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ABSTRACTS FROM „THE SECOND SCIENTIFIC CONFERENCE OF THE CHARLES UNIVERSITY FACULTY OF MEDICINE AND TEACHING HOSPITAL“, 16 -17 DECEMBER 1997, HRADEC KRÁLOVÉ

Abstracts of papers of „The Second Scientific Conference of the Charles University Faculty of Medicine and Teaching Hospital“ contain summaries of research projects completed through different grant agencies.

Differentiation markers in human malignant glioma explant cultures

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Differentiation inducing agents, such as hexamethylene bisacetamide, sodium butyrate, retinoic acid, phenylacetate or lovastatin are agents in experimental use that are capable of inducing morphological and biochemical differentiation in malignant tumor cell lines in culture and are believed to be a promise for the future treatment of glial tumors. Intermediate filament proteins, like GFAP, vimentin and nestin are being used as tumor markers to evaluate the degree of differentiation - malignancy of glial tumors; GFAP being positive mainly in low-grade astrocytomas and nestin in anaplastic astrocytoma and glioblastoma multiforme. In this respect, detection of differentiation antigens in testing the therapeutic effectiveness of differentiation inducing drugs is a methodological prerequisite. In our study, the immunoreactivity for GFAP, vimentin and nestin was detected in human glioma explants grown in vitro. A specimen of tumor obtained at the operation theater was dissected in ice cold glucose-saline medium and put in culture as an explant of 2-3 mm in diameter on poly-L-lysine coated slides. The other part of the tumor was processed for neuropathological diagnosis. Tumor explants were cultured in DMEM supplemented with 10% FCS, L-glutamine (2 mM), and gentamicine (10 mg/ml) at 37 °C in an incubator with humidified atmosphere with 5% CO_2. Immunoreactivity for the above mentioned differentiation markers of fixed monolayers of tumor cells was compared with histological sections of the same resected specimen.

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Consequences of reduced plasma cholesterol fractions during hypolipidemic therapy, lipoperoxidation activity and the distribution of antioxidant vitamin E in lipoprotein fractions

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Introduction: There is evidence that statins may have other anti-atherogenic effects besides their lipid-lowering activity, including effects on oxidability of lipoproteins. Thus the aim of present study was to examine consequences of reduced plasma cholesterol during hypolipidemic therapy, lipoperoxidation activity and the distribution of antioxidant vitamin E in lipoprotein fractions.

Material and Methods: A group of 54 patients with hypercholesterolaemia was treated using simvastatin (14 patients, 20mg daily), pravastatin (15 patients, 40mg daily) or fluvastatin (10 patients, 40mg daily). To investigate antioxidant effects of fibrates, another group of 15 patients was treated with fenofibrate (200mg daily). Blood samples were examined before treatment, after 4 and 8 weeks of therapy. After ultracentrifugation, samples were analyzed for vitamin E content in lipoprotein fractions. Antioxidant status was examined using serum thiobarbituric acid reacting substance (TBARS) activity.

Results: Both simvastatin and pravastatin were effective hypolipidemic agents I. With simvastatin, total serum vitamin E was reduced during hypolipidemic therapy (44.54±3.62 vs. 36.85±1.72 μmol/l; p=0.06). However, the ratio of serum vitamin E/total serum cholesterol (4,86±0.31 vs. 5.63±0.28 μmol/mmol; p=0.09) and ratio of LDL-C vitamin E/LDL-C (3.57±0.31 vs. 3.67±0.31 μmol/mmol; n.s.) did not change, and the ratio of IDL-C vitamin E/IDL-C (4.44±0.32 vs. 5.40±0.61 μmol/mmol; p<0.01) and HDL-C vitamin E/HDL-C (3.78±0.41 vs. 5.83±0.49 μmol/mmol; p=0.01) significantly increased. Serum TBARS significantly decreased (6.97±0.69 vs. 4.72±0.48 μmol/l; p<0.001). II. With pravastatin, serum vitaminu E was decreased in the fractions of total cholesterol, LDL1, LDL2 a VLDL-cholesterol. However, the ration vitamin E/total cholesterol (4.57±0.32 vs. 5.12±0.37 mmol/l/mmol/l; p<0.05) a ratio LDL2-C vitamin E/LDL2-C (3.92±0.07 vs. 4.64±0.37 mmol/l/mmol/l; p=0.08) increased vs. baseline. III., IV. The results obtained with fluvastatin and fenofibrate will be available within one month.

Conclusion: We conclude, that effective hypolipidemic treatment with statins is associated with improved antioxidant status and proportional increase in the serum content of vitamin E in HDL and IDL cholesterol fraction, and that the content of vitamin E in total and LDL-cholesterol did not change despite the decreased concentration of its lipid carrier. With regard to functions of vitamin E, this may be an additional anti-atherogenic effect of such therapy.

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Improvement of practical training in molecular biology at Charles University Faculty of Medicine in Hradec Králové

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Molecular biology is one of the most rapidly growing biomedical disciplines with immediate impact on clinical practice, mainly in the field of diagnosis. Therefore it is essential to accommodate curricula at medical faculties. This is true not only for graduate training but for postgraduate training as well. The curricula will be insufficient without practical courses in important basic techniques of molecular biology.

The main problem connected with the introduction of molecular biology is a high demand for financial resources. Therefore teachers from several departments at our faculty (biology, biochemistry, microbiology, immunology, genetic) joined and prepared a co-ordinated scheme for teaching practicals focused on molecular biology.

The main goal of this project is further improvement of practical classes from molecular biology. In our original proposal we would like to introduce the following new techniques: isolation of genomic DNA from human cells, agarose gel electrophoresis of DNA, PCR, blotting and hybridisation of nucleic acid. All these methods belong to basic standard in modern molecular laboratory. Due to the fact that the proposed budget was substantially lowered (to the 35 % of proposed value), we prepared a new reduced version which will still allow introduction at least some essential techniques. During this year we prepare protocols for new practical classes. This laboratory classes are now obligatory for students of biology. Newly built laboratory facilities are utilised also by several postgraduate students.

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Development and validation of new methods for the assessment of toxic effects of stomatological materials

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The goal of this project is to compare tests recommended for toxicity assessment of stomatological materials by the international standard ISO 10933 (Millipore filtration test, Agar diffusion test) with tests recommended by the Czech Committee for New Dental Materials (Cell proliferation assay, Direct contact assay) with newly developed photometric tests based on cultivation of cells in microtitration plates (96 well plates). During the last three years we have tested the toxicity of metal dental alloys (TI 45), root filling materials (AH26, Dexamethasone), and this year we have focused our attention on composite restorative materials (Evicrol Solar LC) and dental amalgams (ANA 2000).

Our results demonstrate a basic correlation between the different cytotoxicity tests. Nevertheless, in several cases we have found surprising differences. Therefore we recommend using a battery of the following basic tests as a standard: Agar diffusion test, Cell proliferation assay and Mitochondrial activity assessment by WST-1 assay. Based on our results we will prepare a proposal for the Committee for New dental materials. Optimised standard operating protocols for each tested protocol are available.

Results of this project were presented at 15 scientific meetings, six of them abroad, and 25 scientific papers related to the project were published.

Submitted by Grant: IGA MZ No 3263-3.

The Value of Doppler Ultrasonography for Screening of Renovascular Hypertension

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Purposse: To assess the diagnostic accuracy of Doppler ultrasonography (DUS) for the detection of renal artery stenosis (RAS).

Materials and methods: Between January 1995 and July 1997, 142 kidneys in 72 hypertensive patients were studied with DUS. Combined technique of the direct insonation of the renal artery (DDUS) and quantitative analysis of early systolic upstroke of distal Doppler waveform (IDUS) was performed in each kidney. Achieved feasibility for DDUS was 85 % and for IDUS 98 %. The diagnostic criteria for RAS (50 % or more) were as follows: PDUS - maximum systolic velocity more than 150 cm/s, IDUS (according to Stavros) - acceleration time more than 70 ms, acceleration less than 300 cm/s^2. The results were compared tho those from intraarterial digital subtraction angiography (DSA) in a prospective unbiased manner. Forty nine kidneys with RAS were found in DSA. Based on DSA as a gold standard, sensitivity, specificity, positive predictive value (PPH) and negative predictive value (NPH) were calculated for each kidney in order to estimate the diagnostic accuracy of DUS in detection of RAS. In a limited cohort (41 kidneys, 11 RAS), the results of captopril scintigraphy (CS) were also evaluated.

Results: DUS yielded the sens. 94 %, spec. 75%, PPV 67 %, NPV 96%, the results of CS were less favorable : sens. 50 %, spec. 86 %, PPV 60 %, NPV 80 %.

Conclusion: According to our results, Doppler ultrasonography can be used for screning of renovascular hypertension.

Supported by Grant: IGA MZ No 2965-3.

Localization diagnostics of pancreatic islet-cell tumors

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Purpose: To assess the diagnostic value of various diagnostic methods (ultrasonography- US, dynamic incremental contrast enhanced CT - DICECT, intraarterial contrast enhanced CT - CTAG, conventional angiography - AG, arterial stimulation venous sampling - ASVS, endoscopic ultrasonography - EUS, somatostatin receptor scintigraphy - SRS) for detection of neuroendocrine tumors of pancreas (gastrinomas, insulinomas).

Material: We have found 21 surgically (13) and diagnostically (10) proved functioning islet-cell tumors in 14 patients during 3 year period.. There were 7 insulinomas in 4 patients and 14 gastrinomas in 10 patients. The average tumor size was 15 mm.

Results: Accurate localizations were obtained in 3 of 8 (37 %) insulinomas and 5 of 14 (36 %) gastrinomas with US, in 2 of 8 (25 %) insulinomas and 5 of 14 (36 %) gastrinomas with DICECT, in 5 of 8 (63 %) insulinomas and 5 of 6 (83 %) gastrinomas with CTAG, in 1 of 8 (13 %) insulinomas and 3 of 7 (43 %) gastrinomas with AG, in 1 of 8 (13 %) insulinomas and 2 of 2 (100 %) gastrinomas with ASVS, 0 of 1 (0%) insulinoma and 8 of 8 (100 %) gastrinomas with SRS, and 1 of 1 (100 %) insulinoma and 7 of 8 (88 %) gastrinomas with EUS.

Conclusions: In cases of searching for insulinoma, the first step should be the EUS, followed by DICECT and CTAG. On the other hand, SRS is the most sensitive first step method for localization of gastrinomas. When SRS is positive, it should be followed by EUS, DICECT or CTAG for more detailed topical information.

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Lyme Borreliosis: Development of reccombinant DNA as an internal positive control of polymerase chain reaction for molecular detection of borrelia

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At the very begining of PCR introduction into detection methods of pathogens, the false positivity caused by carry over contamination arrised as the problem. As soon as the UNG enzyme was introduced in the reaction, problem of the false negativity rested to be solved. Inhibitors of TAQ polymerase present in the biological sample even after the DNA purification decrease the detection limit of PCR dread fully. The best way how one can be sure that the negativity of the sample is the real negativity is introduction of the internal positive control. Spiking the biological fluids and tissues with arteficially enlarged PCR target molecules makes all the detection more relliable. As amplicons arising from positive controle templates show quite longer DNA fragments than those obtained with speciemens, electrophoresis bands are easily distinquished. Making dicision to construct such a positive control, we have chosen chromosomal DNA target published by Rosa (Rosa PA et al. J Clin Microbiol,1991;29(3):524) for that purpose because of best detection limit achieved in our lab (35 fg DNA). Arteficial sequence 56 bp was synthetized in a common phosphoramidite manner carriing a HIND III cohesive end at the 3'site and the PST I restriction site at the 5'end. Amplicon 365 bp of Borrelia garinii M 192 was cleaved by HIND III into two fragments (210 bp + 155 bp). The smaller one was ligated with arteficial sequence and the molecule 211 bp was selected by PCR. By the means of site directed mutagenesis the new restriction site was introduced into the molecule 210 bp. Both molecules were cleaved by PST I, ligated and the new fragment 421 bp was selected by PCR. Ligation into the pUC 19 plasmid and subsequent cloning in E.coli followed. Recombinant plasmid DNA can be used as an internal positive control of PCR.

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New cytostatic agents - problems of their cardiotoxicity and potential cardioprotective activity

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The study was aimed to investigate cardiotoxic effects of anthracycline derivative (daunorubicin), a new antineoplastic agent - Oracin (6-[2-(2 hydroxyethyl) aminoethyl]-5,11-dioxo-5,6-dihydro-11*H*-indeno[1,2-*c*]isochinoline hydrochloride) and of cardioprotective effects of dexrazoxane in daunorubicin cardiomyopathy in rabbits in vivo. The effects of drugs (administered repeatedly, once weekly,

10 administrations - daunorubicin /50 mg/m^2 i.v./, Oracin /10 mg/kg i.v./, dexrazoxane /60 mg/kg i.p./) were evaluated on the basis of non-invasive polygraphic recordings (PEP:LVET ratio), dP/dtmax., biochemical, haematological and histological parameters. It is possible to conclude that:

1) Results obtained in the daunorubin-induced cardiomyopathy (significant, progressive increase in the PEP:LVET ratio, decrease in dP/dtmax., changes in biochemical, haematological and histological parameters) corresponded with the cardiotoxicity of daunorubicin and confirmed the adequacy of selected methods.
2) A cardioprotective effect of dexrazoxane during daunorubicin treatment was found in the experimental model used in our study.
3) Repeated administration of Oracin (10 mg/kg i.v.) induced mostly non-significant changes in polygraphic, biochemical, haematological and histological parameters and did not induce signs of cardiotoxicity in rabbits in vivo. This observation is considered to be important from the viewpoint of the therapeutic use of the drug.
4) Changes in cholinesterase activities (a significant decrease of the BuChE activity in the cardiac septum and ventricles in the daunorubicin group and a protective effect of dexrazoxane on cholinesterase activity) may reflect their possible role in cardiomyopathy.

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Familiar hypertrophic cardiomyopathy detection of mutations in exon 13 of beta-myosin heavy chain

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Familiar hypertrophic cardiomyopathy (FHC) is genetically and clinically heterogenic disease. From genetic viewpoint, FHC is an autosomal dominant inherited disease with various extent of penetrance. The most dangerous clinical consequence of FHC is heart failure and sudden cardiac death, also in otherwise asymptomatic individuals, often at young age. One third to one half of all cases of FHC is explained as a result of a mistake in the structure of gene coding for β-myosin heavy chain (MYH7) on chromosome 14 q11-q12. Most of all mutations on gene MYH7 is concentrated to several exons, especialy to xon 13.

We performed DGGE (denaturing gel gradient electrophoresis) detection of PCR products comprising tested locus. Methods based on DGGE enable to investigate the whole exon and are convenient for primary search for mutations, especially at a larger number of samples tested parallelly. The method is based on enzymatic amplification (PCR) of tested DNA samples using primers with GC clamps, that enable to test melting behaviour of the whole chain of DNA with all domains including that not detectable without GC clamps.

As we did not have any confirmed positive DNA sample at disposal, we constructed positive controls using mutagenesis protocol. We constructed 3 positive samples with most frequent mutations of exon 13 described on codon 403: G->A: 403 $^{Arg->Glu}$, G->T: 403 $^{Arg->Leu}$, C->T: 403 $^{Arg->Trp}$. Melting behaviour of the constructs were studied on perpendicular DGGE and conditions convenient for testing all samples on parallel DGGE were established. The mutated constructs were loaded on gel parallely with samples of tested patient's amplified DNA.

Until now, we have investigated 54 DNA samples coming from patients with FHC well clinically diagnosed, especialy by ultrasonography. We have not found any positive DNA sample although elsewhere approximately one third of samples is described to be positive. We conclude that two explanations are possible: either Czech population differs from that investigated before and described in literature and most mutations are present on different site of MYH7 gene, or tested patients' phenotype does not result from mutation of MYH7 gene at all.

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Development of computer controlled multithermocouple unit for temperature measurement in hyperthermia

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In the second year of the project we solved these problems:

1) The electro-thermal model of the multithermocouple probe was defined on the basis of analysis of thermal properties of materials and the arrangement of the probe. We created an interactive program that enables the correction of errors of temperature measurements caused by the thermal interaction between successive thermocouples inside the probe. This program enables us to estimate the thermal properties of new designed probes.
2) We developed, tested, and realized the connection between the probe and measuring unit (card in the PC with A-D convertor). The common reference point of the probe, Pt sensor for its temperature measurement and the connectors are situated in the external shielded box with thermal isolation. It gives us an option to place the unit outside the room with HF generator to eliminate possible interactions.

3) We theoretically described the origin of parasitic thermovoltage which occurs in all points behind the measuring point lying in the sharp thermal interface. It was shown and verified that this parasitic thermovoltage can be partly eliminated by the appropriate choice of materials of common wire of the thermocouples. As a positive side effect of this phenomenon we develop a special „continuous" probe for the measurement of temperature maximum in given length of the thermocouple probe.
4) The experiments with HF generator (433.92 MHz, l40 W) on the phantom proved that it isn't necessary to use only on/off mode of heating.

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The development of antioxidant blood activity and the changes of lipoprotein subfractions during hypolipidemic therapy

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The extralipid mechanism whereby hypolipidemic therapy reduces coronary disease risk is not completely known. The changes of lipoprotein subclasses and changes of antioxidant activity of blood during hypolipidemic therapy where studied in connection to this question.

Cholesterol and triacylglyceroles were examined by enzymatic method (kits Lachema, Brno) in lipoprotein classes after ultracentrifugation (Beckman TL 100, Palo Alto, CA). Vitamin E was analysed by HPLC (Hewlett Packard 1084 A, Palo Alto) and fluorescence detector (Perkin Elmer MPF-3, Norwalk, CT). The whole antioxidant status of serum was measured using thiobarbituric acid reacting substances activity (TBARS) by a fluorescence method. The software Solo 4.0 was used for statistical analysis (paring T-test, mean level and standard deviation, criterion of significance $p \leq (0,05)$.

I. Fifteen patients were treated by 40mg of **pravastatin** during the period of 8 weeks. We found favourable significant changes in whole lipoprotein classes: total cholesterol 9,85±2,35, 6,81±1,63, 7,92±2,15 mmol/l, VLDL cholesterol 1,88±0,86, 1,28±0,68, 1,61±0,87 mmol/l, LDL1 cholesterol 4,56±1,58, 3,11±1,07, 3,36±1,43 mmol/l, LDL2 cholesterol (dense subfraction of LDL) 1,86±0,84, 1,42±0,52, 1,26±0,33 mmol/l. The vitamin E /cholesterol ratio significantly increased.

II. Nineteen diabetic hypercholesterolemic patients were treated by 300mg of **fenofibrate** for more than one year long period. In three control investigations during this period we found favourable hypolipidemic effect, which was associated with improving of diabetic hard exsudates of retina. The main decline of cholesterol was also in subfraction of dense LDL 2. Total cholesterol 7,50±1,64, 6,60±1,43, 5,80±1,04, 5,80±1,24 mmol/l, HDL cholesterol 1,40±0,51, 1,26±0,41, 1,20±0,37, 1,07±0,23 mmol/l, a LDL2 cholesterol 2,15±1,38, 1,77±0,84, 1,52±0,96, 1,37±0,77 mmol/l. Vitamin E/cholesterol ratio in subfractions of lipoproteins was not afected. The total/HDL cholesterol ratio decline and the level of apoprotein A increased significantly 1,33±0,38, 1,57±0,28, 1,47±0,35, 1,54±0,27 g/l.

III. The hypolipidemic effect of 20mg **simvastatin** daily treatment was studied in the group l4 hypercholesterolemic patients during eight weeks long period.The favourable effects was found in whole mesured parametres. Total cholesterol 9,28±0,56, 6,64±0,35 mmol/l, IDL cholesterol 1,76±0,15, 1,08±0,09 mmol/l and LDL cholesterol 3,8±0,35, 2,63±0,23 mmol/l. The changes in HDL cholesterol level 1,77±0,28, 1,17±0,41 mmol/l and vitamin E 44,54±3,62, 36,85±1,72 umol/l were not significant. The decline of TBARS was significant 6,97±0,69, 4,72±0,48 umol/l.

Conclusion: The changes of lipoprotein fractions and antioxidant serum activity during hypolipidemic therapy (by simvastatin, pravastatin, fenofibrate) are favourable. It may be part of explanation of non LDL hypolipidemic drugs effect on improving of coronary heart disease risk.

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Amino acid metabolism in different forms of liver injury

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Four separate experimental studies were performed within this project:

1. To assess the effect of pathogenesis of liver injury on the plasma amino acid pattern four models of hepatic injury were studied (partial hepatectomy, liver ischemia, carbon tetrachloride induced acute liver damage and carbon-tetrachloride induced liver cirrhosis). We conclude that a high increase of most amino acids is characteristic of fulminant hepatic necrosis; decreased BCAA/AAA ratio is characteristic of liver cirrhosis; and decrease of BCAA/AAA ratio may not be used as an indicator of the severity of hepatic parenchymal damage. The results were published in *Amino Acids 1996;10:229-41.*

Study 2 and 3: In order to investigate the pathogenesis of reduced plasma levels of BCAA in liver cirrhosis, we

have evaluated the rates of leucine turnover, oxidation and incorporation into proteins in cirrhotic and in partially hepatectomized rats *in vivo* and in the isolated perfused liver. The results indicate that the predominant mechanism decreasing plasma BCAA levels in cirrhosis is an increase in the oxidized leucine fraction. The results were published in *J. Hepatol 1996;24:209-16* and *in J Hepatol 1997; 26:1141-7.*

Study 4: The changes of individual plasma amino acid levels were assessed in relation (1) to the severity of liver damage and (2) to the process of liver recovery. The study demonstrated the more pronounced decrease of BCAA than of AAA during the recovery period of liver damage. The results are accepted for publication in *Amino Acids.*

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Determination of the algorithm for the complex Lyme diseases diagnosis and the humoral and cellular immunity factors exercising influence on the Borrelia persistence in the host organism

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Parameters of cells mediated immunity were evaluated (population and subpopulation of T cells, activated T cells and naive and memory T cells.) Lymphocytes were stimulated in vitro and the presence of membrane activation markers was measured by flow cytometry. The polymerase chain reaction was used to detect DNA of Borrelia burgdorferi sensu lato in synovial fluid, CSF or in plasma. In spite of the fact that detection limit achieved with primers by hybridizing within 16S rRNA gene was 50 bacteria per sample, the sensitivity of the whole reaction based on the retrospective study in patients was not sufficient. Manifestations of Lyme borreliosis is described in 120 patients with direct proof of Borrelia burgdorferi sensu lato (immunoelectron microscopy). Using a commercial kit for the examination of recombinant immunoblot the authors examined serum of 85 patients with direct evidence of Borrelia burgdorferi sensu lato in serum or cerebrospinal fluid or patients with typical dermal form of borreliosis. The results were compared with the results of assessment of specific antibodies by the ELISA test. The specificity and sensitivity of the investigated test were not sufficient. Examination of the immunoblot was negative in 8.8% of patients with direct evidence of the causal agent of Lyme borreliosis.

