CROATICA CHEMICA ACTA CCACAA **71** (3) 591–605 (1998)

ISSN-0011-1643
CCA-2514 *Original Scientific Paper*

Cytogenetic Effects of Met-enkephalin (Peptid-M) on Human Lymphocytes

Nikola Štambuk,[a] Nevenka Kopjar,[b] Karmela Šentija,[c] Vera Garaj-Vrhovac,[b] Dražen Vikić-Topić,[a] Branka Marušić-Della Marina,[d] Vesna Brinar,[e] Milica Trbojević-Čepe,[f] Neven Žarković,[a] Božidar Ćurković,[g] Đurđa Babić-Naglić,[g] Mirko Hadžija,[a] Niko Zurak,[e] Zdravko Brzović,[e] Roko Martinić,[h] Vjera Štambuk,[h] Paško Konjevoda,[i] Nikola Ugrinović,[j] Ivana Pavlić-Renar,[j] Zdenko Biđin,[k] and Biserka Pokrić[a]*

[a]*Rugjer Bošković Institute, Bijenička 54, HR–10001 Zagreb, Croatia*

[b]*Institute for Medical Research and Occupational Health, Ksaverska c. 2, HR–10000 Zagreb, Croatia*

[c]*Clinical Hospital "Merkur", Zajčeva 19, HR–10000 Zagreb, Croatia*

[d]*Childrens Hospital Zagreb, Klaićeva 16, HR–10000 Zagreb, Croatia*

[e]*Department of Neurology, Clinical Hospital Centre, Zagreb, Kišpatićeva 12, HR–10000 Zagreb, Croatia*

[f]*Clinical Institute of Laboratory Diagnosis, Clinical Hospital Centre, Zagreb, Kišpatićeva 12, HR–10000 Zagreb, Croatia*

[g]*Department of Rheumatology and Rehabilitation, Clinical Hospital Centre, Zagreb, Kišpatićeva 12, HR–10000 Zagreb, Croatia*

[h]*Clinical Hospital Split, Spinčićeva 1, HR–21000 Split, Croatia*

[i]*Department of Pharmacology, Zagreb University School of Medicine, Šalata 2, HR–10000 Zagreb, Croatia*

[j]*University Clinic for Diabetes and Metabolic Diseases, Dobri dol 4a, HR–10000 Zagreb, Croatia*

[k]*Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, HR–10000 Zagreb, Croatia*

Received August 11, 1997; revised June 12, 1998; accepted June 15, 1998

* Author to whom correspondence should be addressed.
(e-mail: stambuk@rudjer.irb.hr)

The structure, complementary structure and cytogenetic/proliferative effects of the Met-enkephalin on human peripheral blood lymphocytes were analyzed. Met-enkephalin, *i.e.* Peptid-M (LUPEX®), is a low molecular weight synthetic pentapeptide that corresponds to thymus Met-enkephalin. Its structure was examined by means of NMR spectroscopy. The influence of Met-enkephalin on *in vitro* normalization of chromosomally aberrant lymphocytes of patients suffering from different immune-mediated diseases, was analyzed by the sensitive cytogenetic tests for screening and detection of genome damages in human lymphocytes. The tests showed that *in vitro* stimulation of human lymphocytes with the Met-enkephalin led to dissapearance of different types of chromosome aberrations, reduction in the number of micronuclei, decrease in the frequency of sister chromatid exchange (SCE) and apoptosis as well as a cytostatic effect on mitosis cycles. They have also confirmed normalization of chromosomally aberrant cell findings in patients suffering from different immune-mediated diseases. These results suggest a possible role of Met-enkephalin (Peptid-M) in immunotherapy of different diseases which involve chromosomal aberrations as well as abnormal cell proliferation and offer new approaches to immunotherapy by the use of Peptid-M. Based on the molecular recognition theory and the SCA method, peptide complementary to Peptid-M was designed, synthesized and denoted Peptide-D. Peptide-D is a calpastatin fragment. Predicted ligand-receptor interaction between both peptides is confirmed by the results showing that Peptide-D blocked the Peptid-M induced lymphocyte proliferation in a dose-dependent manner.

INTRODUCTION

In human lymphocytes, gene mutation occurs mostly in dividing cells. Aberrant lymphocyte clones, stimulated by autoantigens have been isolated from subjects with different immune-mediated diseases.[1–6] Mutant oligoclonal T and B cell clones are thought to be involved in the production of oligoclonal immunoglobulins, *i.e.* antibodies of restricted clonal heterogeneity, often directed to self-antigens (*e.g.* retinal S-antigen, myelin basic protein, *etc.*).[1–3,7–10]

Chromosomal investigations of peripheral blood lymphocytes (PBL) in multiple sclerosis (MS), uveitis and other autoimmune diseases revealed different aberrations, particularly chromosomal breaks.[1–6] Cytogenetic data also showed that chromosome abnormalities were more common in subjects with a high frequency of relapse or with a progressive form of the disease.[6] In recent years, new approaches to the treatment of immune-mediated diseases have been considered. Some of them include administration of low molecular substances, *e.g.* peptides, and their effects on cell cycle, cytokine secretion and apoptosis.[2,3,7–9,10]

Met-enkephalin, *i.e.* Peptid-M (LUPEX®), is an ubiquitous pentapeptide found in different tissues, including thymus.[2,3,11–16] Recent reports indicate that it may be a promising new drug for several immune-mediated diseases.[2,3,11–13] It was also shown that Peptid-M modulates lymphocyte[2,3] and other tissues[14–16] cell cycle, and protects cells against chromosomal aberrations *in vitro* and *in vivo*.[2,3]

