal epitope on solid phase (Ac-TGTQ) and in solution (PTGTQ) [2]. The presence of β-turn structure at the N-terminus was important also for efficient recognition in solution [3].

Now we describe the effect of flanking sequence coupled to the N- and C-terminus of the minimal epitope on the recognition by MAb 996 in solution. Three arrays of peptides - 19 peptides each - were synthesized using Boc chemistry: decapeptides with a structure of AAA-TGTQ-AXA, octapeptides (AX-TGTQ-AA) and nonapeptides (AX-PTGTQ-AA). In the position X, one of 19 proteinogen amino acids was built in; cysteine was omitted. The binding properties of the peptides were studied in competitive ELISA. In the case of decapeptides the presence of Ala_3 on the N-terminus of the epitope resulted in the loss of the binding to MAb 996. The different amino acids in the second position from the C-terminus (position X) had no effect on the binding. In the array of octapeptides the antibody recognised only AP-TGTQ-AA (IC_{50} = 89 μmol/dm^3). It shows that the N-terminal proline residue connected to TGTQ within the tandem repeat unit is necessary for the recognition in solution.

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