Supported by Grant: IGA MZ No 2962-3.

Significance of serum interleukin - beta levels in hairy cell leukemia and correlation with tumor mass and sIL-2R levels

Follow up of RdW values and dyserythropoiesis and their correlation with sIL-2R values

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Serum inteleukin-beta levels do not reflect the tumor mass in hairy cell leukemia (HCL). In contrary serum interleukin 2 receptor (sIL-2R) levels were found to be a reliable non-invasive marker of HCL tumor burden. The sIL-2R levels were increased in all 15 patients before the iniciation of the therapy with 2-chlorodeoxyadenosine (median 1350 pM/ml, range 188 to 9000 pM/ml) and decreased after the successful therapy (median 84,3 pM/l range 37,0 to 382 pM/ml).

RdW values which reflect anisocytosis and dyserythropoiesis were avaluated in 18 patients with HCL treated with 2-chlorodeoxyadenosine (2-CdA) and in 5 patients treated with interferon-alpha (IFN-alpha). The mean value of RdW in the group of patients treated with 2CdA was 18,2% before therapy, 14% after 6-12 months and 14,2% after 18 months, with corresponding levels of hemoglobin 119,6 g/l, 145,2 g/l and 143,3 g/l respectively. In 5 patients treated with IFN-alpha the RdW value dropped from the median of 21,3% (range 19,2 to 28,7%) before therapy to the median of 15,3% (range 12,4 to 16,7%).

Bone marrow findings in respect to dyserythropoiesis and incorporation of iron into the erythroblasts were avaluated in 16 patients treated with 2-CdA. Definite dyserythropoietic changes were found in 3 patients, disturbed incorporation of iron with coarse granules or increased number of granules was found in 7 patients. These changes disappeared after successful therapy. In one patient association of HCL with sideroblastic anemia was encountered. These findings show that dyserythropoiesis may participate on the anemia in HCL.

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The endoscopic adenoidectomy

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Contrary to classical adenoidectomy where the operation is blind, endoscopic adenoidectomy is an operation checked as a rule optically on a screen. This led to a qualitative chan-

ge as regards the accuracy of indication as well as the operation proper. The operation must be performed in a standard manner under general anesthesia which substantially reduces the patient's stress, the number of complications and the number of necessary re-adenoidectomies.

We can use two methods: transoral approach with 70° degree optic and transnasal approach with 25° degree optic. Both aproaches enable us to check the whole curettage visually.

First - a child is less stressed during the operation, second - there is complete amnesia of the unpleasant surgery, and finally - safe curettage is checked visually. If the operation team is well organized, the time spent on operation can be greatly shortened, 15 or 20 minutes one endoscopic adenoidectomy.

In the long term follow up it is expected that it will improve the results of treatment of common chronic inflammations of the nasal cavity and paranasal sinuses and otitis media with effusion in children.

We conclude, that the endoscopic adenoidectomy under general intratracheal anaesthesia is a great qualitative progress in ENT.

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Relationship of women hormonal treatment to blood flow of leg-venous with used of duplex sonography

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Introduction: The research explores the relationship between two groups of women with hormonal treatment. We examined 76 women with HRT (hormonal repleace treatment) and 75 women with OC (oral contraceptive) older than 30 year. The examination was made before the treatment, and 3, 6 and 12 months after beginning of the treatment.

We examined biochemical parametres (bilirubin, ALT, AST, GMT, cholesterol, HDLC, TAG and LDLV), electrophoresis of the blood protein (total protein, A1G, A2G, BG, GG and A/G), hemocoagulation's parametres (APTT, APTT patient/control, fibrinogen, d-dimer, antithombin III., APC/APTT, APC, protein C and protein S), Leiden mutation of the factor V. by the women with APC resistance. Next we investigated complete angiologic examination (Trendelenburg, Perthes, circuit of the leg, duplex sonography of the leg-venous, D-PPG, pulsation, murmers, ankle pressure, ultrasonography of the leg-arteries, family history, history, smoke, varix of the leg, job).

Results: We compared two groups of the women with hormonal treatment by use of the pair and unpair statistic test. Some of the results : in the bichemical parametres has the OC group higher level of ALT and AST after 12 months of the treatment than HRT group. The OC group has the changes in the HDLC, TAG a LDL. The results in electophoresis are the same in both groups. We dicovered 8 women with APC resistance in the HRT group and 8 women with APC resistance in the OC group. We found 4 women with Leiden mutation of the factor V in the OC group. In the same group are changes in the angiologic examination.

Conclusion: It is essential to addict the attention to women with OC after 30. We are projecting the questionnaire before starting of the OC by the older women with questions about tromboembolism and leg-venous problems in family history and history and select the high risk group of women. By this group will be urgent to examine APC resistance and leg-venous system by angiology examination and think about prescription of the OC.

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Algorithm of food allergy examination, introducing of DBPCFC (double-blind placebo-controlled food challenge)

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Food allergy is unadequate reaction of the organism on the ingestion of food caused by immunological mechanisms. Its diagnosis and diferential diagnosis is very complicated. Double-blind placebo-controlled food challenge is in the literature described as the gold standart.

The aim of the research project was to elaborate the algorithm of the examination of the food allergy and introduce the double-blind placebo-controlled food challenge (DBPCFC) into the clinical practise.

50 examination in 17 patients with the suspicion on the food allergy was performed. In 22 examinations (44%) was the reaction positive and the elimination diet in these patients was succesfull. In the rest of the examinations (56%) DBPCFC was negative, in 50% of them the reintroduction of the suspected food was possible and remained without reaction. 50% of the false negative give a reason for a necessity of open exposition of the food, if the DBPCFC is negative.

If the food allergy is suspected, the following algorithm of the examination is recommended - anamnesis, physical examination, prick tests, total and specific IgE in the blood, DBPCFC and open exposition of the suspected food, if the previous tests are negative.

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Study of intestinal permeability in dependence on small bowel damage, possibilities of therapeutical influence

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The damage of small bowel mucosa in the course of various diseases is possible to investigate using the measurement of small bowel permeability.

The aim of research project was to introduce test of small bowel permeability into clinical practise. The permeability test with lactulose and mannitol was used, the concentration of both sugars in 5 hours collected urine was measured using cappilary gas chromatography.

Index of small bowel permeability was increased in patients with untreated coeliac disease, after gluten-free diet it returned to the normal value, the same results we found during examination of children. On the contrary this index was not changed in patients with food allergy. The increased value of small bowel permeability was found (in the agreement with literature) in critically ill patients on ICU, in patients with active Crohns disease and ulcerative colitis, in children with cystic fibrosis, especially in homozygotes with alela delta F508.

In patients with Crohns disease and ulcerative colitis the value of small bowel permeability correlated with the other parameters of activity of these diseases, in patients with tumours of gastrointestinal tract is the permeability increased 7 days after the end of the cytostatic therapy.

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Cardiovascular system in healthy and pathological neonates

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The level of serum TnT in the cord blood of term healthy neonates was determined. Normal value = 0.05±0.03 ug/l is comparable with dates published in the literature. These observed dates served as a control group.

The level of serum TnT in venous blood was determined in 10 neonates after severe perinatal asphyxia. The mean value of TnT was significantly elevated. That result confirmed presumed myocardial damage during severe hypoxia. The similar results have been already published in the literature.

We monitored influence of tocolytic therapy (beta-mimetics), used as a routine method to prevent premature labour, on the foetal myocardium. 70 neonates after tocolytic therapy was included to our study. Three various beta-sympatomimetics were used to prevent labour - fenoterol, ritodrine, terbutalin. Results received in this study:

The mean level of serum TnT in the cord blood was significantly elevated after acute infusion tocolysis. TnT values were the most frequently elevated when pregnancy were finished 2-3 days after beginning of therapy. This result corresponds with knowledge that maximum side effects of tocolytic therapy can be observed during the first 3-4 days of tocolysis.

The mean level of serum TnT in the cord blood was not significantly elevated after preventive long term orally given tocolytics drugs. Nevertheless TnT level was elevated above 2 sigma of normal value in 9 neonates.

We did not found any difference in TnT level when various tocolytic drugs were compared.

Conclusion: troponin T could become suitable marker of myocardium damage also in neonatology. It seems that known negative side effect of beta-mimetics tocolytic was proofed in our study with the use of troponin T. Elevated level of troponin T also proofed myocardial damage after severe birth asphyxia in our study. Next studies will be necessary to confirm our result.

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Troponin T in neonatology

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In our study 25 pregnant women were assessed with the respect to potential drug-induced cardiotoxic effects. We used the determination of cardiac troponin T (cTnT) that represents one of the most sensitive and specific method for detection of myocardial damage.

The cTnT concentration was measured in maternal venous blood obtained 24 hours after the beginning of therapy. In case that the therapy was not successful and did not last more than 4 days we tried to compare the levels of cTnT in the maternal venous blood just before labour and cTnT in the cord blood of neonates.

The cTnT activity in 15 healthy pregnant women (control group) without any therapy who were matched for week of gestation (33.5±0.5) was (0.01 ±0.00 ug/l). The cTnT activity in the pregnant women during the first day of treatment was in the physiological range (0.09±0.03 ug/l) and sigificantly increased during the next 2-3 days of the tocolytic therapy (0.27±0.13 ug/l). The cTnT levels in the cord blood of neonates (0.13±0.03 ug/l) did not correspond with cTnT concentration in their mothers.

We tried to verify transport of cTnT across the in situ perfused rat term placenta. Troponin T was found to cross the rat placenta from the maternal-to-foetal direction easily. Time to reach equilibrium was within 30 minutes, when foe-

to-maternal concentration ratio (FMCR) reached maximum value 1.2. Then FMCR exceeded mildly the value 1 and both concentration curves descended with the slope 0.0020.

Conclusion: Our results should give evidence that cTnT can cross through placental barrier both in materno-foetal and foeto-maternal direction in experimental rats.

The infusion tocolytic therapy can increase venous blood concentration of cTnT in the pregnant women.

The fact that cTnT levels in maternal venous blood and cord blood of neonates are not the similar can reflect the limited transfer of cTnT through the human placenta.

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Prediction of vancomycin and aminoglykosides dosage regimen at the preterm neonates in the first week of postnatal age. Part - Pharmacokinetics of vancomycin at neonates

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The purpose of this study was:
- to examine the relationship between vancomycin pharmacokinetics and various indices of maturation in a population of critically ill neonates.
- to investigate a dynamic pharmacokinetic model based on Bayesian algorithm.

Pharmacokinetic population parameter estimates were determined in the 1st Group of neonates (15 preterm and 6 full term neonates, 27-41 wks of postconceptional age).After the first dose of vancomycin hydrochloride (30 mg/kg/day over a 60 min infusion) 4-5 serum vancomycin concentrations were fitted by nonlinear least-squares regression analysis to a one-compartment infusion model. Linear regression was used to determine significant relationships ($p<0.05$) between pharmacokinetic parameters and patient characteristics.

The mean for the apparent volume of distribution Vd was 0.699 L/kg (SD 0.366 l/kg), for vancomycin clearance CL 0.077 L/h/kg (SD 0.054 L/h/kg). The mean population parameter estimate for Vd was set at 0.699 L/kg . We entered the equation that explained the relationship between vancomycin clearance and creatinine clearance Clcr into the model ($r=0.61$, $p<0.01$):

$$CL = (0.527 \times Clcr)+0.0206$$

The Vd and CL were allowed to be fitted during the Bayesian estimation (ABBOTTBASE Pharmacokinetic Systems, Abbott Laboratories). Inputs of initial peak and trough concentrations of vancomycin in Group 2 of neonates (17 preterm and 3 full term, 25-41 wks of postconceptional age) were used as feedback information for the Bayesian estimation of subsequent concentrations. Using population-based parameters and creatinine clearance the mean error ME and the mean absolute error MAE for predicting concentrations were 0.453 mg/L and 2.75 mg/L.

Conclusions: The vancomycin clearance and volume of distribution was directly related to postconceptional age by linear regression analysis.The study found a significant correlation between vancomycin clearance and creatinine clearance. Vancomycin dosage should be prescribed based on postconceptional age and weight. In view of the interpatient variability in pharmacokinetic parameters observed in the neonates, monitoring of vancomycin concentration is necessary to attain safe and effective therapy. The use of population-specific pharmacokinetic parameters and Bayesian forecasting insure an accurate dosage regimen design to achieve steady-state peak concentrations of 20-40 mg/L and trough concentrations of 5-10 mg/L.

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Flow cytometry and double colour immunofluorescence in diagnosis of leukemias

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Flow cytometry was established for the immunophenotypic analysis of malignancies of hematopo- ietic origin. Double and triple immunofluorescence analysis of either unseparated or separated samples of peripheral blood, bone marrow, liquor, pleural and pericardial fluids was performed. Method for the measurment of cytoplasmic and nuclear molecules was developed. Initially, flow cytometry was run simultaneously with standard UV microscopy to compare the results. Totally samples of bone marrow and (or) peripheral blood obtained from 983 individuals were analysed. According to our results following panels of monoclonal antibodies for diagnosis of acute leukemias, lymphoproliferative diseases, hairy cells leukemias, multiple myelomas and myelodysplastic (myeloproliferative) diseases were proposed.

Acute leukemias: isotypic control, CD45/CD14, CD3/HLA DR, CD5/CD19, CD10/CD19, CD38/CD13, CD14/CD33, CD2/CD7, CD15, CD34, CD41, CD65, cTdT, cMPO, cCD3, cμ.

T-ALL: (extended panel) CD7/CD2, CD3/CD4, CD3/CD8, TcRαβ, TcRγδ, CD8/CD38, CD1a.

B-ALL: (extended panel) CD79a, CD79b, cCD22.

Multiple myeloma: isotypic control, CD45/CD14, CD3/HLA DR, CD5/CD19, CD13/CD38, CD14/CD33, CD34, CD15, CD38/CD138/CD56, CD38/CD138/CD54, cκ/CD138/CD56, cλ/CD138/CD56.

Chronic leukemias (lymphomas): isotypic control, CD45/CD14, CD3/HLA DR, CD5/CD19, CD10/CD19,

CD2/CD7, CD20, CD21, CD22, CD23, CD37, CD15, CD34, κ/λ, μ.

Hairy cell leukemias: isotypic control, CD45/CD14, CD3/HLA DR, CD5/CD19, CD10/CD19, CD2/CD7, CD3/CD4, CD3/CD8, CD20/CD25, CD19/CD25, CD20/CD11c, CD103, κ/λ, μ.

We conclude that immunophenotyping is the best approach to diagnose blood malignancies but re- sults have to be interpreted in context with clinical evaluation and other laboratory tests. Extended panels of monoclonal antibodies together with double- and triple staining and determination of intracellular molecules are useful in the delineating of unusual cases of leukemias. Clinical relevance of certain immunophenotypes was sought in our study.

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Adoptive immunotherapy - ex vivo generation of lymphokines activated cytotoxic cells and their clinical applications

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Samples of ascitic fluids and pleural effusions were obtained from 15 patients suffered from metastatic form of breast cancer, ovarial carcinoma and colon carcinoma. Tumor cells and adherent cells were removed from the suspension by nylon wool. Tumor infiltrating cells (TIL) were than expanded in AIM-V medium, therapeutic grade (GIBCO) supplemented by rGM-CSF and rIL-2. Duration of incubation was individual, up to 1 month. Different number of samples were processed from various individuals (up to 10 samples). Cytotoxic activity of TIL as well as their phenotypes were measured at the begining and at the end of cultivation using above mensched methods and panels of monoclonal antibodies.

Clinical aplications of The procedure for the generation of lymphokine-activated killer cells was developed. Briefly, density gradient separated mononuclear cells were incubated in RPMI-1640 supplemented with hu- man albumin up to 14 days. Optimal concentration of rIL-2 also in combination with rGM-CSF was used for the induction of LAK cells. Cytotoxic activity was determined by standard 51Cr release test at the beginning and than at finishing of cultivation. Changes in the expression of membrane molecules during cultivation were measured by flow cytometry using following panel of monoclonal antibodies: isotypic control, CD45/CD14, CD3/HLA DR, CD3/CD4, CD3/CD8, CD3/CD16+CD56, CD3/CD25, CD5/CD19, CD57/CD8, CD8/CD28, CD3/CD45RA, CD3/CD45RO, CD3/CD80, CD95/CD45, CD3+CD14/CD4, CD19/CD86, CD3/CD152 at days 0, 4, 7, 11, and 14. Correlations between particular phenotype and cytotoxic activity of LAK cells were sought.

TIL cells were performed in 1 patient. There were no significant side-effects. Therapeutic efficiency of this adoptive immunotherapy is under study now.

Guidlines for the complex care including immunotherapy (rIL-2, rINFα) or chemoimmunotherapy were developed for patients suffering from metastatic form renal carcinoma and malignant melanoma. Totally 32 patients were treated according to these guidlines. Efficiency of this complex approach to the treatment of cancer is now evaluated.

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Motion related visual evoked potentials (VEPs) and their diagnostic application

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Pattern and motion specific components were identified in the motion-onset VEPs (1). The motion-onset related VEPs were characterized in 94% of the population by a dominant negative peak (motion specific) with a latency in the range of 135 - 180 ms and maximum amplitude in lateral temporo-occipital leads. Stimulation of the lower half of the visual field gave larger responses in comparison with the upper half (1).

Contrast sensitivity of motion-onset VEPs and their dependence on spatial frequency of a moving pattern was tested. Large check sizes (> 30') provide detectable responses up to the minimum contrast (0.3 %), which gives evidence chat the motion related negative peak of the VEPs represents a magnocellular system activity (2,3).

Diagnostic applications of motion-related VEPs based on their specific properties (4,5,6,7) were verified. Motion specific VEPs provide new diagnostic possibilities because in comparison with any other VEPs they predominantly test the magnocellular system. The following profits can be achieved from their examination: higher sensitivity for visual pathway demyelination; better differential diagnosis of retrobulbar neuritis; early detection of subclinical optic nerve changes in glaucoma; peripheral visual field defects verification; objective testing of amblyopic eyes.

References:

1. Kremláček J, Kuba M, Kubová Z. Dependence of motion-onset VEP parameters on stimulus localisation. Proceedings of the XXXVth ISCEV Symposium, Asilomar (USA), July 20 - 24, 1997.
2. Kubová Z, Kuba M, Spekreijse H, Blakemore C. Contrast dependence of motion-onset and pattern-reversal evoked potentials. Vision Res 1995;35:197-205.
3. Kremláček J, Holčík J, Kuba M, Kubová Z, Vít F. Model of contrast sensitivity in visual perception of motion.

Bridging Disciplines for Biomedicine - Conf Proc, IEEE Engineering in Medicine, ISBN 90-9010009-9 (CD-ROM), 1996.
4. Kubová Z, Kuba M, Spekreijse H. VEP evidence for predominant parvocellular pathway impairment in retrobulbar neuritis. Invest Ophthalmol Visual Sci 1995;35:197.
5. Kubová Z, Kuba M, Juran J, Blakemore C. Is the motion system relatively spared in amblyopia? - Evidence from cortical evoked responses. Vision Res 1996;36:181-90.
6. Kubová Z, Kuba M, Svěrák J, Hrochová J. Motion-onset visual evoked potentials improve the diagnosis of glaucoma. Doc Ophthalmol 1996;92:211-21.
7. Kuba M, Kubová Z, Kremláček J, Svěrák J. Visual field examination via motion-onset VEPs. Vision Res 1995;35 (Suppl.): S166.

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New electrophysiological examinations for neuro-ophtalmological diagnostics

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New visual stimuli for visual evoked potentials (VEPs) were developed. Animation technique was used for stimuli generation on a PC monitor with special synchronization pulses (1).

Increase of VEP examination sensitivity in Multiple Sclerosis was achieved and differentiation of Neuritis Retrobulbaris and demyelination on the basis of the motion-onset VEPs seems to be possible (2,3).

Dynamic objective perimetry via motion-onset VEPs was performed (4,5) and a method for early detection of glaucomatous changes in the visual pathway was introduced (6).

Optimum localization of a reference electrode in secondary (associate) visual centers examination was tested (7). Activation of associate cortex excludes a use of any cephalic reference and either zygoma or ear lobes are recommendable.

The presented research results were verified in 700 neuro-ophthalmological patients.

International Society for Clinical Electrophysiology of Vision entitled us to organize the ISCEV Symposium in 1998 on the topic „Electrophysiological and related measures of motion detection".