The aim of this study was to investigate *in vitro* effects of Met-enkephalin (Peptid-M) on the number and types of lymphocyte cromosomal aberrations, cell cycle and apoptosis in subjects suffering from immune-mediated diseases. We also analyzed the structure of Peptid-M by means of NMR spectroscopy. To obtain more data on the effector function of Peptid-M and its interaction with other receptor systems we observed the effects of Peptid-M and its receptor site on lymphocyte proliferation. The receptor site of Peptid-M was recently defined by Štambuk[17,18] according to the molecular recognition theory.[17–22]

MATERIAL AND METHODS

Peptid-M Analysis

Syntethic analogue of thymus Met-enkephalin, *i.e.* Peptid-M (LUPEX®, Biofactor, Germany) was analyzed by one- and two-dimensional NMR spectroscopy on a Varian Gemini 300 spectrometer, operating at 75.46 MHz for the ^{13}C nucleus. The samples were dissolved in DMSO-d_6 solution at 20 °C and recorded in 5 mm NMR tubes. Chemical shifts were referred to TMS. Digital resolution in ^{1}H NMR spectra was 0.28 Hz per point and 0.70 Hz per point in ^{13}C NMR spectra. The techniques used were: broadband proton decoupling, gated decoupling, APT, COSY, NOESY and HETCOR. COSY spectra were obtained in magnitude mode, while NOESY spectra in phase-sensitive mode. In both, COSY and NOESY, 1024 points in F2 dimension and 256 increments in F1 dimension were used. Data in F1 were subsequently zero-filled to 1024 points. Every increment was obtained with 16 scans, using a 2750 Hz spectral width and a relaxation delay of 1 s. The resolution was 5.4 Hz/point and 10.7 Hz/point in F2 and F1 dimensions, respectively. NOESY spectra were measured with several mixing times (0.45–0.70 s). HETCOR spectra were obtained with 2048 points in F2 dimension and 256 increments in F1 dimension, which were zero-filled to 512 points. The increment parameters were as follows: 64 scans, relaxation delay of 1 s, spectral width of 20000 Hz in F2 and 4500 Hz in F1 dimensions. Digital resolution was 19.53 Hz/point in F2 and 17.6 Hz/point in F1 dimension. Proton decoupling was performed by Waltz-16 modulation. Standard one- and two-dimensional pulse sequences were used.

Defining and Synthesis of Peptide-D

The sequence of Peptide-D (IPPKY), complementary to Peptid-M (YGGFM), was derived by Štambuk by means of the SCA procedure based on the molecular recognition theory.[17–19] Briefly, the method defines the most probable complementary pairs that interact, based on the analyses of a large number of complementary motifs belonging to seven different ligand-receptor systems (epidermal growth factor, interleukin 2, transferrin, von Willebrand factor, angiotensin II/III, vitronectin and prolactin).[17,18] Peptide-D was synthesized using the standard solid-phase method and analyzed by HPLC and amino acid analysis (Biofactor, Germany; Lot No. B-0158). Synthesized Peptide-D had a molecular mass of 616.8 D, sequence H-Ile-Pro-Pro-Lys-Tyr-OH, and >97% purity.

Sample Collection

Samples of venous blood for the *in vitro* study of PBL were obtained from 22 subjects (17F/5M) suffering from ocular, demyelinating, rheumatic and allergic autoimmune diseases and type I diabetes (3 intermediate uveitis, 2 optic neuritis and multiple sclerosis, 1 hereditary eosinophilia with front eye segment affections, 1 neurodermitis with keratoconus and atopy, 1 Fuchs' cyclitis with subacute sclerosing panencephalitis, 10 diabetic retinopathy, 4 rheumatoid arthritis). Lymphocytes were cultivated *in vitro*. For each culture heparinized whole blood was added to F-10 medium (Sigma), supplemented with fetal bovine serum, phytohaemagglutinin (Murex) and antibiotics (penicillin and streptomycin). While conducting the investigation, the principles of Helsinki Declaration were observed.

Peptid-M Effects on Cytogenetic Findings

Cytogenetic tests of structural chromosome analysis (CA), sister chromatid exchange (SCE) and micronucleus (MN) assay were carried out in the control and Peptid-M treated cultures (Figures 2 and 3) to determine the mutagenic status *in vitro* before and after lymphocyte stimulation with Peptid-M (LUPEX®). In Peptid-M treated cultures, standard assays[22–26] were modified with 1.2 μg/mL of its aqueous solution for each method, as described by Štambuk *et al.*[2,3]

Analysis of Chromosome Aberrations

Lymphocytes were cultivated for 48 h at 37 °C. In each culture, colchicine was added 3 h before harvesting. Cultures were harvested and slides were prepared according to the standard method.[22] For hypotonic treatment, 0.075 M potassium chloride was used, and for fixation, cold freshly prepared fixative methanol/acetic acid (3:1). Cell suspensions were dropped onto clean dry-cold slides. They were allowed to air-dry and were stained

with 5% Giemsa solution. For structural chromosome aberrations, 300 well spread metaphases for each culture were examined. Cells were analyzed for chromatid and chromosome breaks, acentric fragments, dicentric and ring chromosomes.