References:

1. Kremláček J, Kuba M, Kubová Z, Vít J. Simple and powerful visual stimulus generator. Comput Methods Programs Biomed 1998 - in press.
2. Kubová Z, Kuba M. Motion-onset VEPs improve the diagnostics of multiple sclerosis and optic neuritis. Sb Ved. Pr Lek Fak Univerzity Karlovy Hradec Králové 1995;38:89-93.
3. Waberžinek G, Kremláček J, Kuba M, Kubová Z. Comparative study of electrophysiological methods in diagnostics of multiple sclerosis. Eur J Neurol 1997;4(Suppl. 1):S158.
4. Kuba M, Kubová Z, Kremláček J, Svěrák J. Visual field examination via motion-onset VEPs. Vision Res 1995;35(Suppl.):S166.
5. Kremláček J, Kuba M, Kubová Z. Objective perimetry based on motion-onset VEP examination. Proceeedings of the XXXIVth Symposium ISCEV, Tübingen, July 20-24, 1996, Poster Nr. 70.
6. Kubová Z, Kuba M, Svěrák J, Hrochová J. Motion-onset visual evoked potentials improve the diagnostics of glaucoma. Doc Ophthalmol 1996;92:211-21.
7. Kuba M, Kubová Z, Kremláček J. Relevant reference electrode for motion-onset VEP recordings. Proceeedings of the XXXIVth ISCEV Symposium, Tübingen, July 20-24, 1996, Poster Nr. 71.

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Immunocyt ochemistry of the anterior pituitary and of pituitary tumours. Correlation between immunocytochemical and clinical findings

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Normal human anterior pituitary, 48 pituitary adenomas found at autopsy and 90 tumours removed at surgery were studied immunocytochemically (ICC) using monospecific polyclonal (PABs) and monoclonal antibodies (MABs) against hGH, hPRL, ACTH, hTSH, hFSH, hLH, and alpha-subunit. Both the direct and indirect ICC methods were employed with the antibodies labelled with fluorochromes (FITC, TRITC, AMCA) for the immunofluorescent techniques, enzymes (HRP, AP) for the immunoenzymatic techniques, and with colloidal gold for the immunogold silver staining (IGSS) technique. ICC detection of 3 different pituitary hormones in one single tissue section simultaneously was achieved using antibodies tagged with different fluorochromes or with 2 enzymes and colloidal gold. A combination of immunofluorescent, immunoenzymatic, and IGSS techniques made it possible to detect any number of pituitary hormones in one single tissue section by the successive use of fluorescence and light microscopy. The following conclusions may be drawn from the results obtained: (1) Monospecific PABs are superior to MABs, since false negative results may be obtained with MABs in some adenoma cases, where PABs give a clearly

positive result. (2) For this reason, the number of the null-cell adenomas may also be lower with the use of PABs, especially if the sections have been pretreated in the microwave oven. (3) In plurihormonal adenomas, only double or triple ICC staining of the same tissue section can justify the diagnosis of plurihormonality of the tumour and differentiate these tumours from unihormonal tumours containing other non-tumorous pituitary cells engulfed by the expanding tumour from the surrounding pituitary. While in true plurihormonal adenomas all tumorous cells follow the same histological pattern of the adenoma, false positive plurihormonal tumours contain cells that do not obey this pattern. It may prove misleading to make a diagnosis of a plurihormonal tumour on the basis of findings obtained in adjacent sections of the tumour. (4) The plurihormonal tumours were, for the most part, mixed adenomas associated with the production of two or more hormones in distinct tumorous cells. However, we have also found tumours consisting of one cell type producing both GH and PRL (clinically acromegaly and symptoms of hyperprolactinaemia), GH and gonadotrophins (clinically acromegaly and hypergonadotrophinaemia), and GH and TSH (clinically acromegaly and thyrotoxicosis). (5) The 48 autopsy cases included also 5 cases of multiple pituitary adenomas, of which 2 cases were associated with the production of multiple hormones in each of the adenomas. (6) Of the 48 autopsy cases, there were 17 cases of silent corticotroph cell adenomas. (7) Plurihormonal adenomas often displayed normal blood plasma levels of the respective hormones found in the tumours.

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Children coping with distress

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It is very difficult to study all the problems concerning children's coping with distress because of all the possible diseases and various life conditions. Therefore we decided to choose several medical and school situations. The age distribution of children was from 6 - 18 years. Coping with distress we define as a multidimensional, dynamic, sequential and reciprocal problem. It is necessary to take into account the personality of the child, the specific situation of the child, child s perception of the situation etc.

The results of our project come from three working groups:

J. Křivohlavý collected and evaluated 14 methods describing and analyzing children coping with distress. The methods are: KIIDKOPE (Spirito), CSI (Tobin), SRCMC (Causey, Dubow, Roth, Causey), CI (Zetlin), WCQ (Folkman, Lazarus), GOS-W (Jerisalem, Schwarzer), Hope Scale (Gottschalk, Gleser), LECI (Dise-Lewis), CISS, CBSS (Combo-Richets), RLI (Range). He also evaluated 7 methods for discovering social support in coping with distress: SSQ6 (Sarason), social self-conception (Stevenson-Hinde), PYL (Marzialli), PYL (Furukawa), PCF (Ladd), MMFF (Stevenson-Hinde), SRSC (ROSS, Lehmkuhl). The methods most convenient for the Czech conditions were translated and adapted by him. J.Křivohlavý prepared field research for the year 1998, which should show us events children themselves consider stressing and also find out how children aged 6 - 16 yr. try to cope with them.

A. Furman studied theoretical approaches to children coping with distress at school. He made a large library research, started a survey study and he also introduced an empirical probing for children of middle school.

The group of J. Mareš and M. Králová studied three theoretical models of children coping with distress (Rudolph, Denning, Weisz; Peterson, Oliver, Saldan; Skinner, Wellborn). They translated the questioner of children coping with pain WV/PPCI (Waldron, Varni) and verified it using a sample of 93 healthy and 53 hospitalized children. The research of children perception of hospital distress situation and provided social support by drawing-verbal method of J. Mareš continued. A new study of children coping with dental pain situations and children adapting on locomotor aparatus pain was introduced.

After a theoretical preparation and verifying of chosen methods (the main target of year 1997) all groups start empirical research which is planned for years 1998 and 1999.

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Screening for defects in the metabolism of tryptophan

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An algorithm for screening of defects in the metabolism of tryptophan (Trp) has been evaluated: Thin-layer chromatography at the first stage of examination is followed by two methods, employing high-performance liquid chromatography (HPLC) on reverse phases. Clinical symptoms, indicating for examination in both the „indole-" and the „kynurenine-metabolic pathways", were listed; *Chromatographia 1997;45:195-8, Acta Med (Hradec Králové) Suppl. 1997;1:67-73.*

UV/VIS detector, allowing the scanning in the process of analysis was bought (by the financial means of the grant) and set in motion.

A new procedure for sample pre-treatment before HPLC analysis has been elaborated, utilising the Sep-Pak cartridge, the gradient of dodecylsulphate in methanol for the successive cleaning of sample and the ammonia-methanol mixture for the elution. The manual technique was modified using the vacuum unit „manufold" for 12 samples (the purchase of the equipment was sponsored by IGA).

Our investigations are pointed to the establishment of the excretory pattern of the healthy and the sick controls, where the search for defects in Trp catabolism has not been clinically indicated so far. We intend to screen a biological material (urine above all), collected in a large group of children of various age, symptoms and diagnoses, sent to our laboratory for the general screening of inherited metabolic diseases. At present, 115 children have been examined, using both of the two HPLC methods.

We are going along with the investigations in a group of burn patients and in those suffering from some skin diseases (some changes in the excretion of the Trp metabolites upon those diagnoses have been described before).

Previous co-operation with a unique laboratory (Dept. Immunol. and Gnotobiol., Instit. Microbiol., Acad. Sci., Nový Hrádek, CZ) was renewed, enabling us to proceed in our research program when raking over the origin of some metabolites of Trp in urine of mammals, in connection with their pathological excretion in man. We have processed urine and plasma samples (72), obtained from a group of both conventional and germ-free piglets (Minnesota-derived breed) of different age, bred under various dietetic conditions. The evaluation of all analyses is not yet completed.

Here are we presenting the poster report, given at the *5th Internat. Congress on Amino Acids, Kalithea, Chalkidiki, Greece 25. - 29. 8. 1997* (the abstract published in *Amino Acids 1997;13(1):38*, the paper accepted for publication in extenso).

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New cytostatic and immunosuppressive drugs

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The primary aims of these in vivo and in vitro studies are to obtain further information with respect to antitumor activity, therapeutic index and resistance that could be investigated for its circumvention by resistance modulating agents.

1,4-dihydroxy-5,8-bis{[2-/(2-hydroxyethyl)amino/ethyl] amino}anthraq uinone dihydrochloride, mitoxantrone, is potent antitumor agent and active substance of REFADOR® Inj. SPOFA. Indications are breast carcinoma (including advanced stage of the disease), malignant lymphoma of non-Hodgkin type, acute leukemia in adult patients, chronic myeloid leukemia and hepatocellular carcinoma. Mitoxantrone reveals pronounced inhibitory effects on DNA and RNA polymerase systems. A great attention is given to the relation betveen its cytotoxicity and topoisomerase enzyme activity. Overexpression of P-glycoprotein is referred to be a mechanism of resistance.

In experiment on L1210 leukemia bearing female DBA2 mice (variant developed for resistance to mitoxantrone, L1210/MX) antitumor efficacy and therapeutic synergism of mitoxantrone and levocarnitine was tested. Mitoxantrone was administered intravenously in the doses of 6 and 3 mg/kg (D1 and 7). Levocarnitine was administered subcutaneously in the doses of 200 and 100 mg/kg (D1, 7 and 8). Survival of experimental animals was observed for 60 days after leukemia inoculation. Survival time as a biological response was evaluated and the effective doses were calculated from the dose-response curves and equations according to Carter W.H., Jr. (1). The Cox's proportional-hazards model:

$$\lambda_{(t)} = \lambda_{(t)}\, exp(x'\beta) \qquad (1)$$

with x'β as a second-degree polynomial in the dosage levels of the drug was used to estimate the relationship between treatment levels and length of survival and statistically evaluated using Snedecor F-test. The experiment revealed an antitumor efficiency in combination of mitoxantrone (3 mg/kg i.v., e.g. 9,6 mg/m^2, D1 and 7) and levocarnitine (the optimal dose 78 mg/kg, e.g. 250 mg/m^2) administered on D1, 7 and 8 ($p<0.001$). Mitoxantrone and/or levocarnitine in monotherapy were ineffective.

Acute toxicological study was performed in albinotic NMRI female mice. Ten animals per dose level were used. Behavioural changes and exitus were registered 45 days after drug administration and were evaluated according Berkson (2). LD 50 of mitoxantrone alone was 15.18 mg/kg i.v. and that of mitoxantrone in combination with levocarnitine was 18.00 mg/kg i.v. The relative toxicity of the combination was 76 %.

Efficacy of mitoxantrone in monotherapy and in combination was studied in „in vitro" experiments on short-term cultured L1210/MX leukemia cells. The procedure described by Miko et al. (3,4) with minor modification (5) was used. IC50 values representing the concentration of the drug halving the initial rate of simultaneous incorporation of ^{3}H or ^{14}C from [6-^{3}H]thymidine and [U-^{14}C]amino acid mixture into the acid-insoluble fraction of cells were calculated for 2-deoxy-D-glucose and its combination with mitoxantrone. No therapeutic synergism of 2-deoxy-D-glucose and mitoxantrone was observed.

Total energies of compounds were calculated after optimalization of their structures by MM+ and/or AM1 methods (5,6) using program Hyperchem 2.0 Autodesk,

Inc. and interactions of molecules were simulated. No interactions between mitoxantrone and levocarnitine were found. Interaction between mitoxantrone and cofactor of mammalian telomerase was discussed.

References:

1, Carter WH Jr, Wampler GL, Stablein DM, Campbell ED. Cancer Res 1982;42:2963-71.

2. Berkson J: J Am Stat Assoc 1955;50:529.

3. Miko M, Drobnica L', Chance B. Cancer Res 1979;39:4242.

4. Miko M, Drobnica L'. Neoplasma 1969;16:161.

5. Mattern J. In: Human Tumours in Short Term Culture. Dendy PP, ed. London, New York, San Francisco: Academic Press, 1976.

6. Allinger NL. J Am Chem Soc 1977;99:8127-34.

7. Stewart JJP.J Comp Aided Molec Design 1990;4(1):1-108.

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Composite dermoepidermal graft for burn treatment

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The application of the composite dermo-epidermal graft prepared in vitro from allogeneic acellular deepidermized dermis (DED) and autologous keratinocytes represents one of the possible approaches to treatment of full-thickness burns. In our experiments, we focused on in vitro preparation and the morphological examination of two types of the composite dermo-epidermal graft: 1) using the dermal component and keratinocytes harvested from one dead donor-i.e. „autologous" model of the composite graft, 2) using the dermis and keratinocytes taken from different dead donors - i.e. „allogeneic" model. The dermal component, the DED, with destroyed cellular elements was prepared by the method of Krejci et al. (J. Invest. Dermatol. 1991, 97, 843-848) and stored at -80°C. The primary culture keratinocytes were seeded in suspension on the papillary surface of the DED and were growing as submerged culture in presence of 3T3 cells for 10-13 days. In both in vitro models, confluent epithelial sheets gradually adopting the character of a stratified squamous epithelium were obtained. In sites of increased layering of the epithelium, keratohyalin was present in keratinocytes of the upper cell strata showing differentiation of cells. Nevertheless,some defects in the cultivated epithelia were also detectable (regressive changes in some cells, probably reflecting worsening nutrition conditions in multicellular cultures). The dermal border line showed irregularities (i.e. occasional dermal „papilae" interdigitating with epithelial pegs) and ultrastructurally, various patterns of the attachment of basal keratinocytes to the dermis including that, mimicking the normal dermal-epidermal junction were detected.

Beside the above cultivation procedure the cocultivation of cadaveric keratinocytes and the deep-frozen DED without the presence of 3T3 fibroblast feeders was performed. We also made preliminary trials with cultivation of keratinocytes on the DED stored at +4°C and keratinocytes cultured on a hyaluronic ester membrane.

Our hitherto experience with in vitro preparation of the composite grafts shows that these models, with their structural appearance of the dermal-epidermal junction, to a certain extent recapitulate the in vivo situation. This represents the phenomenon favourable for maintaining cohesion between the epithelium and the underlying tissue bed. On the contrary, formation of the flat dermal-epidermal junction after transplantation of the only keratinocyte sheets on deeply excised burn wounds is described as major disadvantage leading to increased fragility of the restored skin cover. The results we have obtained are encouraging for further experimental and prospective preclinical studies.

The study was presented at scientific meetings of the European Tissue Culture Society (Mainz, FRG, October 1997) and at the international symposium „Current Therapy in Burns" (Bydgoszcz, Poland, October 1997).

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Cultivation of neural precursor cells and modification of their further development in vitro

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The inverted microscope NIKON ECLIPSE TE 300 that was purchased from instrument investments provided by GA CR enabled a detailed study of formation of multicellular spheroids by neural precursor cells (NPCs) in vitro. Dissociated NPCs isolated from the fetal rat brain were cultivated in serum-free medium supplemented with epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) or a mixture containing EGF plus bFGF. Phase contrast microscopy revealed that mitogenic growth factors caused proliferation of NPCs and formation of multicellular spheroids under all the above specified conditions. The inverted microscope enabled a taking of tiny samples of spheroids from cultures in the course of the earliest stages of their cultivation. The spheroids were processed for histology at light and electron microscopical levels. Peroxidase immunohistochemistry utilizing a broad set of primary antibodies provided information on expression of characteristic antigens in EGF-responsive spheroids yielded from

primary cultures at different time-points and confirmed that composition of spheres changed over time in that aspect that EGF-responsive NPCs tended to differentiate inside of spheroids. Although the tissue blocks of bFGF-responsive NPCs embedded in paraffin await for processing, a preliminary observation of different adhesive properties of cultured bFGF- and EGF-responsive spheroids suggests that both subsets of NPCs may reveal different properties.

The study of the inner structure of spheroids composed of EGF-responsive NPCs on semithin resin-embedded sections stained with toluidine blue and paraffin-embedded sections stained with haematoxylin and eosin or propidium iodide confirmed the presence of degenerating cells with pycnotic nuclei. These were abundant even in the tiny early spheroids formed by immature proliferating NPCs. Our finding of segmented nuclei with condensed chromatin is considered to be a criterion of apoptotic cell death; nevertheless, we were unable to identify the DNA fragmentation in these cells using the TUNEL method. The floating spheres can adhere together and form large structures of bizarre shapes. This property is more pronounced in cultures with high density of plated NPCs and in media with reduced concentrations of growth factors. Fusion of neurospheres containing NPCs derived from different regions of the CNS makes this model attractive for confrontation studies. Prior to a further study of interactions between neurospheres and brain tumours we performed preliminary cultivations of explants of human brain tumours. To ensure standard results in experiments it proved necessary to introduce cultures of stable experimental cell lines (C6 glioma and NG108 mixed astroglioma/neuroblastoma). At present we carried out immunophenotypization of these cell lines grown in monolayers.

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Cultivation of neural stem cells and their transplantation into the recipient s brain

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In this project, IGA MZ enabled us to purchase a basic equipment for cultivation of fetal mammalian cells. We adopted the method for isolation and cultivation of neural precursor cells (NPCs) in serum-free medium supplemented with epidermal growth factor (EGF) originally developed by Reynolds et al. (J. Neurosci. Res. 1992, 12, 4565-4574). In the course of this 3-year grant project, we modified the original procedure for processing the fetal rat brain and cultivation of neural cells which enabled us to increase significantly the numbers of EGF-responsive NPCs harvested from cultures.

Under a mitogenic effect of a human recombinant analogue of EGF, Long-EGF, NPCs expressing appropriate receptors were stimulated to proliferate and formed multicellular structures of spheroidal shape, the so called neurospheres. We successfully reproduced experiments with neurospheres plated on polylysine-coated surface in medium supplemented with serum that confirmed the ability of NPCs to emigrate from the core of neurospheres and differentiate. Experiments with passaging dissociated spheroid cells confirmed that some spheroid cells retained their ability to form secondary neurospheres.

Our histological study of inner structure of neurospheres brought original results on changes showing in composition of the neurospheres during the extended cultivation periods. Immunophenotypization of spheroid cells confirmed a gradual maturation of NPCs inside of neurospheres: while the early neurospheres were composed of cells that expressed markers specific for immature neural cells like nestin or vimentin and proliferating cell nuclear antigen (PCNA), the neurospheres yielded after the prolonged cultivation contained only sporadic mitotic cells and many cells expressing antigens characteristic of mature neural cell lines, i.e. glial fibrillary acidic protein (GFAP)-immunoreactive astroglia, myelin basic protein-immunopositive (MBP^+) oligodendroglia and neurofilament$^+$ (NF^+), synaptophysin$^+$ and microtubule associated protein 2^+ ($MAP\text{-}2^+$) neurons. Light and transmission electron microscopy of paraffin- and resin-embedded neurospheres gave evidence that these solid three-dimensional formations consisted of heterogeneous population of neural cells that were able to undergo gradual differentiation; the neurospheres yielded 115 days after establishment of primary culture revealed, besides chemical synapses, sporadic myelinated fibres suggesting maturation of neuronal and oligodendroglial cell lines.

Parallel to the histological study of neurospheres we processed the intact brains of fetal and postnatal rats for histology including immunohistochemistry. To identify low levels of antigens in the developing CNS we embedded the brains and spheres in low-melting paraffin (Polyester wax) and applied the Catalyzed signal amplification (CSA) method which enhances the sensitivity of the immunodetection. These techniques enabled to detect specific markers of mature neural cell lines at earlier stages of development than routine procedures (i.e. formalin-fixed and paraffin-embedded tissue). Gradual appearance of characteristic markers and structures in EGF-responsive neurospheres over extended periods of time mimicked corresponding phenomena occurring in the development of mammalian brain. Our original results of phenotypic changes occurring inside of neurospheres proved that spheroids of EGF-responsive NPCs represent a novel three-dimensional model for the study of neurogenesis in vitro.

Preliminary trials with grafting NPCs into the forebrain of adult rats suggest the cells cease to proliferate early after

transplantation that prevents to increace their numbers. Nevertheless, the applied cultivation method enables to increase the amount of native NPCs in vitro prior grafting. The process of gradual differentiation of NPCs that occurs in primary cultures can be eliminated or interrupted by dissociation of neurospheres and subsequent passaging dissociated spheroid cells in EGF-supplemented serum-free medium.

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Antibodies and cytokines in serum and cell cultures in children suffering from primary immunodeficiencies

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There were prepared protocols to examine the cellular immunity in children. The panels were designed to make the diagnosis of the child on the base of investigated surface makers on the surface of the immunocompetent cells and on the base of the answer to the stimulation of the cells by mitogens, antigens, and specific antibodies to the surface receptors. Indications for the examination are clinical or clinical and laboratory signs of the disease. Therefore the panels are used for clinical praxis as well as for the research purpouses. By this time is required to interpretate the results individualy because there are no reference ranges for most of the examined surface markers (and those which are available are not age corrected). There are also prepared the clinical protocols to reduce the duplication of laboratory tests to reach diagnosis and to reduce the cost of the examination.

There were selected children with severe immunodeficiencies which were diagnosed clinicaly and laboratory and more precious diagnosis took place using the previously mentioned panels. There are patients suffering from both types (humoral and cellular) of the immunity defects (X-linked hypogammaglobulinemia, Common variable immunodeficiency, Wiskott-Aldrich syndrome, DiGeorge syndrome etc.). There are measured serum levels of immunoglobulins in patients who have IVIG substitution and there are studied the changes with predicted and actual serum levels and the influence of acute infections to the followed up parametres. The age of patients varies from the infancy up to early adulthood.

The laboratory approach to reach optimal results of cytokine production in supernatant of separated lymphocytes is investigated. There is used the stimulation by mitogens (PWM, PHA) to prepare the laboratory protocols. Some of the wery common and well known cytokines are used to prepare the laboratory approach (IL2 ets.).

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Children and parents

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The aim of the project was in improvement of the co-operation of children and parents with health services professionals in both - preventive and curative health care. There was the attempt to achieve the higher effectivity of the children care together with decrease of children stress due to contact with healt service. The picture booklet LUCY IS ILL was published and is suitable for the at school as well as at home. It is capable to prepare the child for adequate co-operation with physicians durin g the preventive examinations, vaccinations, and treatment of illnesses.