Sister Chromatid Exchange Analysis

For SCE analysis and studies of cell proliferation, 5-bromodeoxyuridine (BrdU) was added to cultures at the initiation of the experiment and cells were cultivated for 72 h. These cultures were shielded from light. For mitotic arrest, colhicine was added to each culture 3 h before harvest. Slides were prepared according to the standard method.[23] SCE were analyzed in 50 second division cells for each sample, and the proportion of first, second and third mitoses was based on 300 metaphases per experimental point. To define the cellular kinetics, the number of first (M_1), second (M_2), third and more (M_3) mitosis were investigated using SCE analysis and proliferation rate indices (PRI).[24,25]

Micronucleus Evaluation

For the MN assay, the cells were cultivated for 72 h and cytohalasin B (Sigma) was added to the cultures 28 h prior to harvesting, according to the method described by Fenech and Morley.[26] In order to preserve the cytoplasm, binucleated cells were not exposed to hypotonic treatment. Cells were fixed with methanol/acetic acid (3:1). The slides were air-dried and stained with 5% Giemsa solution. Scoring of MN was limited to binucleated lymphocytes only with the preserved cytoplasm. The results are expressed as the number and the distribution of micronuclei per 1000 binucleated cells.

Peptid-M Effects on Cell Apoptosis

The level of lymphocyte apoptosis was evaluated in the cells of patients with diabetic retinopathy. The results were obtained by comparing lymphocyte apoptosis in the Peptid-M treated and untreated 5-day cell cultures. After a 5-day culture period, the cells were stained with propidium iodide (dead cells) and fluorescein diacetate (viable cells) and apoptosis was counted and evaluated using fluorescence microscopy at 490 nm as described by London *et al*.[27–29]

Peptid-M Effects on Cell Proliferation

Lymphocyte proliferation was also performed by means of 5-day cell cultures (sodium citrate anticoagulant, 2×10^5 cells/250 μL well, 5% FCS) and ^{3}H-Thymidine incorporation, as described by deSmet *et al.*[30] Cellular proliferative responses of the untreated, Peptid-M and Peptide-D treated cultures

were compared. Blocking of Peptid-M induced lymphocyte proliferation by means of different concentrations of its complementary Peptide-D is presented in Figure 4.

Statistical methods: Data were compared by the McNemar chi-square test.

RESULTS

NMR Spectroscopy

NMR spectroscopy confirmed that the structure of synthetic Peptid-M (LUPEX®, Biofactor, Germany) corresponds to Met-enkephalin. This analysis was based on chemical shifts, spin-spin coupling constants and connectivities in one and two-dimensional spectra. The assignment of signals was substantiated by heteronuclear $^{1}H–^{13}C$ correlated two-dimensional measurements (HETCOR). In dimethylsulfoxide solution (DMSO), Peptid-M molecule exists in dipolar form. In Figure 1, the COSY spectrum of Peptid-M in DMSO solution is given. Two- and three-bond spin-spin coupling connectivities between amid and methine and/or methylene protons corroborate dipolar structure. The amid proton signal of Phe^{4} is separated from the signal of Gly,[3] which is, in turn, overlapped with the amid proton signal of Met^{5}. In contrast, in the cationic form of Peptid-M, the Gly^{3} and Phe^{4} amid signals would be overlapped, while amid proton signal of Met^{5} would be separated.[31, 32]

Effects on Cromosome Aberration, SCE and Micronuclei

Structural chromosome aberrations observed in lymphocytes of all subjects before treatment with Peptid-M (LUPEX®) were chromatid breaks, chromosome breaks, acentric fragments, dicentric and ring chromosomes (Figure 2). The types and frequency of total chromosomal aberrations significantly decreased after treatment with Peptid-M in all subjects (Figure 2a, $p = 0.0027$). Severe chromosome aberrations, *i.e.* dicentric (3) and ring chromosomes (2), observed before treatment, disappeared after *in vitro* stimulation with Peptid-M.

The data on the frequency of SCE are given in Figure 2b. *In vitro* stimulation with Peptid-M caused a reduction of SCE frequencies per cell, as well as a decline in the SCE range (Figure 2b, $p < 0.001$).

After *in vitro* stimulation with Peptid-M, the total number of micronuclei significantly decreased in all cell cultures (Figure 2c, $p < 0.0001$). Distribution of micronuclei per 1000 binucleated cells was also changed, as compared to untreated lymphocytes. We observed mainly binucleated cells with one micronucleus in the Peptid-M treated cells, since the distribution of mi-