A videoprogramme was recorded to describe it is possible to make good conditions for small patients respecting The Chart of Richts of the Ill Child with very small amount of financial support. The prexence of parents during the treatment and examination of children in the departments and professionaly organised games casuse that the psychical state of children and knowledge of parents are improved. There are also enriched the skills and habits of parents to provide optimal home care, rehabilitation, and prevention. Due to them the whole life style of the family is well changed. The video mentioned earlier can become a useful instrument of motivation and education.

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Diagnosis of proarrhythmic effect of antiarrhythmic drugs

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Proarrhythmia can be a life-threating complication of antiarrhythmic therapy. The aim of this study was to determine if programmed stimulation of ventricles (PVS) is useful in recognizing proarrhythmia that may not be noted using noninvasive techniques.

The patients (52 men, 6 women, mean age 58±11,5) underwent in summary 123 PVS. The cause of antiarrhythmic therapy was CPR (21 pts), syncopy (15), preasyncopy (6), sustained VT (21). The drug administered was amiodaron (33 pts) and propapheron (12), the other used some of combination of antiarrhythmics. All patients underwent initial PVS and in 51 of them a subsequent PVS was repeated after an interval at least of 6 weeks after the treatment was started.

Criteria for proarrhythmia during PVS were present in 47% of pts (15 pts conversion of induced non-sustained VT to sustained VT or increase of the frequency of VT, 5 pts induction of arrhythmias with less agressive stimulation protocol, 8 pts new requirement for electrical cardioversion to

terminate tachycardia - 7 pts showed combinations of these criteria). Ambulatory Holter ECG was positive in 9 pts (only in 12% in association with positive PVS). There were no significant correlation between PVS and late potentials, ejection fraction or ergometry. We observed proarrhythmias in 33% of pts after amiodaron treatment and in 66% after propaphenon treatment. During follow up (mean 35±27 month) the pharmacotherapy was succesfull in 77%. There were 3 sudden seaths and 3 implantations of ICD - without any difference between pts with positive or negative PVS.

Conclusions: This study confirms that PVS is a usefull method for recognition of early patterns of proarrhytmia. The patients with higher cardiovascular risk should undergo this examination during antiarrhythmic treatment. Follow up data show that early diagnosis of proarrhythmia markers may be of very important value in guiding therapy.

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Development of programs for learning of medical chemistry and biochemistry

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Computer programs of three types for teaching medical chemistry and biochemistry are in development. The first type of such programs is graphic and interactive information from selected areas of chemistry and biochemistry. At present time the „Titration" is finished. This program gives basic information about acid-base titration and makes possible to try „virtual" titration of three model solutions (HCl, acetic acid or acetate buffer) or two „unknown" samples (HCl and HAc). Students can also try to calibrate interactively the pH-meter. The representative of the second type of teaching software is the interactive computer aid for statistical and visual evaluation of experiments using photometry. This program is in development now. Computer program for testing of chemical knowledge is of the third type of teaching software. This aid makes possible computer testing according to practice in the department of medical biochemistry of our school.

Remark: In consequence of the decision of the Ministry of Finance about allocation of money the work on development of educational software could start in october 1997. So, the planned time for programming was dramaticaly shortened.

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The changes of energy metabolism and body compartments during absolute fasting

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The aim of present study was to assess the influence of prolonged total fasting on resting energy expenditure (REE), thermic effect of nutrition (TEN), body compartments and plasma protein and lipid parameters.

Nine patients accepted to metabolic care unit because of obesity were studied before and after fourteen days of absolute fasting. During the period of fasting they received noncaloric fluids (water, tea, coffee), vitamins and potassium. Before and after fasting period their REE and TEN after mixed liquid meal (4 kcal/kg body weight; 55% CHO, 30% fat, 15% protein) were measured by indirect calorimetry (MMC Horizon, Sensor Medics). At the same time intervals serum samples were analysed for protein and lipid levels. During the fasting period balance trends were also followed.

The patients lost 9.98±1.6 kg of body weight during the fasting period. The total loss of 570.6±118.8mmol Na corresponded to loss of 3.96±0.83 kg of extracellular fluid. Protein loss reached to 0.89±0.20 grams which corresponded 3.54±0.80 kg of muscle tissue. Urinary sodium and nitrogen outputs decreased exponentially during the fasting period. Calculated reduction in fat stores reached 3.64±2.34 kg of fat tissue. This results corresponded to energy expenditure measured by the indirect calorimetry. The relationship of protein loss to fat tissue loss was higher during the first week of fasting.

REE decreased from 2334.4±171.7 kcal/d to 2000.5± 88.26 kcal/d, however TEN to mixed meal (4 kcal/kg b.w.) was not influenced by the fasting period.

These data shows us that during the first week of absolute fasting the body weight decrease is associated predominantly with water, mineral and muscle tissue loses. Adipose tissue decline can reach maximally 200 - 300 g per day. Energy expenditure decreases during fasting period without any changes in thermic effect of nutrition.

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The influence of antioxidant balance on clinical outcome and biochemical parameters in the aged - open prospective clinical trial

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The aim of the project was to evaluate the role of overall biochemical parameters in predicting mortality and morbidity in aged population. Thirty-eight nonagenarians aged 92±2 (range 90 - 100) years, followed at the Department of Gerontology and Metabolism entered the study. At the start of the study, a sample of peripheral blood and urine were obtained for analysis of 50 basic biochemical, hematological and biological parameters. The samples of urine and blood were then obtained in 6 - 12 months intervals.

The significance of difference between surviving subjects and those who died was examined by Mann-Whitney U test. Correlation was studied by Spearman rank correlation coefficient. The decision on significance was based on P = 0.05 level.

During the period of observation, 21 subjects died, leaving 17 persons still alive at the end of the study. The mean time from the first measurement to the death was 12±10 (range 0 - 33) months. The mean follow-up time in surviving subjects was 31±12 (range 4 - 45). The summary of results of the project can be seen in the table. Serum vitamin E and calcium were significantly higher, and serum ALT and urinary neopterin were significantly lower in survivors compared to the subjects who died. No other parameters were significantly different in survivors and in persons who died. Urinary neopterin exhibited a significant correlation with serum sodium concentration (rs = - 0.50, P<0.01), but the other parameters did not correlate significantly.

Summary of statistically important biochemical parameters based on survival

Variable	Unit of Measurement	Survivors			Died			P
		n	Mean (±SD)	Range	n	Mean (±SD)	Range	
Age	years	17	92 (2)	90 - 100	21	93(2)	90-98	N.S.
serum vit. E	µmol/l	17	27,9 (8,6)	10,7 - 41,2	21	21,0 (5,6)	8,3 - 30	0,02
ur. neopterin	µmol/mol crea	16	370 (131)	159 - 633	20	701 (552)	203	2479
serum calcium	mmol/l	17	2,28 (0,13)	1,93 - 2,46	17	2,21 (0,15)	2,09 - 2,71	0,02
serum ALT	µkat/l	17	0,30 (0,11)	0,11 - 0,55	21	0,42 (0,20)	0,20 - 1,07	0,03

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Three-dimensional organization of synapses and endoplasmic reticulum of dendritic spines

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Dendritic spines of cerebral cortex, synapses placed on them and changes of their number, shape and size (so called synaptic plasticity) are supposed to represent a structural basis for processes of learning and memory. That is why these structures have been in the centre of interest of many neurobiologists during last two decades. Results of our new observations on the spines and synapses were as follows:

A new model of morphologically inhomogeneous synapse is presented in which apart from an active zone associated with synaptic vesicles, an adherent zone free of vesicles appears. 45 % of synapses localized on dendritic spines (rat, hippocampal area CA1) contains this punctum adhaerens-like zone or vesicle free transitional zone.

64 % of all remaining puncta adhaerentia (i.e. of those not associated with synapses) are localized between large dendritic spines and astrocyte processes in their vicinity. A close functional relationship between synapses and astrocytes is thus morphologically fixed.

A smooth endoplasmic reticulum is associated with puncta adhaerentia and synapses. The amount of endoplasmic reticulum in parent dendrites is proportional to a number of spines and synapses originating along their lengths. On the small spines only small macular synapses are placed and only a small amount or no reticulum is present. The large spines contain a cisternal spine apparatus derived from reticulum and directed to the extensive „perforated" synapse.

The results strongly support the role of smooth reticulum in regulation of ionic microenvironment of synapse and adherent zones. The spine apparatus also appears to produce a building material for puncta adhaerentia.

References:

Špaček J, Harris KM. Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J. Neurosci 1997;17:190-203.

Špaček J, Harris KM. Three-dimensional organization of cell adhesion junctions at synapses and dendritic spines in area CA1 of the rat hippocampus. J. Comp Neurol 1997 (In print).

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Recurrent and chronic myotic vulvovaginitis. Improvement of diagnosis, therapy and prophylaxis

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Recurrent vulvovaginal candidiasis particularly its chronic form (four or more episodes per annum) is an unsolved problem in gynecology. Risk factors are usually absent in these women and there is often a remarkable discrepancy between symptoms (itching, burning, discharge) which are usually intensive and clinical findings which are mostly poor (absence of inflammation). In women with idiopathic RVVC, antifungal therapy is highly effective for eliminating topical clinical symptoms but not mycological eradication. This is the reason for failing to prevent next attack. Vaginal Candida infection is distinctly hormone dependent, occurring rarely in premenarcheal girls as well as in postmenopausal women.

In our set of patients (n=50) Candida albicans was prevalent in etiology of RVVC because it accounted for 86,9% of strains isolated from vagina. The remaining species were represented Candida glabrata (10% isolates), C. tropicalis, C. parapsilosis and Saccharomyces cerevisiae. It seems that the microscopic examination has little use for diagnosis of RVVC

in contrast to acute episodes. Culture is more sensitive method which in addition enables identification of a isolate at species level. Identification of etiologic agent and evaluation of its antifungal susceptibility in vitro is an important part of therapeutic management of RVVC. The decreased susceptibility of some non-albicans Candida especially to azole derivatives (fluconazole, ketoconazole) can contribute to failure of treatment in some cases of RVVC. Our experience suggests that for successful management of RVVC is necessary elimination of underlying factors. Gestagen administration (Depo-Provera) is one of the possibility as a alternative approach to management of women with RVVC. Our study is based on a group of 12 patients with chronic vulvovaginal candidiasis treated with Depo-Provera (administration 150 mg every 3 months by intramuscular injection). 6 patients (50%) are successfully treated = no symptoms, normal clinical and microbiological findings; 2 patients (16,66%) = improvement of symptoms, normal clinical and microbiological findings; 1 patient (8,33%) = unsuccessful treatment; 3 patients (25%) = beginning of the treatment.

The immunological examination of peripheral blood was performed. Among 27 parameters, 22 were normal, the other were slightly changed. Only IgE levels and CRP concentrations as well as monocyte count were very low, while neutrophile counts were often elevated as well as IgM. No remarkable changes between period of recrudescence and remission in individual patients were found. No patognomic changes in immunological parameters appeared. The parameters of local immunity are now under investigation.

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To the health risk of asbestos and others fibrous materials

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Thanks to the physical, chemical, and functional properties the asbestos fibres were commonly used in industry till the end of the last century. Nevertheless, asbestos has been gradually recognized as an agent laden with numerous high risks (proved complex human carcinogen). That is also why it has been replaced by natural, or more often, even artificial substances - MMMF (Man Made Mineral Fibres), which were considered to have a lower genotoxicity (carcinogenity) when compared with asbestos. The goals were to evaluate delay effects of fibrous materials and compare the genotoxic risk of MMMF with those of asbestos.

Occupational environmental monitoring and biological - medical monitoring of persons with present of previous exposition to fibrous materials have been performed. Four groups of investigated persons were established: 8 living persons with asbestosis as proved occupational disease, 21 persons formerly working with asbestos but not suffering from any illness, 15 persons who presently work with MMMF and 17 persons as a control group.

Conclusions: Cytogenetic survey of peripheral blood lymfocytes proved to be the most sensitive well illustrating biological exposition test for genotoxic load caused by fibrous material.

MMMF were found to be far more dangerous than expected. Their genotoxic effects are similar, or even worse, when compared with asbestos.

Workers formerly exposed to asbestos and those currently exposed to MMMF both belong into the group with a „high genetic risk“ (% of aberated cells -AB.C. > 4).

Persons with asbestosis have lower frequency of chromosomal aberrations after several years lasting exposition interruption.

Finding of an increased AB.C. percentage in the control group seem to witness that the whole enterprice belong into the zone of increased genotoxic risk.

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High dose chemotherapy with supportive care by whole blood rich in stem cells

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To support multicyclic, dose-intensive chemotherapy in breast cancer, we assessed the effects of reinfusing hematopoietic progenitors either as a leukapheresis product or as mobilized unprocessed whole blood. We tested three mobilisation regimens, standard FAC (fluorouracil 400 mg/m^2, cyclophosphamide 400 mg/m^2 and adriamycin 40 mg/m^2), HD-FAC (fluorouracil 400 mg/m^2, cyclophosphamide 1200 mg/m^2, adriamycin 40 mg/m^2) and EC regimen (epirubicin 150 mg/m^2 and cyclophosphamide 1250 mg/m^2). In regimens FAC and HD-FAC were yields of PBPC in whole blood insufficient for clinical use. In our study, 16 consecutive female breast cancer patients were given six cycles of chemotherapy regimen EC (epirubicin 150 mg/m^2 and cyclophosphamide 1250 mg/m^2 on day 1). In the firs cycle 24h after chemotherapy, mobilization of the peripheral blood progenitor cells (PBPC) was started with growth factor G-CSF (Neupogen, Amgen Roche) at a dose of 5 μg/kg/day for 13 days. In all other cycles G-CSF had been given at the same dose from day 7. On days 11,12 and 13 the leukaphereses were performed and their products cryopreserved. On day 14 whole blood was collected. The median peak incidence of CFU-GM (granulocyte-macrophage co-

lony-forming unit) in peripheral blood was approximately 50 times the baseline level. The leukaphereses PBPC were divided into portion and reinfused after the fourth, fifth and sixth chemotherapy courses. The support with mobilized whole blood was given after the second and third cycles. the best yields of leukaphereses were achieved on day 13 after initiation of chemotherapy. The mean number of CD34+ cells was $4.93x10^6$/kg (range 0.36-$10.54x10^6$/kg) the amount of CFU-GM was $2.18x10^5$/kg (range 0.07-$4.2x10^5$/kg). The yields of CFU-GM in 450 ml whole blood collected on day 14 reached $0.51x10^6$/kg (range 0.05-$1.5x10^6$/kg and CD34+ cells were $1.3x10^6$/kg (range 0.18-$2.58x10^6$/kg). PBPC yields in 450 ml of unprocessed whole blood were in some cases not sufficient for good hematopoietic recovery after EC cycles. Grade 4 leukopenias and thrombocytopenias were two times higher in cycles with whole blood support than in cycles with cryopreserved PBPC support. An increase of PBPC harvest can be simply achieved by collecting larger amount of unprocessed blood, as used by some authors. Hematologic effects of G-CSF and EPO combination after priming intensive chemotherapy in the treatment of female breast carcinoma were tested. We found that the administration of G-CSF and EPO combination following intensive chemotherapy reduces hematologic toxicity and induces large amount of hemopoietic progenitros suitable for autologous transplantation in women with breast carcinoma.

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Evoked potentials in early diagnosis of multiple sclerosis

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A scheme for multiple sclerosis (MS) examination was designed on the basis of results obtained from a group of more then four hundred patients investigated by various techniques of evoked potentials. The observations were completed with CT or MRI findings and cerebrospinal fluid analysis results.

The entire group of patients was examined for the parvocellular subsystem of the visual pathway by means of pattern-reversal visual evoked potentials (P-VEPs) and about 20% of the group underwent motion-onset VEPs (M-VEPs) examination of magnocellular subsystem.

Next to the visual pathway we inspected auditory pathway in about three hundred patients through brain stem evoked potentials (BAEPs) and in ninety patients we recorded somatosensory evoked potentials (SSEPs).

A single type of the evoked potentials yielded diagnostic sensitivity up to 70%. The evaluation already two types of evoked potentials increased the sensitivity up to 90% (1). The study confirmed generally accepted recommendation to combine various kinds of evoked potentials and suggest combine the P-VEPs and the M-VEPs as the first choice. In special questions concerning brain stem lesions the BAEPs should to be examined. Brain and spinal cord white matter lesions (demyelinisation) can be detected by SSEPs.

The electrophysiological methods seemed to be very important when the imaging techniques brought no positive findings especially in the early stages of MS.

References:

1. Waberžinek G, Kremláček J, Kuba M, Kubová Z. Comparative study of electrophysiological methods in diagnostics of multiple sclerosis, Eur J Neurol.1997;4(Suppl. I).

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The degree of cytokinenetwork activation and quality of renal allograft

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The aim of this project is to study the role of selected laboratory tests in prediction of renal graft quality. The study had two parts: methodological and experimental.

1) Methodological part: The possibilities of cytokine estimation in urine samples.

Patients and methods: This part of study included 11 children (6 boys, 5 girls) without renal disease. TNF-α, IL-1α, IL-6, IL-2 and sIL-2R in plasma and urine were measured using EIA kits (Immunotech). Estimations were performed without previous concentration of urine samples, except TNF-α (samples concentrated 5times with Minicon concentrators).

Results: TNF-α was not detected in urine of healthy children, IL-6 concentration was very low (0.2±0.1 pg/ml), IL-1α, IL-2 and sIL-2R were detected in urine of healthy children.

2) Experimental part: Comparison of blood and urine cytokine levels in kidney transplant donors and recipients. Patients and methods: 5 renal graft donors and relevant recipients were enrolled in this study. Blood and urine samples were taken in donors at the time of brain death diagnosis and then every 4 hours. Samples from recipients were taken approximately 6,24 and 72 hours after transplantation. Blood and urine concentrations of sIL-2R, IL-6, IL-1β were measured using ELISA kits (R&D Systems, USA). Selected results are presented as mean(SEM in pg/ml. Statistical analysis was made using Jandel Scientific software.

Results: Blood levels of IL-6 in donors (245.1±40.5) were higher than in recipients (37.9±7.7, $p<0.05$). IL-6 in urine was 11.1±2.4 in donors and 100.8±27.3 in recipients. Blood levels of sIL-2R in donors (60.4±30.0) were lower than in recipients (122.8±10.1, $p<0.05$).

Conclusion: These preliminary results and comparison with clinical findings in renal transplant recipients suggest that blood IL-6 concentrations could predict graft quality.

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ORIGINAL ARTICLE

ACTIVITY OF ALKALINE PHOSPHATASE IN THE MAJOR SALIVARY GLANDS OF MICE AT VARIOUS AGES OF POSTNATAL LIFE, AND DURING PREGNANCY AND LACTATION

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Summary: Activity of alkaline phosphatase in the major salivary glands of male and female mice at various ages of postnatal life, and in females during pregnancy and lactation was studied histochemically. Enzyme activity was not detected on the day of birth, but was found in the terminal tubules of all major salivary glands during the first postnatal week. Alkaline phosphatase activity was increasing gradually with age and a definitive enzymatic pattern was observed by the age of 6 weeks. No difference in enzyme activity was found among the major salivary glands of young adult and old animals. The parenchyma of fully differentiated submandibular glands showed clear sexually dimorphic patterns of alkaline phosphatase activity. During pregnancy, a significant increase of alkaline phosphatase activity was detected in submandibular gland. From gestation day 15 to the end of pregnancy, enzymic pattern of granular convoluted tubules of pregnant females was the same as in the adult males. Histochemical masculinization of the submandibular gland during pregnancy suggests that besides androgens also progesterone exerts masculinization of the murine submandibular salivary gland.

Key words: *Major salivary glands; Submandibular gland; Sublingual gland; Parotid gland; Histochemistry; Alkaline phosphatase; Sexual dimorphism; Mouse*

Introduction

The major salivary glands (MSGs) of mouse are the submandibular, sublingual and parotid glands. The submandibular gland (SMG) is situated on both sides of the midline on the floor of the oral cavity; the sublingual gland (SLG) closely adjoins at the anterolateral surface of the submandibular gland; the parotid gland (PG) lies dorsolaterally behind the ear (Fig.1). In mice, major salivary glands are not fully developed at birth and continue to differentiate during several postnatal weeks. The parenchyma of the salivary glands in newborn mice is composed of rudimentary secretory units known as terminal tubules (TTs) (30).The fully differentiated parenchyma of the rodent major salivary glands comprises morphological units composed of acini (Ac), intercalated ducts (IDs), striated ducts (SDs), and excretory ducts (14,25). In addition, the SMG of mice contains granular convoluted tubules (GCTs) representing secretory structures between the intercalated and striated ducts - they differentiate from the upper parts of the SDs postnatally (25,30) (Fig. 2). The SMG of mice is an androgen-dependent organ (8) showing sexual dimorphism in adult animals (1,7,14,17,23,28). This sex dimorphism is morphologically characterized by three features: 1) larger and more frequent GCTs in males, 2) fewer SDs in males, 3) granular IDs in females. The cells of mouse GCTs contain a lot of biologically active polypeptides, much more in males than in females (2).

Although MSGs of mice have been extensively studied morphologically (1,7,9,10,14,17,25,26,28,30,31), histochemical and immunohistochemical studies of mouse salivary glands are less frequently found in the literature (1,14,15,26,29).

The present study was aimed to determine the time of appearence of alkaline phosphatase (AP) activity in murine MSGs, as well as enzyme activity changes in the parenchyma and the capillary endothelial bed of murine MSGs during various periods of postnatal life, and during pregnancy and lactation. However, to our knowledge no reports are available on the distribution of AP activity in murine MSGs during postnatal glandular parenchyma differentiation, or during pregnancy and lactation.

AP (non-specific alkaline phosphatase, orthophosphoric-monoester phosphohydrolase) (EC 3.1.3.1), one of the most important enzymes in histochemistry, hydrolyzes various phosphate esters at alkaline pH. AP has been investigated histochemically, imunohistochemically or biochemically in many tissues and organs of various mammals: e.g., in the rat bone (24) and intestine (34), mouse uterus and placenta (16), rat uterus (6), and rat, cat, dog and man

salivary glands (3,12,13,18). Only two histochemical studies have demonstrated the distribution of AP in SMG of adult mice of both sexes (1,14).

AP is also known as an excellent marker of capillary endothelial cells (CECs) (19,27), usually in the arterial part of the capillary bed (19). Membrane localization of this phosphatase strongly suggests its function in membrane active transport, but the mechanism is not known as yet (5). Analysis of AP in mice with defective vitamin B-6 metabolism suggests involvement of AP in vitamin B-6 metabolism in the central nervous system (32), however, its biological and physiological roles in other tissues remain unknown.

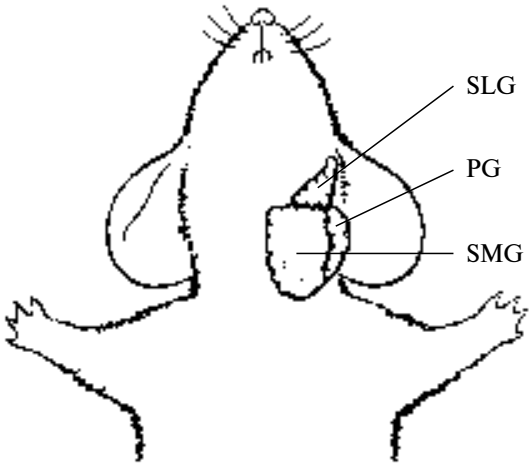

Fig. 1: Position of murine major salivary glands.
SLG - sublingual gland, PG - parotid gland, SMG - submandibular gland.

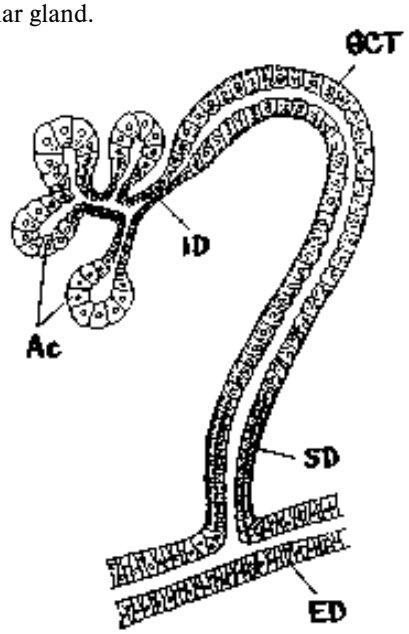

Fig. 2: Schematic presentation of a major salivary gland.
Ac - acini, ID - intercalated duct, GCT- granular convoluted tubule (in submandibular gland only), SD - striated duct, ED - excretory duct.

Materials and methods

Experimental animals

Sixty ICR mice, 20 males and 40 females, were used in this study. Experimental mice were assigned to six groups of 10 animals each (5 males and 5 females in groups N, P, Y, O; 10 females in groups G, L), and classified as follows:

group N: early postnatal period (newborn males and females aged 0-7days; the day of birth was counted as day zero)
group P: prepubescent and pubescent period (prepubescent males and females aged 1-3 weeks; pubescent males and females aged 4-6 weeks)
group Y: young adult period (males and females aged 7-20 weeks)
group O: old adult period (males and females aged 24-26 months)
group G: pregnancy (5-day-, 10-day-, 15-day-, 21-day-pregnant females; 21st day of gestation = birth of offspring)
group L: lactation (5-day-, 10-day-, 15-day-lactating females; 21st day of lactation = day of weaning)

Histochemistry

SMG, SLG and PG were removed bilaterally in each animal and rapidly frozen. For light microscopy, cryostat sections (20 μm) were used. Histochemical detection of AP was carried out by the simultaneous coupling method of Burstone, 1962 (4); the succedaneous enzymatic method in the combination AP/ AChE (acetylcholinesterase) was sometime used. Parallel detection of AP and AChE (direct-colouring thiocholine method of Karnovsky and Roots, 1964) (21) in the same section was used for better topical orientation in histochemical sections and for complex mapping of the capillary bed.

Results

Histochemical patterns of AP activity in the SMG, SLG and PG of mice of both sexes at various ages of postnatal life, and in the MSGs of female mice during pregnancy and lactation are shown in Table 1.

AP activity in MSGs during the early postnatal period (group N) (Fig. 3, 4)

On day zero, all components of the gland parenchyma and likewise the CECs were entirely unreactive for AP. On day 2, apical cell membranes of TTs in the SMG and SLG began to react weakly positive. On day 4, first signs of AP activity were seen in the TTs of PG and in IDs of SMG. AP activity was slowly increasing during the next days of the 1st week. Slight AP activity in the CECs of the arterial part of the capillary bed of all MSGs was detected between day 1 and day 7. No sex difference in enzyme activity pattern was seen in the glands during the early postnatal period.

Tab. 1: Histochemical patterns of AP activity in the major salivary glands in several groups

	GROUP N *newborn animals*	**GROUP P** *prepubescent and pubescent animals*	**GROUP Y** *young adult animals*	**GROUP O** *old adult animals*	**GROUP G** *pregnant females*	**GROUP L** *lactating females*
SMG	**TT** (2nd day) **ar** **ID** (4th day) **ar** ± → + + **CEC** +	**TT, ID ar** + + → – /3rd week/ **AC br** (2nd week) ♀ ± + + → or + + ♂ ± + + → + + **GCT bm** (4th week) ♀ – → – or ± ♂ ± → + + + **CEC** + → + + + +	**AC br** ♀ + or + + ♂ + + + **GCT bm** ♀ – or ± ♂ + + + **CEC** + + + +	**AC br** + or + + + + + **GCT bm** ♀ – or ± ♂ + + + **CEC** + + + +	**AC br** + + **GCT bm** – or ± → + + + **CEC** + + + +	**AC br** + + → + **GCT bm** + + + → ± or – **CEC** + + + +
SLG	**TT** (2nd day) **ar** ± → + + **CEC** +	**TT ar** + + → – /3rd week/ **AC br** (2nd week) ± → + **CEC** + → + + + +	**AC br** + **CEC** + + + +	**AC br** + **CEC** + + + +	**AC br** + **CEC** + + + +	**AC br** + **CEC** + + + +
PG	**TT** (4th day) **ar** ± → + **CEC** +	**TT ar** + → – /3rd week/ **AC br** (2nd week) ± → + **CEC** + → + + + +	**AC br** + **CEC** + + + +	**AC br** + **CEC** + + + +	**AC br** + **CEC** + + + +	**AC br** + **CEC** + + + +

SMG: submandibular gland
SLG: sublingual gland
PG: parotid gland

ar: apical cell region
br: basal cell region
bm: basal cell membranes

(day, weak): date of the 1st signs of activity
/weak/: date of disappearance of activity
→: increase /decrease of activity

-: negative ++: moderate
+-:trace +++: intense
+: slight ++++: extremely intense

TT: terminal tubules
ID: intercalated ducts
Ac: acini
GCT: granular convoluted tubules
CEC: capillary endothelial cells

AP activity in MSGs during the prepubescent and pubescent period (group P) (Fig. 5)

AP activity in TTs and IDs was gradually decreasing to disappear around week 3. However, enzyme activity in the region of developing Ac appeared during the 2nd postnatal week. In all MSGs reaction product outlined basal parts of acini. AP activity in Ac was gradually increasing with age (more rapidly in SMG). No sexual differences in AP activity in SMG of prepubescent (2-3-week-old) animals were observed, but histochemical sexual differences in SMG of pubescent mice (from the 4th week of age on) were seen. During the 4th week, the male SMG Ac displayed rapidly increasing activity, while AP activity in female Ac increased very slowly. The first signs of AP activity in male SMG GCTs were detected during the course of the 4th postnatal week. However, female GCTs stayed enzyme negative at the same time. Between the 4th and 6th postnatal weeks enzyme activity in male GCTs was gradually increasing. During this period, female GCTs displayed none or only sporadic, very slight AP positivity. No enzymic sex differences were seen in SLG and PG. A definitive enzymic pattern was observed in the glands of animals aged six weeks. AP activity in the CECs during the first six postnatal weeks increased also. When histochemically examined by the succedaneous

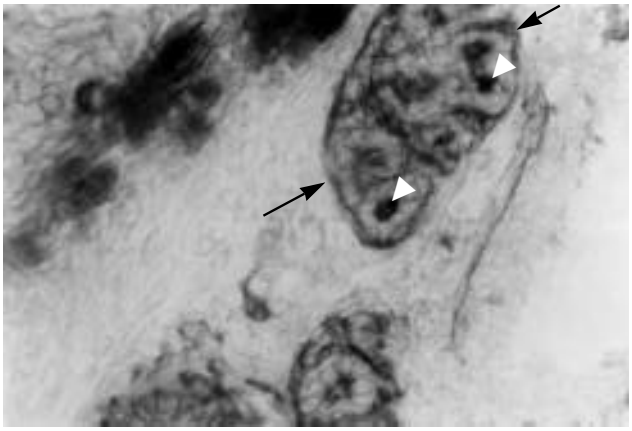

Fig. 3: Parallel detection of AP/AChE in parotid gland of newborn mouse (6-day-old male). The micrograph shows the moderate AP activity in apical parts of terminal tubules (small arrows). Basal parts of terminal tubules stains for AChE (large arrows). 230x.

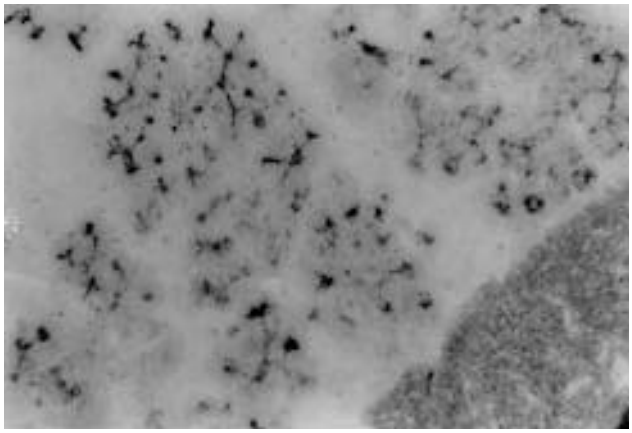

Fig. 4: AP activity in apical parts of terminal tubules and intercalated ducts of submandibular gland of 6-day-old female mouse. 150x.

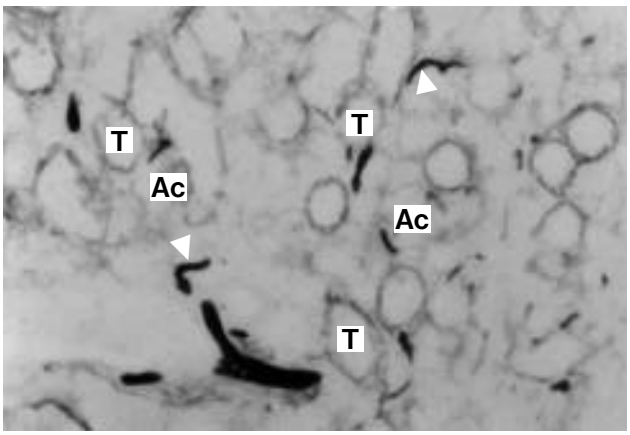

Fig.5: In the submandibular gland of thirty-five-day-old pubescent male, basal cell membranes of developing granular convoluted tubules (T) and basal parts of acini (Ac) show moderate staining for AP. Strong AP reaction is present in arterial parts of numerous capillaries (arrows). 150x.

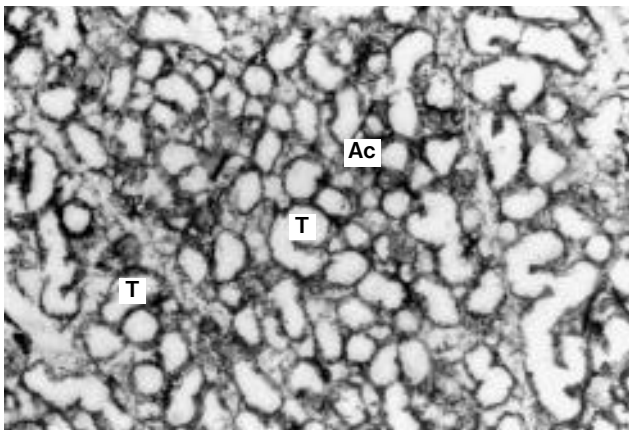

Fig.6: Strong AP activity in basal parts of granular convoluted tubules (T) and acini (Ac) of submandibular gland of young adult male (9 weeks of age). 100x.

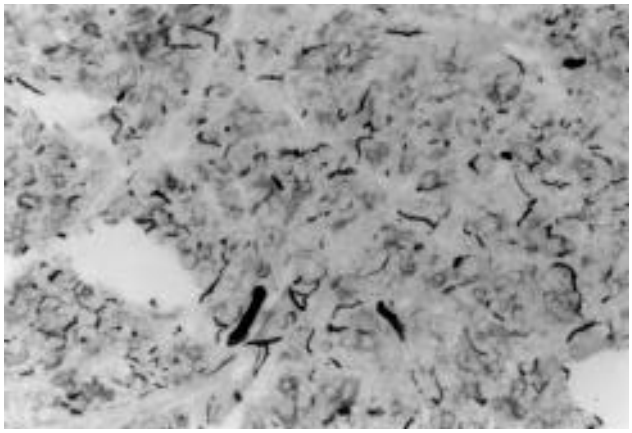

Fig. 7: In submandibular gland of young adult 9-week-old female, basal parts of acini show only weak or moderate AP activity. 100x

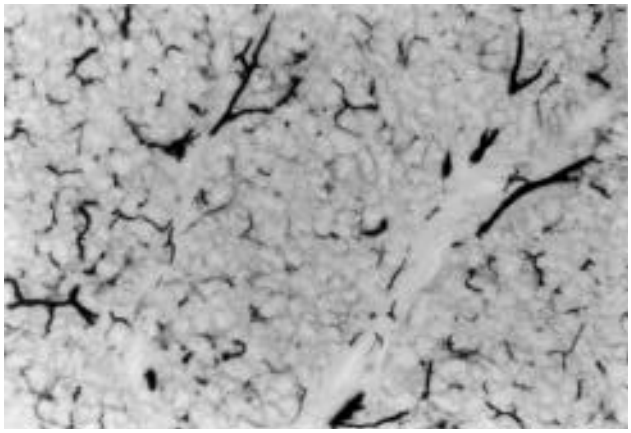

Fig.8: The AP activity in the sublingual gland of young adult 10-weak-old male. The reaction in basal parts of acini is weak, whereas in arterial parts of numerous capillaries there is an intense pattern of enzymic activity. 100x.

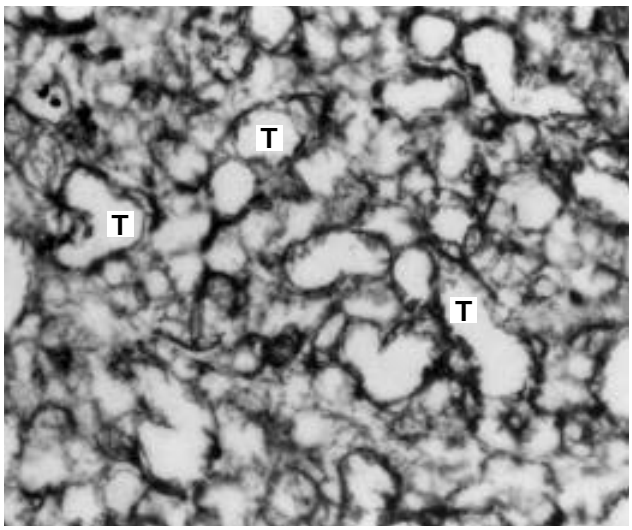

Fig. 9: The submandibular gland of 21-day-pregnant female shows clear pattern of histochemical masculinization. Strong AP reaction is seen in numerous granular convoluted tubules (T). The enzymic pattern is the same as in adult male SMG (see Fig. 6). 150x.

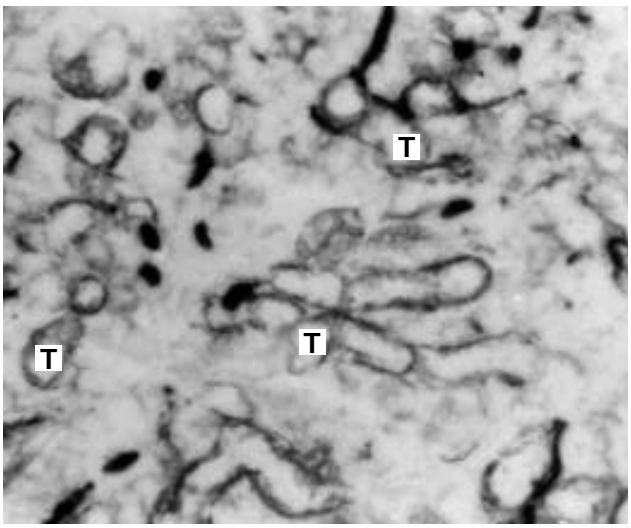

Fig. 10: During lactation, AP activity in the submandibular gland gradually decreases. However at the 10th day of lactation, the moderate AP activity in basal cell membranes of granular convoluted tubules (T) is still present. 150x.

enzymatic methods for AP and AChE, the arterial segment of capillary bed revealed the AP activity in contrast with the AChE-stained venous segment of the capillary bed. The middle part of the capillary bed between those segments displayed activity of both enzymes.

AP activity in MSGs during young and old adult periods (groups Y, O) (Fig. 6, 7, 8)

No difference was found in AP activity of the parenchyma of MSGs between young adult (group Y) and old animals (group O), and no enzymic sex differences were detected between AP activity of the SLG and PG. The parenchyma of the SMG demonstrated a more intense reaction than the parenchyma of the SLG or PG. AP activity of fully matured SMG showed clear signs of histochemical sexual dimorphism. Strong enzymic reaction was found in the basal cell membranes of male GCTs, but none (or only sporadic trace reaction) in the female GCTs. Moreover, the GCTs were more frequent and larger in the male than in the female animals. AP activity in the basal cell region of mature SMG Ac was also more intense in males than in the females. Arterial parts of the capillary bed displayed strong AP activity without gland and sex differences.

AP activity in MSGs during pregnancy (group G) (Fig. 9)

In the SLG and PG of pregnant females, the enzymic pattern was the same as in adult males or adult non-pregnant females (groups Y, O). However, dramatic histochemical changes were detected in SMG during the course of pregnancy. On the 5th day of gestation, slight AP activity was seen in the basal cell membranes of all GCTs. From gestation day 15 throughout the end of pregnancy till the 21pregnancy day (birth of offspring), enzyme activity of GCTs reached a maximum. At this time the enzymic pattern of pregnant GCTs was the same as in adult male GCTs.

AP activity in MSGs during lactation (group L) (Fig. 10)

During lactation, AP activity of GCTs significantly decreased and the SMG of lactating females gradually lost its „masculine" enzymic pattern. However, between lactating day 5 and 15, the GCTs retained some AP activity. At the 10th lactating day, the AP activity of GCT was intermediate between that seen in pregnant females (or adult males) and non-pregnant adult females, whereas on the 21st lactating day (day of weaning) the gland already displayed a „female" type of AP activity (with histochemically negative or only a few positive GCTs).

Discussion

The histochemical demonstration of alkaline phosphatase in the parenchyma of salivary glands of various mammals has been reported by a number of authors (1,13,14,18). Andrews and Bulock (1) reported strong AP activity in the male granular convoluted tubules of murine SMG. Hill and Bourne (14) described a slight AP reaction in some acinar cells of MSGs of mice. Garrett and Harrison (13) noted AP activity in the myoepitelial-cell plasma membranes adjacent to the secretory acini of the MSGs of cats and also in non-mucous acini of the SLG of dogs. Leeson (18) demonstrated AP activity in the myoepithelial cells of rat salivary glands.

The present study describes the light microscopic localization and intensity of AP activity in MSGs of laboratory mice during postnatal gland differentiation, and during pregnancy and lactation. A survey of literature has noted no reports on AP distribution in developing murine salivary glands, or in the glands during pregancy and lactation as yet. Our investigation also documents that AP is a good marker for developing and mature Ac, male GCTs, as well as the arterial part of the capillary bed of MSGs.

We have shown that AP activity in MSGs was undetectable histochemically on the day of birth, appeared in the TTs and SMG IDs during the 1st postnatal week, and then gradually decreased in these structures to disappear at the end of the 3rd week. However, we showed that during the 2nd week, AP activity appeared in region of developing Ac of all three investigated glands. From the 2nd to 6th week of age, AP activity of basal parts of Ac slowly increases in the SLG and PG.

The postnatal development of AP activity in murine SMG can be divided into two phases. In the 1st phase in 2-3-week-old prepubescent animals, the enzymic pattern in developing male SMG is the same as in females. In the 2nd phase in 4-6-week-old pubescent animals, the enzymic pattern is sexually different, the female glands exhibit generally less activity than male ones. In fully differentiated SMG of adult young or old animals this histochemical sexual dimorphism is more pronounced than in SMG of adolescent animals. The enzyme reaction is strong in adult male Ac and GCTs, whereas the reaction is only slight in female Ac, and negative (or sporadically very weakly positive) in female GCTs. It has been known for more than five decades that the SMG of laboratory mice (Mus musculus) exhibits morphological sex dimorphism (17). Number of studies has demonstrated differences between male and female SMG in mice (1,7,14,23). We have shown that sexual differences in intensity and localization of AP activity in adolescent, young and old adult mice exist too.This finding confirms an androgenic dependence of GCTs and indicates a high metabolic activity of male GCTs (which are massive producers of biologically active peptides) (2) and probably plays a different biologic role in males and females. Corresponding to our observations, Andrews and Bullock (1) described strong AP activity in the male GCTs but none in female GCTs of SMG of adult mice. On the other hand, Hill and Bourne (14) found no histochemical sexual differences of AP activity in adult murine SMG.