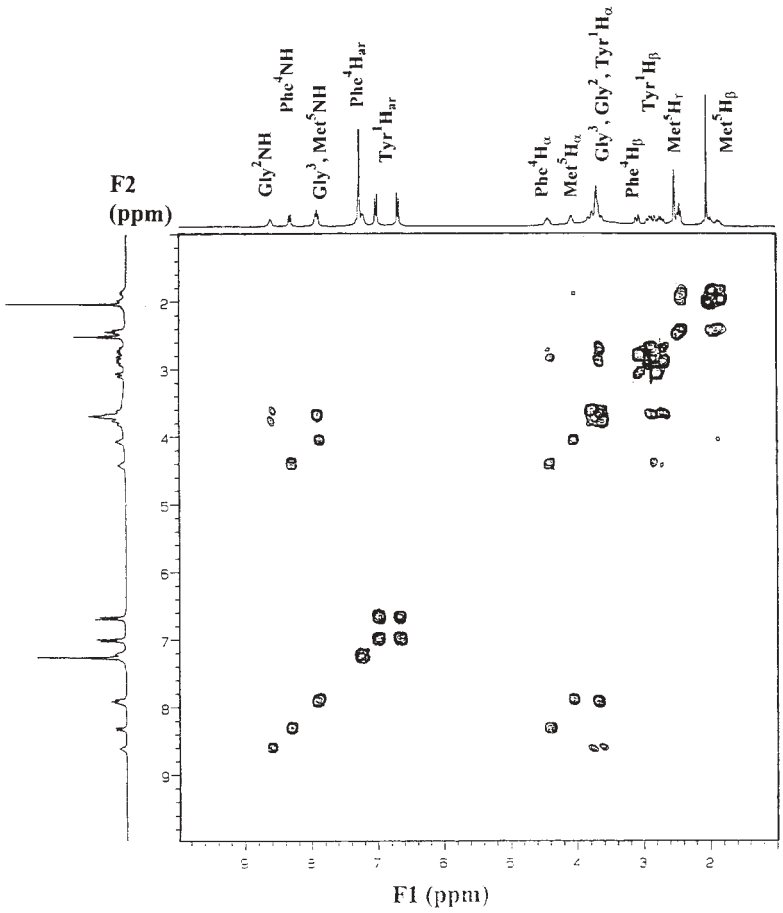

Figure 1. The COSY spectrum of synthetic Met-enkephalin (Peptid-M) measured in DMSO solution.

cronuclei was shifted towards normalization, when compared to the untreated control samples of the same subjects (Table I, $p < 0.0001$).

Effects on Cell Cycle and Cell Apoptosis

Cell kinetics was examined following three cell cycles, the first (M_1), second (M_2) and third (M_3) mitosis in lymphocyte proliferation. After *in vitro* treatment with Peptid-M, we observed a significant reduction in the number of cells that reached the third or more metaphase (M3; Figure 3c, $p < 0.0011$) and a significant increase in the number of first metaphase (M1; Figure 3a, $p < 0.0001$). The number of cells im second mitotic division remained the same (M2; Figure 3b, $p = 0.5829$). Decreased mitotic indices and

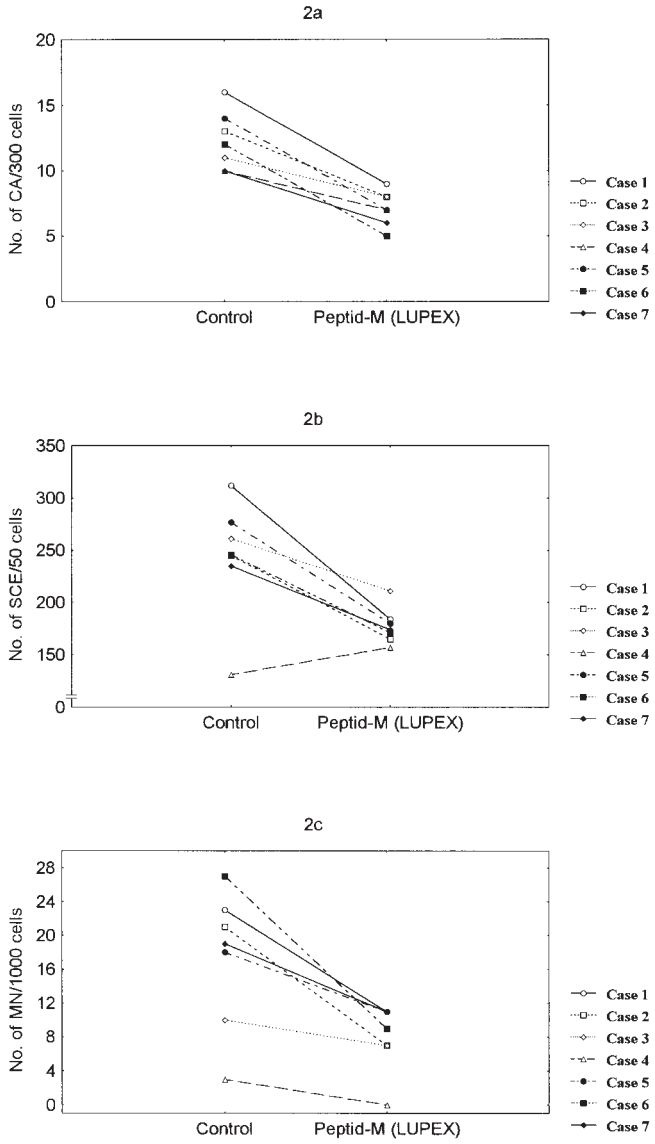

Figure 2. (a) Chromosomal aberrations, (b) sister chromatid exchange and (c) micronuclei in the control and Peptid-M (Met-enkephalin) treated cells.

previously mentioned mitosis division findings confirmed suppressive effects of Peptid-M on mitotic activity *in vitro*.

The results presented in Figure 4 show that apoptosis was significantly reduced in the Peptid-M treated lymphocyte cultures ($2.9 \pm 1.1\%$) when

TABLE I

Peptid-M (Met-enkephalin) treated cell cultures show significantly less micronuclei and a shift towards normalization in contrast to the control untreated samples of the same subjects

No. micronuclei	0	1	2	3
Observed *(Peptid-M treated)**	6444	54	1	0
Expected *(From control values)*	6377	118	4	0

*$\chi^2 = 37.66$; $p < 0.0001$ (Poisson distribution)

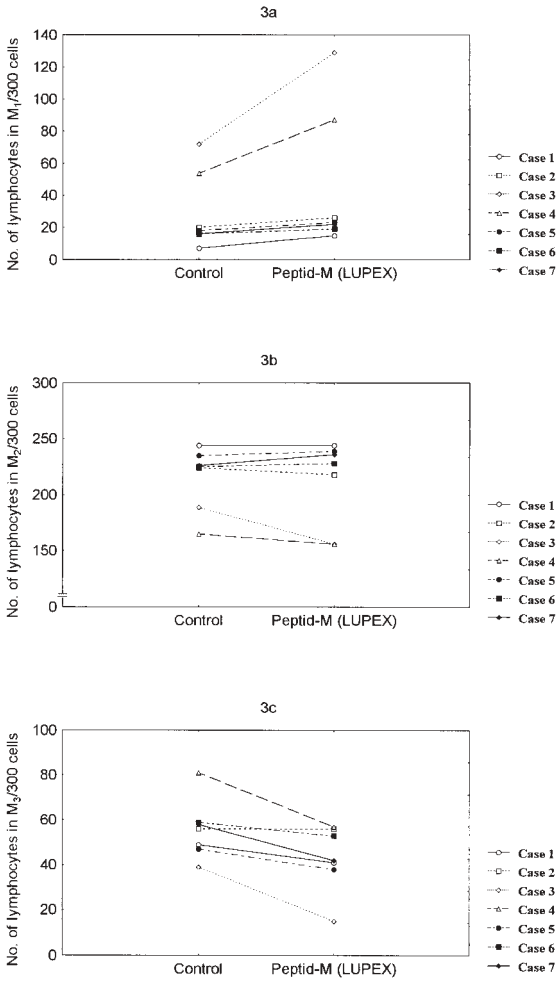

Figure 3. Number of cells in the first (a), second (b) and third (c) mitotic division in the control and Peptid-M (Met-enkephalin) treated cells.

compared to the untreated control cultures ($7.7 \pm 2.1\%$; $p < 0.01$) of the same samples.

Evaluation of Ligand-Receptor Interaction

Ligand-receptor interaction between Peptid-M and its complementary Peptide-D is presented in Figure 5b. It was detected by means of cellular proliferative bioassay based on ^{3}H-Thymidine DNA incorporation. Peptide-D blocked Peptid-M, *i.e.* Met-enkephalin, induced lymphocyte proliferation in a dose-dependent manner (Figure 5b), and exhibited suppressive effects on cell proliferation in a low concentration range.

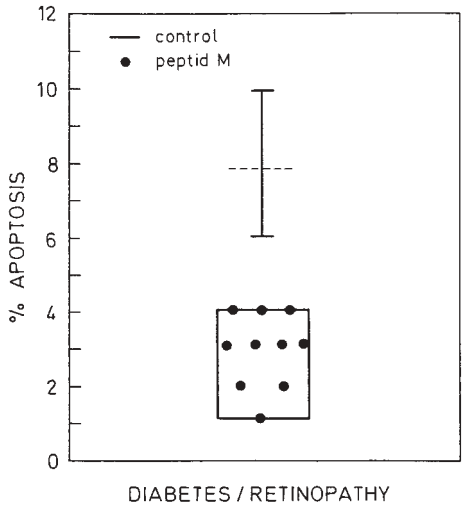

Figure 4. Apoptosis in the control and Peptid-M (Met-enkephalin) treated lymphocytes.

DISCUSSION

Immune-mediated and other diseases may be accompanied by chromosomal rearangements and aberrations.[1–3] Chromosomal investigations carried out on peripheral blood lymphocytes of subjects suffering from multiple sclerosis and other severe diseases revealed different structural aberrations, particularly chromosomal breaks, whose frequencies were significantly higher than in healthy persons.[4] Our results indicate that cytogenetic tests are sensitive methods for the screening and detection of genome damages in human lymphocytes obtained from the subjects suffering form different ocular and other immune-mediated diseases.

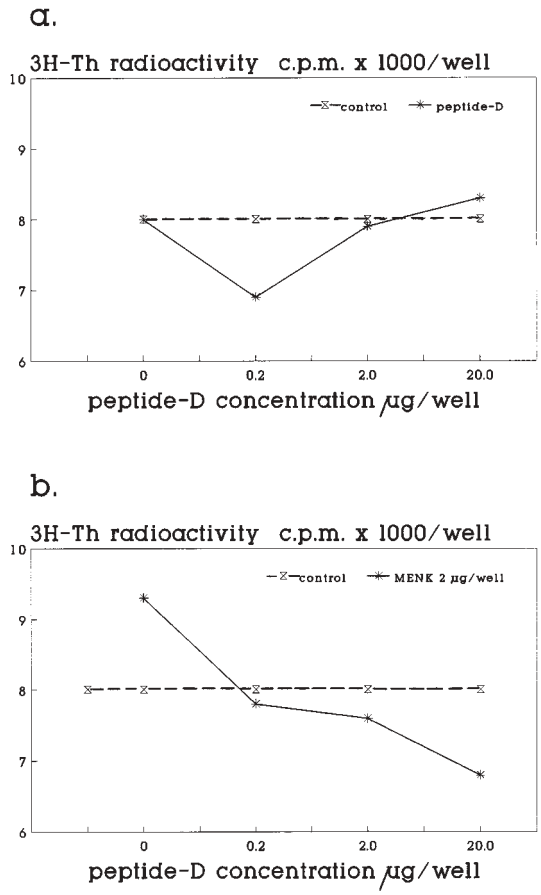

Figure 5. (a) Proliferation of the control and Peptide-D treated lymphocytes. (b) Effects of Peptide-D on the Peptid-M (MENK) induced cell proliferation.