We have observed that the SMG of mice shows histochemical „masculinization" during pregnancy. This SMG masculinization is characterized by numerous, large and histochemically strongly positive GCTs of pregnant fema-

les. The maximum of this masculinization occurs between pregnancy day 15 and day 21 (birth of offspring). During lactation, AP activity of GCTs is gradually decreasing and on lactating day 21 (the day of weaning), GCTs already displays the „female“ type of AP activity. Although Desclin (11) reports that progesterone has no masculinizing effect on the morphology of murine female SMG, other authors have shown that progesterone stimulates the SMG (26, 31). The SMG of mice is a typical androgen target organ and contains receptors for androgens that can be characterized and quantified (33). Studies in Tfm/y mice also show that SMG of these animals is insensitive to progestagens, suggesting that progesterone acts via androgenic receptors. Progesterone could interact directly with the receptors for androgen, or after its biotransformation into steroid C 19 (26). In fact, progesterone can be converted into 5-alpha-dihydrotestosterone in some androgen target organs such as the SMG of mice, showing that the masculinization effect of progesterone takes place after conversion into androgens (20). While the concentration of progesterone in blood starts to decrease from the 17th day of pregnancy, progestin is very high in the rat SMG till delivery (22).This hormone retention in the glandular tissue could enable the histochemical masculinization of SMG till the 21st day of pregnancy, which our study seems to support.

In the MSGs, AP appears as a good marker of the CECs. Enzyme activity of CECs develops during the first postnatal weeks. In a complex delineation of the capillary bed (with the aid of AP and AChE in the same section), AP maps out the arterial parts of capillaries, whereas AChE depicts the venous part of the capillary bed (the middle part displays activity of both enzymes).This finding documents a heterogeneity of CECs of the capillary bed in murine MSGs.

Conclusion

1) A complex topo-histochemic picture of AP activity in murine MSGs during various periods of postnatal life (in newborn, prepubescent, pubescent, young adult and old animals of both sexes), as well as during pregnancy and lactation is presented. In our study, AP appears as a good marker of developing and mature Ac, male GCTs and CECs.
2) AP activity is absent on the day of birth in the parenchyma of all MSGs and appears during the 1st postnatal week in TTs, transitional structures of gland parenchyma. During week 2, AP activity in basal region of developing Ac of all three glands is recognized. During the 4th postnatal week the first signs of AP activity in male GCTs are revealed.
3) AP activity in basal parts of Ac and male GCTs gradually increases with postnatal differentiation of the glandular parenchyma. Definitive enzymic pattern is present in the MSGs at the end of the 6th week of age. There is no difference in enzymic activity between young adult and old animal glands. The parenchyma of SMG demonstrates a more intense AP reaction than the parenchyma of SLG and PG.
4) Distinct sexual differences of AP activity are observed in the SMG. Histochemical sexual dimorphism is not obvious until 3 weeks of age. In adolescent animals (from the 4th week on) a clear histochemical sex dimorphism is evident but is not yet as pronounced as in the mature SMG of adult mice. AP activity in basal parts of Ac is more intense in adult males than in females. GCTs of fully differentiated SMG of adult males are strongly positive, whereas GCTs of adult females are negative or only sporadically very weakly positive. This finding confirms an androgenic dependence of GCTs (primarily known from histological studies) and indicates a high metabolic activity of male GCTs.
5) „Masculinization“ of female SMG occurs during pregnancy (with a maximum from the 15th day of gestation till delivery) with the development of numerous, large, histochemically strongly positive GCTs. Progesterone produced during pregnancy apparently stimulates the transformation of the „female“ type of GCTs into a metabolically highly AP positive „male“ type. This histochemical „masculinization“of GCTs gradually decreases after delivery, and at the end of lactation the gland again assumes the „female“ type of activity (with histochemically negative or only sporadically very weakly positive GCTs).This histochemical masculinization of the SMG during pregnancy indicates that not only androgens but also progesterone exerts masculinization of this gland.
6) AP appears as a good marker of the arterial parts of capillaries in the MSGs.

References

1. Andrews EJ, Bulock LP. A morphological and histochemical evaluation of sexual dimorphism in androgen -intensive pseudohermaphroditic mice. Anat Rec 1972;174:361-70.
2. Barka T. Biologically active polypeptides in submandibular glands. J Histochem Cytochem 1980;28:836-59.
3. Bogart BI. The fine structural localization of alkaline and acid phosphatase activity in the rat submandibular gland. J Histochem Cytochem 1968; 16:572-81.
4. Borgers M, Firth JA, Stoward PJ. Histochemical methods for phosphatases. In: Stoward PJ, Pearse AGE, eds. Histochemistry. Theoretical and Applied. Edinburgh, London, Melbourne, New York and Tokyo: Churchill Livingstone, 1991: 591-606.
5. Borgers M, Firth JA, Stoward PJ, Verheyen A. Phosphatases. In: Stoward PJ, Pearse AGE, eds. Histochemistry. Theoretical and Applied. Edinburgh, London, Melbourne, New York and Tokyo: Churchill Livingstone, 1991: 187-218.
6. Bucci M, Murphy CHR. Alkaline phosphatase distribution in the plasma membrane of uterine epithelial cells is

markedly altered during pregnancy in the rat. Cell Biol Int 1995;19:921-8.
7. Chai Y, Klauser D, Denny PA, Denny PC. Proliferative and structural differeneces between male and female mouse submandibular glands. Anat Rec 1993;235:303-11.
8. Chrètien M. Action of testosterone on the differentiation and secretory activity of a target organ: The submaxillary gland of the mouse. Int Rev Cytol 1977; 50:333-96.
9. Denny PC, Chai Y, Pimprapaiporn W, Denny PA. Three-dimensional reconstruction of adult female mouse submandibular gland secretory structures. Anat Rec 1990; 226:489-500.
10. Denny PC, Chai Y, Klauser DK, Denny PA. Parenchymal cell proliferation and mechanism for maintenance of granular duct and acinar cell population in adult male mouse submandibular gland. Anat Rec 1993; 235:475-85.
11. Desclin J Jr. Action de la progestérone sur la structure des glandes sous-maxillaires chez la souris. C R Hebd Séanc Acad Sci 1967; 264:367-9.
12. Fukuda M. Histochemical studies on the rat submaxillary gland during postnatal development. Histochemie 1967; 8:342-54.
13. Garrett JR, Harrison JD. Alkaline phosphatase and adenosine triphosphatase histochemical reaction in the salivary glands of cat, dog and man, with particular reference to the myoepithelial cells. Histochemistry 1971;24:214-29.
14. Hill CR, Bourne GH. The histochemistry and cytology of the salivary gland duct cells. Acta Anat 1954;20:116-28.
15. Hoshi K, Amizuka N, Oda K, Ikehara Y, Ozawa H. Immunolocalization of tissue non-specific alkaline phosphatase in mice. Histochem Cell Biol 1997;107:183-91.
16. Johanson S, Wide M. Changes in the pattern of expression of alkaline phosphatase in the mouse uterus and placenta during gestation. Anat Embryol 1994; 190:287-96.
17. Laccassagne A. Dimorphism sexuel de la glande sous - maxillaire chez la souris. C R Soc Biol 1940;133:181-226.
18. Leeson CR. Localization of alkaline phosphatase in the submaxillary gland of the rat. Nature 1956; 178:858-9.
19. Lojda Z. Heterogenita endotelu mikrocirkulace se zvláštním zřetelem k mikrocirkulaci CNS. In: Mitro A, Pogády J, eds. Histochémia a morfológia atruktúr nervového systému a cirkulácie. KÚNZ Bratislava, 1988:1-8.
20. Mowszowiecz I, Bieber DE, Chung KW, Bullock LP, Bardin CW. Synandrogenic and antiandrogenic effect of progestis: Comparison with non-pregestational antiandrogens. Endocrinology 1974; 95:1589-92.
21. Oliver C, Lewis PR, Stoward PJ. Histochemical methods for esterases. In: Stoward PJ, Pearse AGE, eds. Histochemistry. Theoretical and Applied. Edinburgh, London, Melbourne, New York and Tokyo: Churchill Livingstone, 1991:591-606.
22. Paulo E, Szoltys M. Steroid hormone content in the submandibular gland of normal and pregnant rats. Arch Oral Biol 1982;27:887-95.
23. Pardini LC, Taga R. Stereological study of the sexual dimorphism in mouse submandibular glands. Okajimas Folia Anat Jap 1996;73:119-24.
24. Pinero GJ, Farach CM, Devoll RE, Aubin JE, Brunn JC, Butler WT. Bone matrix proteins in osteogenesis and remodelling in the neonatal rat mandible as studied by immunolocalization of osteopontine, bone sialoprotein, alpha 2HS-glycoprotein and alkaline phosphatase. Arch Oral Biol 1995;40:145-55.
25. Pinkstaff CA. The cytology of salivary glands. Int Rev Cytol 1980;63:141-261.
26. Rossi JA, Bruschi LC. Alteration of the submandibular glands of female mice during pregnancy and lactation. A histological, histometric and histochemical study. Acta Morphol Hung 1989;37:135-45.
27. Roux F, Durieu-Trautmann O, Chaverot N et al. Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. J Cell Physiol 1994 ;159:101-13.
28. Sawada K, Noumura T. Sexually dimorphic duct system of the submandibular gland in mouse with testicular feminization mutation (Tfm/y). Acta Anat 1992;143:241-5.
29. Sims-Samson G, Gresik E, Barka T. Histochemical localization of ouabain-sensitive, K+-dependent p-Nitrophenylphosphatase (Na+- K+- ATPase) activity in the submandibular gland of the mouse: Effect of androgen, thyroid hormone, or postnatal age. Anat Rec 1984;210:53-60.
30. Srinivasan R, Chang WWL. The postnatal development of the submandibular gland of the mouse. Cell Tiss Res 1979;198:363-71.
31. Travill A.The effect of pregnancy on the submandibular gland of mice. Anat Rec;1966;155:217- 25.
32. Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, Mac Gregor GR. Mice lacking tissue nonspecific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat Genet 1995;11: 45-51.
33. Wilson JD, Goldstein JL. Evidence for increased cytoplasmatic androgen binding in the submandibular gland of the mouse with testicular feminization. J Biol Chem 1972;247: 7342-9.
34. Zhang Y, Shao JS, Xie QM, Alpers DH. Immunolocalization of alkaline phosphatase and surfactant-like particle proteins in rat duodenum fat absorption . Gastroenterology 1996;110:478-88.

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ORIGINAL ARTICLE

IMMUNOHISTOCHEMICAL DETECTION OF INTERMEDIATE FILAMENT NESTIN

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Summary: Using Rat-401 monoclonal antibody and peroxidase immunohistochemistry we have detected IF nestin in developing and adult rat tissues. Although epitope recognized by Rat-401 antibody is relatively resistant to aldehyde fixation and paraffin embedding, the embedding of tissue samples into polyester wax and microwave antigen retrieval of histological sections enabled us to enhance sensitivity of immunohistochemical detection and to identify cells expressing low levels of nestin. Our findings confirm that nestin is predominantly distributed in developing neural, myogenic and mesenchymal cells, i.e. cell types that have been previously described to express this intermediate filament. Furthermore, we made original findings on identification of nestin expression in additional cell types, e.g. newly formed endothelial cells of extra- and intraembryonic blood vessels, epithelial cells of the developing lens, and cells apposed to hair follicles.

Key words: *Dermis; Development; Endothelium; Lens; Mesenchyme; Nervous tissue; Nestin immunohistochemistry; Rat; Skeletal muscle development*

Introduction

Intermediate filaments (IFs) together with microtubules, microfilaments and microtrabecules represent the major components of the cytoskeleton. Based on sequence analysis (conservation of heptad motif and spacer positions), six different classes of intermediate filaments (IFs) have been defined: acidic keratins (class I); basic keratins (class II); desmin, glial fibrillary acidic protein (GFAP), peripherin and vimentin (class III); the neurofilament triplet (class IV); nuclear lamins (class V); and nestin (class VI) - (18,25,30). Postulated functions for IFs include the organization and maintenance of cell shape (17). Therefore certain IFs can be identified exclusively in certain cell types, eg. GFAP is specific for astrocytes, neurofilaments for neurons, peripherin for peripheral neurons, desmin for muscles, keratins for epithelia etc. (5,19,25).

Genes encoding IFs are sequentially expressed in tightly controlled cell-type specific patterns. During development, transition from one IF protein to another occurs at major differentiation steps. Before neurulation, ectodermal cells express cytokeratins. During neural tube induction, specific type of ectodermal cells, the neuroectoderm, starts to express nestin (18). Nestin is then expressed by multipotential neuroepithelial stem cells and their progeny which gives rise to neuronal and glial cells (8). Following terminal differentiation, nestin in neuronal cells is replaced with α-internexin (20), peripherin (19,20) or with neurofilaments (3) whereas in glial cells transient expression of vimentin precedes expression of GFAP which identifies fully matured astroglia (5). Another example of transition in IF expression can be seen in developing skeletal muscles: at early developmental stages, nestin is expressed in presomatic mesoderm as well in the myotome layer of the somites and then it is gradually replaced by vimentin and desmin which is specific for differentiated muscle (18,27,29).

This article is devoted to immunohistochemical localization of nestin in fetal, perinatal and mature tissues using mouse monoclonal antibody (MAb) Rat-401. In agreement with observations made by other investigators, we confirm that the developing nervous tissue and skeletal muscle contain cell types that express the highest levels of nestin. Examination of histological sections of whole rat fetuses enabled us to study distribution of nestin throughout tissues. Examination of tissues taken at different developmental stages allowed us to describe a temporal pattern of nestin expression in distinct cell types. Utilization of different fixatives, embedding and pretreatment protocols as well as sensible detection systems permitted us to observe cell types that express low levels of nestin and moreover, to identify this IF in cells that have not been previously reported to be nestin-immunoreactive.

Material and Methods

Experimental animals

All studies were performed using Wistar rats (VELAZ, Prague, Czech Republic) that were maintained under standard conditions (housed in groups of 2 rats per cage, natural day/night cycle, food and water available *ad libitum*). Female rats were mated with males overnight and the following day was designated as embryonic day 0 (E0). Since then, female rats had been bred separately from males. E14, E15, E18 and E19 rat fetuses were obtained after a median laparotomy carried out in deeply anaesthetized (pentobarbital 50 mg/kg, i.p.) pregnant rats. At the end of surgery, pregnant rats were killed by overdose. Neonatal and postnatal rats were anaesthetized with ether and killed by decapitation. The experiments performed in this study were approved by the Animal Ethical Committee of Charles University Medical Faculty in Hradec Králové.

Histology

Rat fetuses as well as tissue blocks taken from newborn, postnatal and adult rat tissues were immersed in a neutral buffered formalin solution, 4% paraformaldehyde, 70% ethanol containing 5% acetic acid or zinc fixative (1,2) for 24 h at room temperature. Tissue blocks were then dehydrated in increasing alcohols and embedded in polyester wax (PEW; melting point 36 °C; 26) or paraffin (melting point 56 °C). Seven-micron thick coronal sections were cut from PEW and paraffin blocks and attached to the slide with poly L-lysine. Some sections were stained with haematoxylin and eosin.

Immunohistochemistry

For immunohistochemistry, all paraffin embedded sections were deparaffinized with xylene and rehydrated in decreasing ethanols to water. PEW sections were dewaxed with decreasing alcohols. Sections were incubated for 20 minutes in methanol containing 1% H_2O_2 to reduce „endogenous peroxidase activity". Since microwave pretreatment enables to re-establish an original conformation of epitopes modified after fixation (28), we exposed sections to microwaves in sodium citrate solution for 2x5 minutes at 700 watts. After thorough washing in 0.2M Tris-HCl buffer containing 0.5% Triton X-100 (Sigma, Prague, Czech Republic), the sections were exposed to a primary monoclonal antibody (Mab) anti-nestin (12; 1:4, clone Rat-401) for 45 min. Rat-401 Mab was obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. After thorough washing, sections were incubated with anti-mouse secondary biotinylated antibody (Sigma, Prague, Czech Republic) for 45 min and then with streptavidin labelled with horse radish peroxidase (BioGenex, San Ramon, CA, USA) or alkaline phosphatase (Sigma, Prague, Czech Republic) diluted 1:100. After rinsing, the reaction was developed using DAB (3,3'-diaminobenzidine tetrahydrochloride (Sigma, Prague, Czech Republic) and hydrogen peroxide. The whole procedure was carried out at room temperature. Sections processed with omission of the primary antibody were used as controls. Sections were dehydrated, mounted in DPX (Sigma, Prague, Czech Republic), and examined by the Orthoplan light microscope (Leitz, Wien, Austria).

Results

In immunohistochemical staining Rat-401 monoclonal antibody reacted only with certain cells (e.g. radial glia, skeletal muscle, endothelial cells) whereas other structures (e.g. chondrocytes, adult connective tissue or epidermis) were completely devoid of any staining. Such a signal distribution is characteristic of a specific staining that gives no false positivity and minimum background. Omission of the primary antibody in our immunohistochemical procedure did not detect any signal revealing the fact that background and non-specific staining was kept to a minimum level. IF protein nestin was detected in all tissues irrespective of the fixation that was used. Microwave pretreatment significantly enhanced an intensity of a detected signal. When this pretreatment was applied to PEW-embedded tissue samples, the subsequent peroxidase immunohistochemistry provided a signal of the highest intensity.

Nestin in neural tissues

The neuroepithelium of fetal rats exhibited a strong and ubiquitous immunoreactivity for nestin. In E14-18 rat fetuses, intense immunopositivity was seen in cells situated in the ventricular zone, floor plate, proliferating zone as well as in radial fibres that spanned the whole thickness of the neural tube. High levels of nestin were detected in pial and perivascular endings of radial glial cells. In the fetal spinal cord, the proliferating zone contained more nestin$^+$ elements than the marginal zone (Fig. 1). In the neural tissue located outside the neural tube, IF nestin was identified in dorsal root ganglia and peripheral nerves. Ependymal cells as well as nervous cells, e.g. motoneurons and ganglionic cells were devoid of any specific labelling. In the prenatal (E19) rat CNS, nestin expression ceased in postmitotic neural elements of the cortex reflecting the fact that only few remnants of radial fibres were found; persisting nestin$^+$ fibres were apposed to blood vessels. Nestin-immunoreactivity remained preserved in certain regions only, e.g. in the subventricular zone (SVZ), developing hippocampus, cerebellum and pial endfeet. In the SVZ, nestin was expressed in the cytoplasm of small oval cells and in slender processes of radial glia. The same pattern of distribution of nestin was observed in the neonatal (P0) CNS where only few positive profiles were seen. The highest level of nestin was expressed in the SVZ. Due to general downregulation of

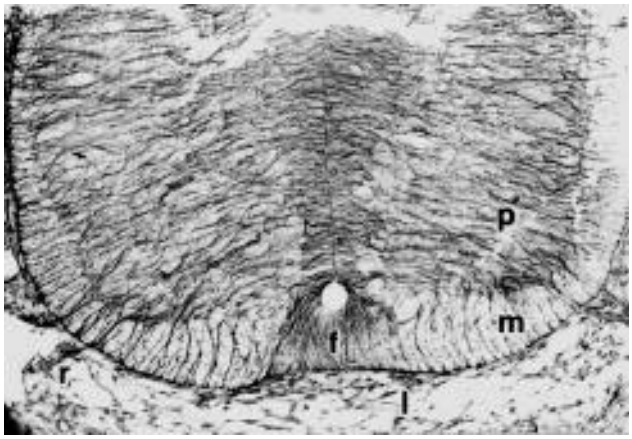

Fig. 1: In the E15 rat spinal cord, nestin was expressed by long and slim processes of radial glia cells spanning the whole thickness of the neural tube. Intense immunostaining was detected in the floor plate (f); the immunostaining was less intense in the marginal zone (m) than in the proliferating zone (p). Outside the CNS, nestin was expressed by glial cells of the ventral roots (r) and mesenchymal cells forming the leptomeninges (l). *Peroxidase immunohistochemistry, x 90.*

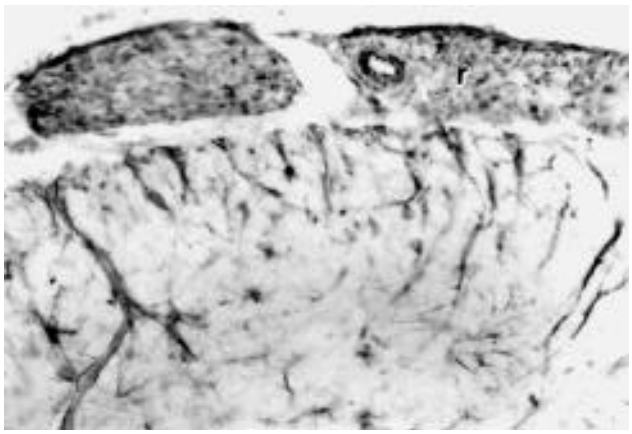

Fig. 2: In the neonatal spinal cord, nestin immunoreactivity was exhibited by endothelial cells and perivascular astrocytic endfeet. In the subarachnoid space, nestin was detected in Schwann cells of the ventral roots (r), the perineurium that enveloped peripheral nerves and accompanying blood vessels. *Peroxidase immunohistochemistry, x 225.*

nestin in surrounding neural elements, capillaries lined by nestin$^+$ endothelium became apparent (Fig. 2). In the peripheral nervous system (PNS), immunopositivity was seen in ganglia and peripheral nerves, e.g. in intercostal nerves or ventral and dorsal roots (Fig. 2). In the second and third postnatal week, IF nestin in the brain parenchyma was downregulated; relatively distinct immunostaining was seen in vascular endothelia. Completely different staining pattern was specific for brains of adult pregnant rats. Brain surface was lined with nestin$^+$ pial endfeet forming the marginal pial membrane; nestin$^+$ perivascular endfeet were apparent in pial funnels and underlying blood vessels in the cortical molecular layer (I). Deep cortical layers (II-VI) were devoid of any immunoreactivity for nestin. Nestin immunopositivity found in deep cerebral structures, e.g. in the corpus callosum, striatum, and optic nerve, was limited mainly to fibrous astrocytes and their perivascular processes. Intense immunostaining for nestin was observed in neural precursor cells in lateral walls of the subependymal zone lining both lateral cerebral ventricles. In the wall of the lower horn of the third ventricle, anti-Rat-401 immunohistochemistry visualized slender processes of tanycytes. Ependymal cells did not reveal any staining. In the lesioned brain, e.g. after a suction lesion or injection of kainic acid, astrocytes became activated throughout the brain and expressed nestin. Whereas occurrence of nestin in activated astrocytes was only transient, upregulation of nestin in reactive astrocytes apposed to the lesioned area was longlasting (Fig. 3). In the PNS, non neural cells including satellite cells in dorsal root ganglia and Schwann cells in peripheral nerves were immunopositive.