Met-enkephalin (Peptid-M) induced modulation of the cell cycle, its cytostatic effects with prevention of mutations, and reduction of apoptosis (programmed cell death) suggest possible applications of this pentapeptide in different diseases which involve lymphocyte dysfunctions, chromosomal aberrations and/or abnormal lymphocyte proliferation. *In vitro* disappearence of abnormal lymphocyte clones in Figure 2 during Met-enkephalin (Peptid-M) treatment has also been confirmed by a recent study of Štambuk *et al.*[3] reporting *in vivo* disappearance of mutant peripheral blood lymphocytes after Peptid-M immunotherapy in an 11-year-old girl.[3,11,33] The latter is to our knowledge the first report indicating that selection of white blood cells may be therapeutically programmed with short peptides.[3,11,33] It remains to be determined if this also holds for other cells types.

The effects of Met-enkephalin (Peptid-M) on cell apoptosis presented in Figure 4 may be explained by the fact that its YGG sequence, which is a part of the calmodulin-binding apoptosis associated protein kinase (DAPK_HUMAN, 1127-1129 aa) binds to the sequences IPP and MPP[1,7,18] belonging to the cellular apoptosis susceptibility protein CAS_HUMAN (758–760 aa). In this way, both apoptotic pathways in proliferating lymphocytes treated with Met-enkephalin (Peptid-M) are disturbed and apoptosis in proliferating lymphocytes may be supressed (Figure 4) regardless of the cytostatic effects of the peptide on the mitosis cycle (Figure 3a–c). The observation of reduced apoptosis in normal lymphocytes is in line with the fact that suppressive effects of Met-enkephalin (Peptid-M) on cell cycle do not result in lymphopenia *in vivo*.[10–13]

Peptide-D is a complementary transcript of met-enkephalin, *i.e.* its possible receptor.[17,18,34] It does not seem to exhibit acute toxicity *in vivo* since its application in 1 μg/kg and 10 μg/kg doses (–30 min) did not induce toxicity or changes of the lesions when compared to the untreated controls in the standard rat cysteamine model (400 mg/kg cysteamine i.p., 0 min; strain: Wistar, F).[35] Theoretically predicted ligand-receptor interaction of Met-enkephalin (Peptid-M) and peptide-D was based on the molecular recognition theory. [17,18,34] Similar results have been obtained by several authors for different short peptide motifs and DNA/RNA transcripts.[1,8–13] Results presented in Figure 5 confirm that Met-enkephalin (Peptid-M) receptors may correspond to human calpastatin location (ICAL_HUMAN, residues 201–205). This short peptide sequence represents the core of the septapeptide TIPPKYR, which is a central conserved sequence generally associated with the inhibitory activity of calpastatin.[36,37] The latter also indicates that immunopathologic mechanisms related to the Met-enkephalin-opioid receptors and calpain-calpastatin system may interact (and interfere), especially since we measured specific and dose-dependent neutralization of the Met-enkephalin (Peptid-M) induced lymphocyte proliferation with Peptide-D.

We additionally evaluated the Met-enkephalin (Peptid-M) induced effects on δ and μ opioid receptors[13] *in vivo* by the Straub tail response[38–40] in mice (male NMRI, Pliva), in a dose of 1 mg/kg, which is therapeutically relevant in animal and in human models.[10,13] Since we could not detect significant positive Met-enkephalin (Peptid-M) effects on the Straub tail behaviour, which is thought to be the *in vivo* marker of the effects of the drug on δ and μ opioid receptors, and we obtained a typical dose-response curve in the Peptide-D neutralization of Met-enkephalin (Peptid-M) effects in bioassay (Figure 5b), it is tempting to postulate that naloxone-dependent opioid mechanism related to Met-enkephalin modulation may not neccessarily be receptor specific.

Peptide-D sequence shares molecular homology to short sequences of rapamycin-selective 25 kD immunophilin FKBP 25, *i.e.* Rapamycin and

FK506 binding protein (FKB3_HUMAN, sequence PPKY, residues 108–111). This explains the fact that immunosuppressive effects of Met-enkephalin (Peptid-M) on mitotic division and cell cycle[2,3,11,33] may be reversed by short peptides containing PPK sequence.[3,11] The fact that the IPPK sequence of Peptid-D corresponds also to the sequence of common cytokine receptor γ chain (CYRG_HUMAN, 335–338 aa), IL-8 receptor (IL8A_HUMAN) and IL-2β receptor indicates that immunologic mechanisms of IL-2 and IL-4 interaction through common cytokine γ chain, may be responsible for a number of effects of both peptides on the immune system, as discussed by Štambuk.[41] Results of Noguchi *et al.*[42] recently confirmed the involvement of common cytokine γ chain in calpain-calpastatin system. Evaluation of interactions between Met-enkephalin (Peptid-M) and calpastatin could be important for drug design related to several immune-mediated, degenerative and genetic diseases,[34,36,37] including: cataract, uveitis, optic neuritis, multiple sclerosis, Alzheimer's disease and rheumatoid arthritis.

Acknowledgements. – The authors wish to thank Mrs. Nevenka Hiršl for the contribution to the experimental work. The financial support by the Ministry of Science and Technology of the Republic of Croatia is highly appreciated (research grants No. 00981108 and No. 00980802).

REFERENCES

1. M. Allegretta, J. A. Nicklas, S. Sriram, and R. J. Albertini, *Science* **247** (1990) 718–721.
2. N. Štambuk, K. Šentija, M. Rudolf, R. Mažuran, T. Marotti, V. Šverko, I. Svoboda-Beusan, S. Rabatić, M. Trbojević-Čepe, S. Seiwerth, V. Garaj-Vrhovac, M. Banović, and M. Č. Pešić, *Int. J. Thymology* **3** (1995) 322–327.
3. N. Štambuk, K. Šentija, B. Marušić-Della Marina, M. Trbojević-Čepe, M. Rudolf, V. Garaj-Vrhovac, and B. Pokrić, *Ocular Immunol. Inflamm.* **5** (1997) S52–S53.
4. P. Mansour, R. D. Adams, P. E. Isaacs, J. C. Ridway, N. G. Flanagan, and. C. J. Harrison, *J. Clin. Pathol.* **43** (1990) 694–695.
5. W. Vormittag, E. Maida, A. Totseva, E. Kunze-Muhl, and K. Summer, *Wien. Klin. Wochenschrift* **93** (1981) 724–729.
6. E. D'Allesandro, M. Di Cola, M. L. Lo Re, C. Vaccarella, F. D'Andrea, C. Marini, and M. Prencipe, *Am. J. Med. Genet.* **37** (1990) 406–411.
7. L. Adorini (Ed.), *Selective Immunosuppression: Basic Concepts and Clinical Applications*, Karger, Basel, 1995, pp. 20–31.
8. D. A. Hafler and H. L. Weiner, *Chem. Immunol*. **60** (1995) 126–149.
9. V. Kumar and E. Sercarz, *Chem. Immunol.* **60** (1995) 1–19.
10. N. Štambuk, V. Brinar, V. Štambuk, M. Rudolf, I. Svoboda-Beusan, S. Rabatić, N. Zurak, Z. Brzović, B. Marušić-Della Marina, R. Mažuran, T. Marotti, V. Šverko, M. Trbojević-Čepe, R. Martinić, B. Malenica, A. Gagro, K. Karaman, Z. Sučić, I. Dujmov, and. B. Pokrić, *Period. Biol.* **98** (1996) 527–532.
11. N. Štambuk, V. Brinar. V. Štambuk, I. Svoboda-Beusan, S. Rabatić, R. Mažuran, B. Marušić-Della Marina, Z. Brzović, N. Zurak, T. Marotti, V. Šverko, M. Rudolf,

R. Martinić, K. Karaman, N. Mašić, Z. Sučić, and B. Pokrić, *Int. J. Thymology*, **5** (1997) 448–464.
12. N. Štambuk, V, Brinar, V. Štambuk, I. Svoboda-Beusan, R. Mažuran, S. Rabatić, B. Marušić-Della Marina, N. Zurak, Z. Brzović, T. Marotti, V. Šverko, M. Rudolf, M. Trbojević-Čepe, R. Martinić, B. Malenica, N. Mašić, A. Gagro, K. Karaman, Z. Sučić, I. Dujmov, and B. Pokrić, *Peptid-M (LUPEX®) Effects on the Immune Response and Clinical Status in Uveitis, 0ptic Neuritis and Multiple Sclerosis*, in: *Uveitis Today,* S. Ohno, K. Aoki, M. Usui, and E. Uchio (Eds.), Excerpta Medica ICS 1158, Elsevier, Amsterdam, 1998, pp. 319–322.
13. N. P. Plotnikoff, R. E. Faith, A. J. Murgo, R. B. Herberman, and R. A. Good, *Clin. Immunol. Immunopathol.* **82** (1997) 93–101.
14. I. S. Zagon, J. W. Sassani, and P. J. McLaughlin, *Am. J. Physiol.* **268** (1995) R942–R950.
15. I. S. Zagon, Y. Wu, and P. J. McLaughlin, *Am. J. Physiol.* **267** (1994) R645–R652.
16. I. S. Zagon, S. D. Hytrek, C. M. Lang, and P. McLaughlin, *Am. J. Physiol.* **271** (1996) R511–R518.
17. N. Štambuk, *Int. J. Thymology* **5** (1997) 487–491.
18. N. Štambuk, *On the Optimization of Complementary Protein Coding*, in: *Uveitis Today,* S. Ohno, K. Aoki, M. Usui, and E. Uchio (Eds.), Excerpta Medica ICS 1158, Elsevier, Amsterdam, 1998, pp. 315–318.
19. N. Štambuk, *Klin. Monatsbl. Augenheilkd.* **211** suppl. 5 (1997) 4.
20. J. E. Blalock and K. L. Bost, *Biochem. J.* **234** (1986) 679–683.
21. J. E. Blalock, *Nature Medicine* **1** (1995) 876–878.
22. IAEA. *Biological Dosimetry: Chromosome Aberration Analysis for Dose Assesment. Technical Reports Series No 260.* International Atomic Energy Agency, Vienna, 1986, pp. 59–63.
23. P. Perry and S. Wolff, *Nature* **261** (1974) 156–158.
24. L. Lamberti, P. Bigatti Ponzetto, and G. Ardito, *Mutation Res.* **120** (1983) 193–199.
25. R. Tice, E. L. Schneider, and J. M. Kary, *Exp. Cell. Res.* **102** (1976) 232–236.
26. M. Fenech, A. A. Morely, *Mutation Res.* **147** (1985) 29–36.
27. N. J. London, H. Contractor, S. P. Lake, G. C. Aucott, P. R. F. Bell, and. R. F. L. James, *Horm. Metab. Res.* **25** suppl. (1990) 82–87.
28. N. Štambuk, M. Hadžija, N. Ugrinović, I. Pavlić-Renar, M. Šunjić, T. Marotti, and V. Šverko *Ocular Immunol. Inflamm.* **5** (1997) S53–S54.
29. M. Hadžija, D. Šupe, L. Rumora, E. Pape-Medvidović, I. Pavlić-Renar, Ž. Metelko, and M. Slijepčević, *Period. Biol.* **97** (1995) 313–316.
30. M. deSmet, J. H. Yamamoto, M. M. Mochizuki, I. Gery, V. K. Singh, T. Shinohara, B. Wiggert, G. J. Chader and R. B. Nussenblatt, *Am. J. Ophthalmol.*, **110** (1990) 135–142.
31. O. Jardetzky and G. C. K. Roberts, *NMR in Molecular Biology*, Academic Press, New York, 1981, p. 154.
32. W. H. Graham, E. S. Carter II, R. P. Hicks, *Biopolymers* **32** (1992) 1755–1764.
33. N. Štambuk, N. Kopjar, K. Šentija, V. Garaj-Vrhovac, M. Hadžija, B. Marušić-Della Marina, N. Ugrinović, Z. Svoboda, K. Karaman, and V. Štambuk, *Effects of Peptid-M (LUPEX®) on Cellular Cytogenetic Findings and Apoptosis in Uveitis and Retinopathy*, in: *Uveitis Today*, S. Ohno, K. Aoki, M. Usui, and E. Uchio (Eds.), Excerpta Medica ICS 1158, Elsevier, Amsterdam, 1998, pp. 323–324.