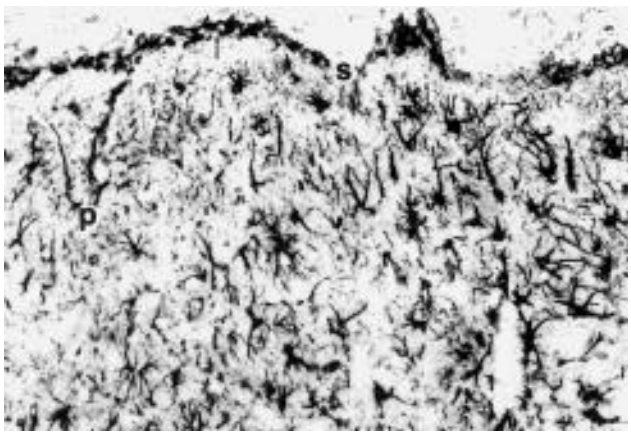

Fig. 3: Nestin immunohistochemistry revealed stellate-like reactive astrocytes that developed in the adult rat brain parenchyma that was compressed with a growing C6 glioma. Astroglial endfeet forming the superficial (s) and perivascular (p) limiting membranes expressed high levels of nestin. *Peroxidase immunohistochemistry, x 115.*

When we cultured dissociated cells isolated from the fetal forebrain in a chemically defined medium supplemented with epidermal growth factor that stimulates proliferation of neural precursor cells, the dividing cells formed multicellular spheroids (23). After embedding the spheroids in paraffin or PEW, cutting the sections and processing them for immunohistochemistry, we found that virtually all cells of early spheroids (8 days in vitro) expressed nestin. Despite all cells expressed IF nestin,

they differed in their morphology: cells situated in the inner zone exhibited long cytoplasmic processes whereas peripheral cells possessed relatively voluminous cytoplasm and short processes (Fig. 4). Cultivation of spheroids for extended periods was accompanied with gradual differentiation of neural precursor cells into neurons, astroglia and oligodendroglia and downregulation of nestin (23).

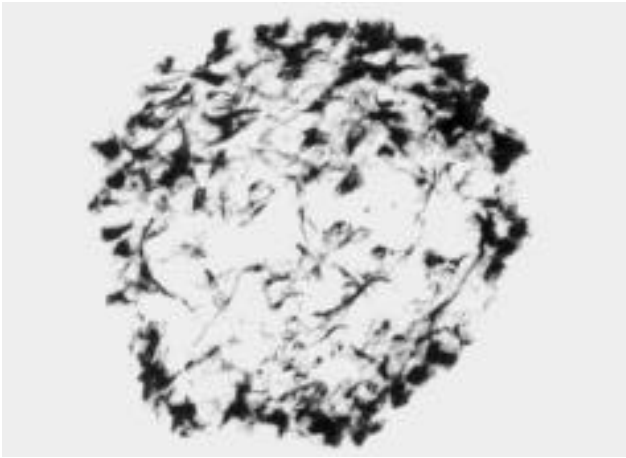

Fig. 4: Neural precursor cells forming 8-day spheroids expressed high levels of nestin. Immunohistochemistry revealed that inner cells exhibited long cellular processes whereas peripheral cells possessed short processes and voluminous, darkly stained cytoplasm. *Peroxidase immunohistochemistry, x 500.*

Nestin expression in muscular tissue

The skeletal muscle represented another source of distinct nestin immunoreactivity. Longitudinal sections of E14 rat fetuses revealed strong immunostaining of paravertebral muscles (Fig. 5). In these muscles, nestin was found in the cytoplasm of mononuclear myoblasts and exhibited a pronounced perinuclear accumulation. Centrally located nuclei of myoblasts were devoid of any staining. Myoblasts were arranged into longitudinal bands but cellular boundaries were still apparent. Transverse sections of E15 fetuses demonstrated the same pattern of nestin-immunostaining in developing muscles of the limb buds. In E18-19 rats, nestin-immunoreactivity was observed in all skeletal muscles, e.g. extraocular, pharyngeal, lingual (Fig. 6), masticatory as well as pre-, para- and postvertebral muscles. Nestin was detected in the cytoplasm of elongated myotubes that were arranged into bundles. Nestin$^-$ nuclei were still located in the centre of myotubes. A level of nestin expression slightly differed among myotubes; some of them were intensively stained whereas the others were immunostained faintly. In longitudinally-sectioned myotubes, cross-striations became apparent at these developmental stages. Neonatal (P0) skeletal muscles consisted of multinucleate muscle fibres that revealed intense nestin$^+$ striations and nuclei displaced peripherally. Individual skeletal muscle fibres situated, e.g. in the intercostal muscle, differed again in the level of IF nestin they expressed. In adult animals, mature skeletal muscle fibres were not stained with Mab Rat-401.

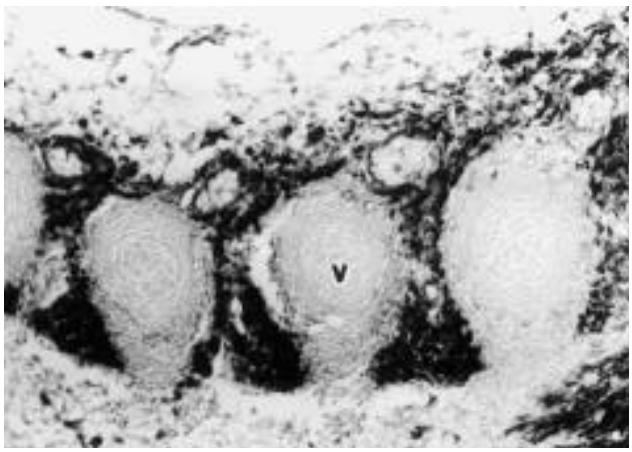

Fig. 5: Paravertebral skeletal muscles and paraxial mesenchyme of E14 rat fetuses expressed high levels of nestin. Developing vertebral bodies (v) were devoid of any staining. *Peroxidase immunohistochemistry, x 320.*

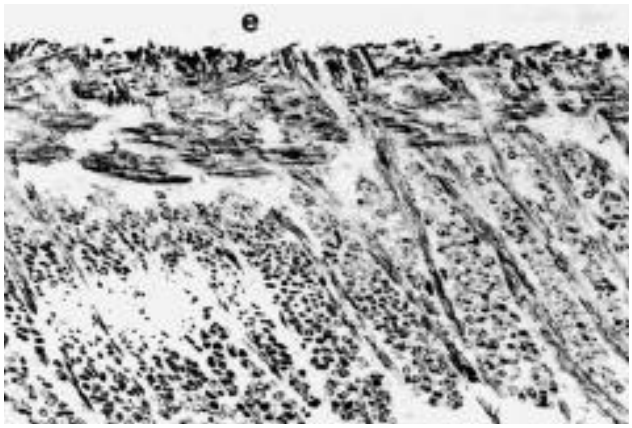

Fig. 6: Skeletal muscle of the E19 tongue. Myotubes expressed high levels of nestin; no immunoreactivity was detected in overlying stratified squamous non-keratinized epithelium (e), connective tissue and nuclei of myotubes. *Peroxidase immunohistochemistry, x 115.*

At developmental stages examined in this study (i.e. starting on day E14), no immunoreactivity for nestin was revealed in the myocardium of the developing heart. Regarding distribution of nestin in smooth muscle cells, we could observe a faint staining in cellular elements surrounding arteries and hollow organs. However, a precise identification of the cell type expressing this faint immunoreactivity requires a further analysis.

Nestin immunoreactivity of other mesenchymal elements

Immunoreactivity for nestin was identified also in mesenchymal cells. A specific signal in the cytoplasm of these cells was lower when compared with intensity of immunostaining in developing skeletal muscles but it could be clearly distinguished from other nestin$^-$ cells, e.g. chondrocytes. Nestin$^+$ mesenchymal cells were distributed, e.g. in the E14 paraxial mesoderm, mesonefric mesoderm, meninges, limb buds or choroid of the eye. In the course of development, nestin was downregulated in these cells. In newborn animals, nestin$^+$ cells were found in the dermis apposed to hair folicles (Fig. 7).

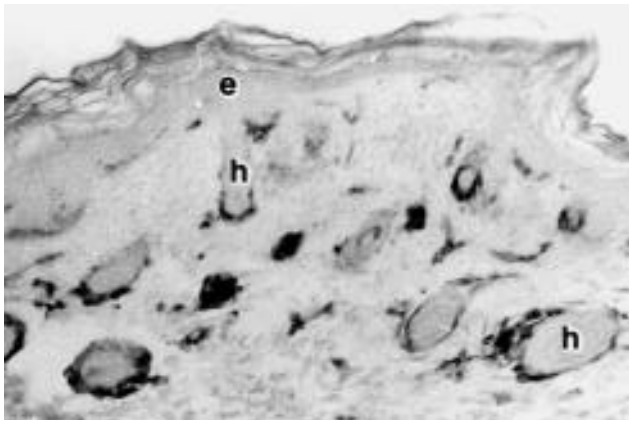

Fig. 7: In the skin of neonatal rats, no immunopositivity for nestin was observed in the epidermis (e). In the underlying dermis, nestin was expressed by vascular endothelial cells and by cells surrounding the root of hair follicles (h). *Peroxidase immunohistochemistry, x 140.*

A prominent immunoreactivity for nestin was detected in newly formed endothelial cells in both extra- and intraembryonic tissues. In extraembryonic tissues of E14 rats, nestin$^+$ endothelial cells were observed in blood vessels of the chorion, umbilical cord and placenta. In intraembryonic tissues, endothelial cells expressing nestin were identified not only in the mesenchyme but also in organ primordia. In the E14 mesenchyme, nestin$^+$ endothelia lined capillaries running in the paraxial mesenchyme, in primitive meninges, dermis (Fig. 7), in the mesenchyme layers of the developing gut (Fig. 8), choroid of the developing eye, in the mesenchyme of limb buds. Reactivity was not confined to small blood vessels but was ubiquitous; nestin was expressed also by endothelial cells lining the largest vessels like the aorta etc. In developing organs, immunohistochemistry utilizing the anti-Rat-401 antibody identified endothelia of blood vessels supplying e.g. the forebrain, spinal cord, lung, gut, liver, spleen or heart. In the E15 heart, nestin$^+$ endothelial cells were found in capillaries supplying the myocardium as well as in endothelial cells that lined the endocardium. In P0 animals, nestin-immunopositivity became evident in the nervous tissue because of cessation of nestin expression in neural elements. At this stage, nestin was downregulated in meningeal vessels. In the choroid plexus, endothelial and perivascular cells were labelled whereas epithelial cell were nestin-negative. Outside the CNS, nestin$^+$ endothelial cells were still present in growing organs, e.g. in the spleen, positivity was observed in sinusoids of the red pulp, in trabecular vessels, central arteries inside of malpighian corpuscles as well as in the splenic vessels situated in the hilum. Postnatally nestin expression by endothelial cells decreased; only occasional endothelial cell were immunoreactive.

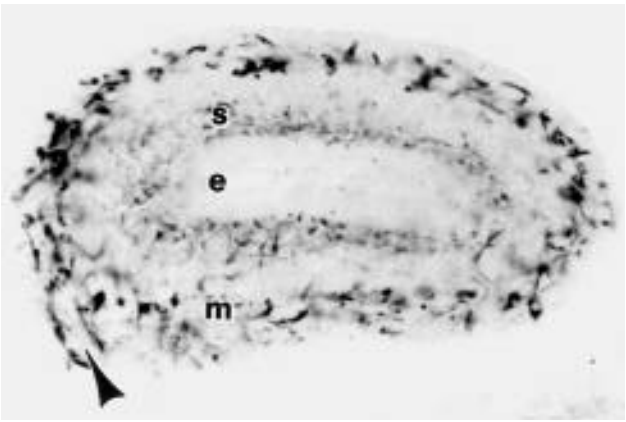

Fig. 8: In the hindgut of E14 rats, no immunoreactivity was detected in epithelium (e) covering the luminal surface. The cells expressing nestin were located in the submucosa (s) and the muscularis externa (m) layers. Nestin$^+$ cells included endothelium lining blood vessels (arrowhead) and neuroectodermal cells forming the submucosal and myenteric nervous plexuses. *Peroxidase immunohistochemistry, x 170.*

Nestin expression in the developing eye

In the developing eye, IF nestin was detected in the neural retina, lens and vascular endothelium. In E14 rats, a strong specific signal was identified in the inner thick layer of the optic cup which represents the primordium for development of the neural retina (Fig. 9). In this layer, nestin was distributed in long fibres stretching across the neuroepithelium layer (i.e. cells analogous to radial glia of the neural tube) as well as in small cellular elements; the highest levels of nestin being concentrated toward the inner surface of the optic cup. Inside the optic cup, the endothelium of the hyaloid artery and vein running through the vitreous body exhibited immunoreactivity for nestin. The lens vesicle that was situated near the edges of the optic cup also exhibited immunostaining for nestin (Fig. 9). Intense immunostaining was detected in the cytoplasm of the anterior epithelium of the lens. Nestin immunoreactivity was also expressed by cells of the posterior wall of the primitive lens. In these cells, nestin was observed in the

cytoplasm of the cell body as well as in extremely elongated cellular processes stretching between the anterior and posterior poles of the lens and obliterating the cavity of the lens vesicle. Outside the optic cup, nestin was seen in endothelial cells lining blood vessels in the choroid. In the eye of E18 rats, faint nestin-positivity was detected in long cytoplasmatic processes of Müller cells in the retina. Strong expression of nestin was found in extraocular muscles. In the eye of adult animals, nestin-immunoreactivity was confined to fibrous astrocytes of the optic nerve and it could be distinuished starting from the area of the lamina cribrosa.

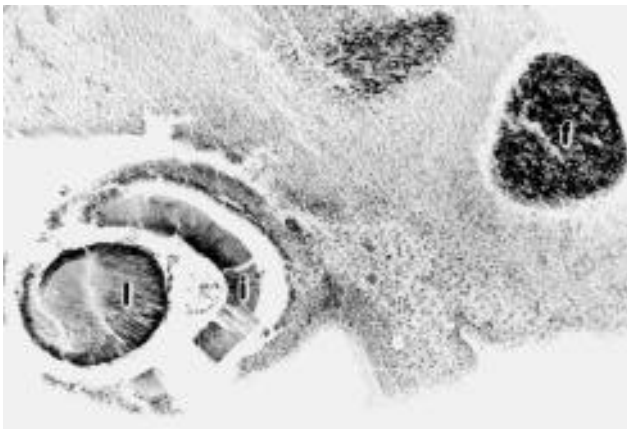

Fig. 9: In the E14 eye, nestin was expressed by cells of the inner (i) thick layer of the optic cup, epithelium of the lens vesicle (l) and choroidal blood vessels. A strong immunopositivity was observed in the neural tissue of the adjacent forebrain (f). *Peroxidase immunohistochemistry, x 50.*

Discussion

Hockfield and McKay (12) who produced Rat-401 Mab identified first cell types immunoreactive for nestin, e.g. developing skeletal muscle, radial glia, satellite and Schwann cells. A further research confirmed that nestin was abundantly expressed by developing muscles (myotomes: 16, 18, 34; myoblasts, myotubes, developing muscle fibres: 12, 27, 29; midembryonic cardiomyocytes: 15) and neural cells (neural precursor cells: 8, 18; pre-O-2A cells, O-2A progenitors, pro-oligodendroblasts: 11; subependymal cells: 24, 33; type 1 astrocytes: 11; reactive astrocytes: 4, 9, 13; neuroectodermal tumour and glioma cell lines: 6, 32). When anti-nestin immunohistochemistry was applied to other tissues, it appeared that immunoreactivity was not restricted to neural and muscular tissues only but was more widespread. Cells that have been found to express nestin include somites, presomitic mesoderm (34), odontoblasts (31), mesonephric mesenchyme and Sertoli cells (10) - see Table 1.

Tab. 1: Cellular distribution of intermediate filament nestin

Nestin-expressing cell type	References
neural precursor cells	Frederiksen and McKay, 1988;
radial glia cells	Hockfield and McKay, 1985; Fredericksen and McKay, 1988
precursors of oligodendroglia	Gallo and Armstrong, 1995
premitotic neuroblasts	Frederiksen and McKay, 1988
reactive astrocytes	Clarke et al., 1994; Frisén et al., 1995; Holmin et al., 1997
Schwann cells	Frisén et al., 1995; Jaeger, 1995
satellite cells	Hockfield and McKay, 1985
developing skeletal muscle	Hockfield and McKay, 1985; Sejersen and Lendahl, 1993; Sjöberg et al., 1994
developing cardiomyocytes	Kachinski et al., 1990
presomitic mesoderm	Zimmerman et al., 1994
myotome	Lendahl et al., 1990; Kachinski et al., 1994; Zimmerman et al., 1994
mesonephric mesenchyme	Frojdman et al., 1997
Sertoli cells	Fjordman et al., 1997
odontoblasts	Terling et al., 1995
newly formed endothelium	Mokrý and Němeček, 1998a; 1998b
lens vesicle	*This article
gliomas	Dahlstrand et al., 1992; Tohyama et al., 1992

Utilization of sensitive immunohistochemical detection systems, e.g. labelled streptavidin-biotin, enabled to identify lower amounts of IF nestin than two-step indirect methods used in the first studies. A further increase in sensitivity of anti-nestin immunohistochemistry was reached by embedding tissue samples in low melting medium (e.g. PEW), cutting thin sections, suppression of non-specific background staining, permeabilization of sections with detergents and microwave antigen retrieval. Combination of these approaches allowed us to confirm previous findings made by other authors and give a clear evidence of nestin expression by endothelial cells of extra- and intraembryonic blood vessels (21, 22). Application of this detection method to sections of whole rat fetuses enabled us to localize nestin in other cells that have not been previously described to express nestin, e.g. in the epithelium of the developing lens.

Most developing cells, that have been found to contain protein nestin, express this IF only for a limited period of their development. A transient expression of nestin has been thoroughly described in the developing CNS (8, 12, 18). The onset of nestin expression in the rat brain was first detected in pial end foot on E10. Its level rapidly increased and on E12 all neuroepithelial cells expressed nestin. Fredericksen and McKay demonstrated that nestin expression occured mainly in proliferating neural cells (8). Maximum nestin expression in the cerebrum was detected on day E16 whereas in the cerebellum on P5 (18). The loss of nestin expression coincided with terminal differentiation of NPCs. In agreement with this observation, we detected low levels of nestin in P14 forebrains and almost none in the adult cerebral cortex. Transient expression of nestin has

been described also in other cell types, e.g. in cardiomyocytes, developing skeletal muscle, mesenchymal cells, neural cells, endothelial cells (e.g. 8,12,14,15,18,21,22,27,29,34).

After these nestin$^+$ cell types, undergo their terminal differentiation, nestin is downregulated and replaced with another type of IF, eg. α-internexin (20), neurofilaments (3), or peripherin (19,20) in nervous cells, vimentin and glial fibrillary acidic protein in astroglia (5) or vimentin and desmin in myogenic cells (27,29). Actually in these cell types nestin is not abruptly downregulated but is co-expressed with another IF (e.g. GFAP, vimentin, neurofilaments, desmin) for a transient period of development (20,29). Nestin has been reported to copolymerize with other IFs, e.g. with vimentin and GFAP (20).

Possible role of IF nestin must be associated with maintenance of transient functions of undifferentiated cells, e.g. their proliferation and migration. The cytoskeleton of endothelia in developing vessels must permit mitotic divisions of endothelial cells and their movement towards primordia of avascular organs. It is likely that the presence of nestin in the cytoskeleton confers on the endothelial cell a necessary flexibility and elasticity that is required for performance of these funcions (i.e. cellular division and migration). After the cells mature, they stop to divide and move, and then establish definitive junctions with neighbouring resting endothelial cells which is accompanied with reconstruction of their cytoskeleton and loss of immunoreactivity for nestin. A possible role of class VI IF nestin in the epithelial cells of the developing lens may be linked with formation of extremely elongated lens fibres. Nestin has been demonstrated to participate in forming a structure of other extremely long fibres, e.g. in radial glia (8, 12) and odontoblasts (31). After the columnar epithelial cells of the developing lens differentiate, they form junctions with other fibres, lose their nuclei and other organelles and stop to express IF nestin.