34. N. Štambuk, B. Pokrić, D. Vikić-Topić, S. Kazazić, V. Garaj-Vrhovac, N. Kopjar, P. Konjevoda, J. Pavan, A. Della Marina, *Ocular Immunol. Inflamm.* **6** (1998) S15.
35. P. Sikirić, D. Mikuš, S. Seiwerth, Ž. Grabarević, R. Ručman, M. Petek, V. Jagić, B. Turković, I. Rotkvić, S. Miše, I. Zoričić, J. Perić, P. Konjevoda, D. Perović, L. Jurina, M. Hanževački, J. Šeparović, M. Gjurašin, S. Jadrijević, N. Jelovac, P. Miklić, G. Buljat and A. Marović, *Dig. Dis. Sci.* **42** (1997) 1029–1037.
36. H. A. Menard and M. El-Amine, *Immunol. Today* **12** (1996) 545–547.
37. K. K. Wang and P. W. Yuen, *Trends Pharmacol. Sci.* **15** (1994) 412–419.
38. L. F. Tseng, J. H. Tsai, K. A. Collins, and P. S. Portoghese, *Eur. J. Pharmacol.* **277** (1995) 251–256.
39. S. J. Oneill, M. A. Collins, H. O. Pettit, R. W. McNutt, and K. J. Chang, *J. Pharmacol. Exp. Therapeut.* **282** (1997) 271–277.
40. C. Nath, M. B. Gupta, G. K. Patnaik, and K. N. Dhawan, *Eur. J. Pharmacol.* **263** (1994) 203–205.
41. N. Štambuk, *Graefe's Arch. Clin. Exp. Ophthalmol.* **232** (1994) 572–573.
42. M. Noguchi, A. Sarin, M. J. Aman, H. Nakajima, *Proc. Natl. Acad. Sci. USA* **94** (1997) 11534–11539.

SAŽETAK

Citogenetski učinci Met-enkefalina (Peptida-M) na ljudske limfocite

Nikola Štambuk, Nevenka Kopjar, Karmela Šentija, Vera Garaj-Vrhovac, Dražen Vikić-Topić, Branka Marušić-Della Marina, Vesna Brinar, Milica Trbojević-Čepe, Neven Žarković, Božidar Ćurković, Đurđa Babić-Naglić, Mirko Hadžija, Niko Zurak, Zdravko Brzović, Roko Martinić, Vjera Štambuk, Paško Konjevoda, Nikola Ugrinović, Ivana Pavlić-Renar, Zdenko Biđin i Biserka Pokrić

Analizirani su građa, komplementarna građa i citogenetski/proliferativni učinci Met-enkefalina na limfocite iz periferne ljudske krvi. Met-enkefalin, odnosno Peptid-M (LUPEX®), nisko je molekularni sintetski pentapeptid koji odgovara timusnom Met-enkefalinu. Utjecaj Met-enkefalina na *in vitro* normalizaciju kromosomski aberantnih limfocita bolesnika s raznim autoimunim bolestima ispitan je osjetljivim citogenetskim testovima za pregled i otkrivanje oštećenja na ljudskim limfocitima. Testovi su pokazali da *in vitro* stimulacija ljudskih limfocita Met-enkefalinom dovodi do nestajanja raznih oblika kromosomskih aberacija, smanjenja broja mikronukleusa, smanjene učestalosti sestrinskih kromatidnih izmjena i apoptoze te citostatskog učinka na diobene cikluse. Također je potvrđeno da je došlo do normalizacije kromosomski aberantnih stanica kod pacijenata s raznim autoimunim bolestima. Ovi rezultati ukazuju na moguću ulogu Met-enkefalina (Peptida-M) u imunoterapiji raznih bolesti kod kojih dolazi do kromosomskih aberacija kao i nenormalne stanične proliferacije, a ujedno omogućuju nove pristupe imunoterapiji Peptidom-M. Sukladno teoriji molekularnog prepoznavanja i SCA metodi uobličen je i sintetiziran peptid, komplementaran Peptidu-M, koji je nazvan Peptid-D. Peptid-D je dio kalpastatina. Pretpostavljena interakcija tipa ligand-receptor između dva peptida potvrđena je rezultatima koji su pokazali da se, ovisno o dozi dodanog Peptida-D, smanjuje proliferacije limfocita izazvana Peptidom-M.