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References

1. Beckstead JH. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. J Histochem Cytochem 1994;42:1127-34.
2. Beckstead JH. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues: Addendum. J Histochem Cytochem 1995;43:345.
3. Cattaneo E, McKay R. Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature 1990;347:762-5.
4. Clarke SR, Shetty AK, Bradley JL, Turner DA. Reactive astrocytes express the embryonic intermediate neurofilament nestin. NeuroReport 1994;5:1885-8.
5. Dahl D. The vimentin-GFAP protein transition in rat neuroglia cytoskeleton occurs at the time of myelination. J Neurosci Res 1981;6:741-8.
6. Dahlstrand J, Collins VP, Lendahl U. Expression of the class VI intermediate filament nestin in human central nervous system tumors. Cancer Res 1992;52:5334-41.
7. Fraichard A, Chassande O, Bilbaut G, Dehay C, Savatier P, Samarut J. In vitro differentiation of embryonic stem cells into glial cells and functional neurons. J Cell Sci 1995;108:318-8.
8. Frederiksen K, McKay RDG. Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. J Neurosci 1988;8:1144-51.
9. Frisén J., Johansson CB, Török C, Risling M, Lendahl U. Rapid, widespread, and lonlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. J Cell Biol 1995;131:453-64.
10. Frojdman K, Pellieniemi LJ, Lendahl U, Virtanen I, Eriksson JE. The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse. Differentiation 1997;61:243-9.
11. Gallo V, Armstrong RC. Developmental and growth factor-induced regulation of nestin in oligodendrocyte lineage cells. J Neurosci 1995;15:394-406.
12. Hockfield S, McKay RDG. Identification of major cell classes in the developing mammalian nervous system. J Neurosci 1985;5:3310-28.
13. Holmin S, Almqvist P, Lendahl U, Mathiesen T. Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury. Eur J Neurosci 1997;9:65-75.
14. Jaeger CB. Isolation of enteric ganglia from the myenteric plexus of adult rats. J Neural Transplant Plast 1995;5:223-32.
15. Kachinsky AM, Dominov JA, Miller JB. Intermediate filaments in cardiac myogenesis: Nestin in the developing mouse heart. J Histochem Cytochem 1995;43:843-7.
16. Kachinsky AM, Dominov JA, Miller JB. Myogenesis and the intermediate filament protein, nestin. Dev Biol 1994;165:216-28.
17. Lazarides E. Intermediate filaments as mechanical integraters of cellular space. Nature 1980;282:249-56.
18. Lendahl U, Zimmerman LB, McKay RDG. CNS stem cells express a new class of intermediate filament protein. Cell 1990;60:585-95.
19. Leonard DGB, Gorham JD, Cole P, Greene LA, Ziff EB. A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. J Cell Biol 1988;106:181-93.
20. Liem RKH. Molecular biology of neuronal intermediate filaments. Curr Opin Cell Biol 1993;5:12-16.
21. Mokrý J, Němeček S. Angiogenesis of extra- and intraembryonic blood vessels is associated with expression of

nestin in endothelial cells. Folia Biol (Prague) 1998a; in press.
22. Mokrý J, Němeček S. 1998b, unpublished results.
23. Mokrý J, Šubrtová D, Němeček S. Differentiation of epidermal growth factor-responsive neural precursor cells within neurospheres. Acta Medica (Hradec Králové) 1996;39:7-20.
24. Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. Neural Stem Cells in the adult mammalian forebrain: A relatively quiescent subpopulation of subependymal cells. Neuron 1994;13:1071-82.
25. Nagle RB. Intermediate filaments: A review of the basic biology. Am J Surg 1988;12:4-16.
26. Ourednik J, Ourednik W. Preservation of the structural integrity of a freshly lesioned or transplanted mouse neocortex and the immunoreactivity of cell-specific marker proteins in demineralized histological material. J Neurosci Meth 1995;62:55-63.
27. Sejersen T, Lendahl U. Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci 1993;106:1291-300.
28. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 1991;39:741-8.
29. Sjöberg G, Jiang WQ, Ringertz NR, Lendahl U, Sejersen T. Colocalization of nestin and vimentin/desmin in skeletal muscle cells demonstrated by three-dimensional fluorescence digital imaging microscopy. Exp Cell Res 1994;214:447-58.
30. Steinert PM, Roop DR. Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 1988;57:593-625.
31. Terling C, Rass A, Mitsiadis TA, Fried K, Lendahl U, Wroblewski J. Expression of the intermediate filament nestin during rodent tooth development. Int J Dev Biol 1995;39:947-56.
32. Tohyama T, Lee VM-Y, Rorke LB, Marvin M, McKay RDG, Trojanowski JQ. Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. Lab Invest 1992;66:303-13.
33. Zerlin M, Levison SW, Goldman JE. Early patterns of migration, morphogenesis, and intermediate filament expression of subventricular zone cells in the postnatal rat forebrain. J Neurosci 1995;15:7238-49.
34. Zimmerman L., Lendahl U, Cunningham M et al. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 1994;12:11-24.

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ORIGINAL ARTICLE

AMBIVALENT EFFECT OF LONG LASTING TERGURIDE TREATMENT ON GENETICALLY BASED GLYCIDE AND LIPIDE METABOLISM ABNORMALITIES IN SHR/N-cp KOLETSKY RATS

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Summary: Experiments were carried out in the genetically hypertensive obese rats of Koletsky type (SHR/N-cp obese), in their lean siblings (SHR/N-cp lean) and in the rats of Han-Wistar strain. The effect of long lasting terguride treatment was monitored when the animal represents a control for itself. Blood was sampled to heparinized capillaries from retrobulbar plexus in the same animal before and after the terguride treament. Long lasting terguride treatment shows decrease in „area under the glucose tolerance curve" in all groups of animals,increase in glycaemia in normotensive males,increase in insulinemia in normotensive rats of both sexes and in SHR/N-cp lean males and decrease of insulinemia in SHR/N-cp obese males, increase of triglyceridemia in normotensive rats of both sexes and in SHR/N-cp lean males, and decrease of triglyceridemia in SHR/N-cp lean females and SHR/N-cp obese of both sexes, increase in cholesterolemia in normotensive and SHR/N-cp lean males,decrease in cholesterolemia in normotensive, SHR/N-cp lean and obese females. Thus ambivalent effect of terguride treatment is more expressive in glucose tolerance and in plasma triglycerides.Ambivalent effect of long lasting terguride treatment is profoundly expressed in correlation between pre-treament state of individual parameters and the effect of treatment expressed in percentage changes in post-treatment state. In all cases statistically significant correlation coefficients show negative mark .Thus it is apparent that terguride increases monitored parameter when this parameter is low before treatment, and vice versa.When it is considered „area under the glucose tolerance curve",significance was attained in all groups,when judging basal glycaemia, significance was attained in normotensive and SHR/N-cp lean rats of both sexes,taking into account insulinemia,significance was attained in normotensive and SHR/N-cp obese rats of both sexes,when analysing plasma triglycerides,significance was attained in normotensive rats of both sexes and in SHR/N-cp lean females and obese males,when we consider total plasma cholesterol, significance was attained in normotensive rats of both sexes and in SHR/N-cp lean males. From the clinical point of view it must be underlined that terguride is potent to increase insulinemia. Thus there is open a possibility of the other clinical indication of the mentioned drug.

Key words: *SHR of Koletsky type; Triglycerides; Total plasma cholesterol; Basal glycaemia; Glucose tolerance; Insulinemia; Ambivalent effect of Terguride*

Introduction

In our previous paper (3) we have documented ambivalent effect of terguride when the drug was applied in normotensive rats of Han-Wistar strain and in SHR/N-cp obese and lean Koletsky rats.The mentined ambivalent effect of terguride was found when plasma triglycerides, total plasma cholesterol and glucose intolerance were considered, i.e., in the group with low parameters terguride shows increase, and vice versa.

While in the above mentioned study the effect of terguride was considered when the results in control and experimental groups were compared, then in the other study where the patients suffering from hyperlipemia accompanied by glucose intolerance the effect of the mentioned drug was monitored in the same patient before treatment and after three months terguride treatment (4),the patient represented a control for himself. Terguride remained without effect or was very low in the patients where no or minimal abnormalities in glucose tolerance were found, and vice versa. For instance, the most profound alleviating effect of terguride was found when before treatment the greatest glucose intolerance was detected. Similar ambivalent effect of terguride was documented in the dislipemia (4).

In the recent study we are verifying the last mentioned results but the measurements were performed in animals where hyperlipemia and the glucose intolerance is based genetically, i.e., in SHR/N-cp obese and lean Koletsky rats.

The normotensive rats of Han-Wistar strain were monitored as well. In recent experiments the animal represents a control for itself.

Material and methods

Animals

Experiments were carried out in the normotensive Han-Wistar rats as well as in obese and lean genetically hypertensive SHR/N-cp rats (5) of both sexes. Lean SHR/N-cp rats represents dominant non-obese homozygotes and heterozygotes whereas their obese siblinggs are recessive homozygotes (cp/cp). The abnormal animals were obtained by Koletsky (75) when mating a female spontaneously hypertensive rat (Okamoto-Aoki strain) with normotensive Sprague-Dawley male rat. The genetically obese animals appeared after several generations of selective inbreeding of hypertensive offsprings of the original cross.

The blood pressure (measured by an indirect method) attained in lean genetically hypertensive SHR/N-cp males 24.61±2.22 kPa (n=l5), l7.60±l.32 kPa (n=8) in females, and in Han-Wistar males l5.90±0.62 kPa (n=7), l4.38±l.22 kPa in females (2) The obese genetically hypertensive SHR/N-cp rats show comparable blood pressure (5).

After weaning at the age of 30 days the animals were kept in groups of four and supplied with water and DOS-2b pelleted diet ad libitum. During the experiment the animals were kept in groups of two. Body weight, water and pellet intake was daily controlled (except Saturdays and Sundays).

Plasma lipids

Blood was sampled to heparinized capillaries (from retrobulbar plexus under light ether anaesthesia at 07.00 a.m.after l4 h starvation) before and after 2l day terguride treatment.Blood was centrifuigated and the serum stored at -20°C.Enzymatic colorimetric method was used for the determination of total plasma cholesterol (CHOD-PAP-Boeringer)as well as for determination of plasma triglycerides (GPO-PAP-TRIG-Boehringer). Estimation was made by Hitachi analyzer.

Plasma insulin

Plasma insulin was estimated by radioimmunoassay.

Glucose tolerance and basal glycaemia

Blood was sampled to heparinized capillaries (from retrobulbar plexus under light ether anaesthesia) before glucose loading (basal glycaemia) as well as 30,60,l20 and l80 min after glucose loading. Glucose (3g/kg b.w., 30% solution) was applied intragastrically after l4 h starvation. Glycaemia was estimated enzymatically (Oxochrom glucose, Lachema). Glucose tolerance is expressed as a sum of glycaemia obtained 30,60,120 and l80 min after glucose loading („area under the glucose tolerance curve").

Terguride treatment

The drug was applied i.p. in two daily doses (07.00 and l4.00) for 21 days. Terguride maleate was administered at a dose of 0.l mg/kg.

Statistics

The data were analyzed by the Wilcoxon test for matched pairs when we were looking for the long lasting terguride effect in individual groups and individual parameters. Moreover, correlations were computed between pre-treatment level of individual parameters and post-treatment level which was monitored in the same animal.

Thus the animal serves as a control for itself when the effect of terguride treatment was calculated. The effect was expressed in percent when the value obtained in pre-treatment was considered as l00%. Statistical significance of value remoteness in the individual groups was controlled by the Dixon test (1). The values reaching statistical sinificance of remoteness from the other values in a group we not considered in statistical evaluation.

Results

Glucose tolerance (Table l)

Terguride decreases the „area under the tolerance curve" in all groups of animals.

Basal glycaemia (Table l)

Terguride increases basal glycaemia in normotensive males.

Table l: Effect of long lasting terguride treatment on glucose tolerance and basal glycaemia

Group	n	drug	glucose tolerance	glycaemia
NR-M	l0	Co	24.48±2.53	3.03±0.39
	l0	Te	21.12±1.49^{d}	3.52±0.55^{b}
NR-F	8	Co	28.05±2.53	3.63±0.50
	8	Te	22.39±1.16^{d}	3.14±0.66
SHR-M	8	Co	33.22±3.41	5.01±0.66
	8	Te	25.96±0.21^{d}	4.62±0.33
SHR-F	8	Co	33.94±3.96	4.57±0.61
	8	Te	26.07±3.25^{d}	4.90±0.33
SHR-O-M	8	Co	52.64±16.67	4.29±0.94
	8	Te	31.74±12.16^{d}	4.51±1.05
SHR-O-F	12	Co	36.74±4.02	5.12±0.88
	12	Te	25.08±2.04^{d}	4.95±0.88

Table l. Means and standard deviations. Abbreviations: Co- control animals, Te - animals under terguride treatment. a - P<0.l0, b - P<0.05, c - P<0.02, d - P<0.01. NR - Wistar strain, SHR - SHR/N-cp lean, SHR-O - SHR/N-cp obese, M - males, F - females.

Insulinemia (Table 2)

Terguride increases insulinemia in the normotensive rats of both sexes and in SHR/N-cp lean males. Insulinemia is decreased at the level of trend (P<0.l0) in SHR/N-cp obese males.

Triglycerides (Table 2)

Terguride increases triglycerides in normotensive rats of both sexes and in SHR/N-cp lean males. It decreases triglycerides in SHR/N-cp lean females and in SHR/N-cp obese rats of both sexes.

Total plasma cholesterol (Table 2)

Terguride increases cholesterol in normotensive males and in SHR/N-cp lean males and decreases SHR/N-cp obese as well as lean females.

Table 2: Effect of long lasting terguride treatment on insulinemia, triglyceridemia and cholesterolemia.

Group	n	drug	insulinemia	triglyceridemia	cholesterolemia
NR-M	10	Co	127±23	1.11±0.09	1.82±0.29
	10	Te	219±55[d]	1.29±0.19[b]	2.02±0.16[b]
NR-F	8	Co	114±18	0.87±0.14	2.24±0.22
	8	Te	153±18[d]	1.12±0.08[d]	1.96±0.2[a]
SHR-M	8	Co	157±20	0.74±0.13	2.31±0.17
	8	Te	202±34[d]	1.36±0.28[d]	2.59±0.16[d]
SHR-F	8	Co	140±22	1.64±0.76	3.84±0.77
	8	Te	140±40	1.03±0.19[d]	3.23±0.55[d]
SHR-O-M	7	Co	857±461	3.81±0.59(6)	2.85±0.30
	7	Te	457±141[a]	3.28±0.35(6)[b]	2.97±1.03
SHR-O-F	12	Co	438±168	7.22±l.30	3.52±0.27
	12	Te	397±128	3.06±0.66[d]	2.30±0.45[d]

Table 2. Means and standard deviations. Abbreviations are the same as in Table l and in Table 3.

Drug state dependent effect - glucose tolerance (Table 3)

In all groups of animals there are statistically significant correlations (irrespective of test which was used, i.e., parametric or non-parametric). In all cases there is a negative correlation coefficient.

Table 3: State dependent effect of long lasting terguride treatment on glucose tolerance and glycaemia

Group	n	drug	glucose tolerance	glycaemia
NR-M	10	Spe	-0.7234[b]	-0.8580[d]
		Pea	-0.8549[d]	-0.7065[b]
NR-F	8	Spe	-0.8434(9)[d]	-0.5334
		Pea	-0.9416(9)[d]	-0.709[5 b]
SHR-M	8	Spe	-0.8434[c]	-0.8051[b]
		Pea	-0.9671[d]	-0.9381[d]
SHR-F	8	Spe	-0.7857[b]	-0.8295[b]
		Pear	-0.7256[b]	-0.8529[d]
SHR-O-M	9	Spe	-0.8619[d]	-0.3810(8)
		Pear	-0.7678[c]	-0.4218
SHR-O-F	12	Spe	-0.7088[c]	-0.3322
		Pear	-0.7581[d]	-0.4349

Table 3. Correlation coefficients. Abbreviations: Spe - Spearman nonparametric correlation, Pear - Pearson pararametric correlation. n = number of rats in group. The other abbreviations are the same as in Table l and in Table 2.

Drug state dependent effect - basal glycaemia (Table 3)

Statistically significant correlations with negative correlation coefficients (irrespective of the test) were found in normotensive as well as in SHR/N-cp lean rats of both sexes. Significance is missing in the SHR/N-cp obese rats of both sexes.

Drug state dependent effect - insulinemia (Table 4)

Statistically significant correlations with negative correlation coefficients were found in SHR/N-cp obese rats of both sexes (irrespective of the test). Statistically significant correlations were found also in normotensive rats of both sexes when parametric Pearson test was used.

Drug state dependent effect - triglycerides (Table 4)

Statistically significant correlations with negative correlation coefficients were found in the normotensive rats of both sexes and in SHR/N-cp lean females and SHR/N-cp obese males (irrespective of test).

Drug state dependent effect - cholesterol (Table 4)

Statistically significant correlation with negative correlation coefficients were found (irrespective of test) in normotensive males and in SHR/N-cp lean males, and in normotensive females when parametric test was used.

Table 4: State dependent effect of long lasting terguride treatment in insulinemia, triglyceridemia and total plasma cholesterol

Group	n	drug	insulin.	triglycer.	cholesterol
NR-M	10	Spe	-0.6121	-0.6544[b]	-0.9030[d]
	10	Pea	-0.6833[b]	-0.6358[b]	-0.8694[d]
NR-F	8	Spe	-0.6946[a]	-0.8133[b]	-0.6190
	8	Pea	-0.7292[c]	-0.8566[d]	-0.7068[b]
SHR-M	8	Spe	-0.4286	-0.4048	-0.8614[d]
		Pear	-0.5658	-0.2843	-0.8208[c]
SHR-F	8	Spe	+0.0476	-0.9048[d]	-0.5476
		Pear	+0.2391	-0.8629[d]	-0.5667
SHR-O-M	7	Spe	-0.9643[d]	-0.8286(6)[a]	+0.6429
		Pear	-0.8846[d]	-0.8580(6)[b]	+0.3586
SHR-O-F	12	Spe	-0.7483[c]	0.3462	+0.0911
		Pear	-0.7358[d]	0.4362	+0.0861

Table 4. Correlation coefficients. Triglycer. - plasma triglycerides, Cholester. -total plasma cholesterol. The other abbreviations are the same as in Table l and Table 3.

Discussion

Data obtained in the recent series of experiments are consistent with the findings mentioned in the introduction (3,4),i.e., long lasting terguride treatment of lipide and glycide metabolic abnormalities shows ambivalent effect. We demonstrated this ambivalent effect of the mentioned drug in the SHR/N-cp obese and lean Koletsky rats when the control animals were compared with experimental animals

(3) as well as in the patients suffering with hyperlipemia acccompanied by glucose intolerance (4),where the patient was a control for himself.

In the recent series of experiments the animal represents a control for itself, i.e., we are using the same experimental arrangement as it was used in the monitoring the effect of long lasting effect of terguride in the patients suffering with glycide and lipide abnormalities (4) with three exceptions,i.e., terguride in the patients was applied for three months (in our series of experiments the drug was applied for three weeks only) moreover, the dose per day was in the patients 0.6 mg and was applied per os (in our rats drug was applied i.p.).

When we compare intergroup differences in the effect of long lasting terguride treatment the most expressed ambivalent effect can be found in glucose tolerance and in plasma triglycerides (see Table l and 2).When pre-treatment „area under the glucose tolerance curve" represents in normotensive males 24.48 mmol/l, in SHR/N-cp lean males 33.22 mmol/l and in SHR/N-cp obese males 52.64 mmol/l, then the terguride effect shows in normotensive males-l4%, in SHR/N-cp lean males -22% and in SHR/N-cp obese males -32%.Similarly the ambivalen effect of terguride treatment is expressed in plasma triglycerides. When pre-treatment plasma triglycerides represent in normotensive females 0.87 mmol/l, in SHR/N-cp lean females l.64 mmol/l and in SHR/N-cp obese females 7.22 mmol/l, then the terguride effect is represented in normotensive females by +30%, in SHR/N-cp lean females by -28% and in SHR/N-cp obese females by -57%.

The above mentioned ambivalent effect of terguride treatment when individual groups of animals are considered can be explained by strain and/or substrain effect. There is also apparent the sex dependence of ambivalent effect which is more expressed in males when glucose tolerance is considered, and in females when plasma triglycerides are analyzed. When the results in individual groups are taken into consideration, then ambivalent effect of terguride is problematical in insulinemia and in total plasma cholesterol. Under these computation arrangement,i.e., when pre- and post-treatment data are compared, the effect of terguride in basal glycaemia is limited to the normotensive males.

The ambivalent effect can be proved also by computation of correlations between the pre-treatment values in some parametr and the terguride effect expressed in per cent (l00% is equal to pre-treament value). The results are summarized in Table 3 and 4.

First, it must be stressed that all statistically significant correlation coefficient show negative marks, i.e., it is in agreement with the data presented in our previous papers (3,4) as well as in the recent study when the results of individual groups were considered (see glucose tolerance and plasma triglycerides, Table l and 2),i.e., the greater pre-treatment value the greater effect of long lasting terguride treatment, and vice versa.

Moreover, statistically significant correlation coefficient can be found also in the cases where differences between control and experimental group do not attained statistical significance. Most apparent differences in the statistical significance can be demonstrated in basal glycaemia. While Wilcoxon test for matched pairs (pre-treatment versus post-treatment values) showed no statistical differences in any group of rats except normotensive males, then statistically significant correlations were attained in normotensive as well as in SHR/N-cp lean rats of both sexes.

On the other hand, when plasma triglycerides are taken into consideration and we are analyzing data in SHR/N-cp lean males and in SHR/N-cp obese females, then statistically significance of correlation coefficient is missing, the statistically significance in Wilcoxon test for matched pairs was attained and this significance is in accordance with the ambivalent princip, i.e., pre-treatment x= 0.74±0.13 mmol/l in SHR/N-cp lean males is accompanied by +84% increase which is done by terguride treatment, and vice versa, pre-treatment x=7.22±l.30 mmol/l in SHR/N-cp obese females is accompanied by -57% decrease induced by terguride treatment.

The above mentioned results suggest that the effect of long lasting terguride treatment is predominatly expressed in the glucose tolerance and in plasma triglycerides.

It remains to be solved the cases where profound effect of terguride is present (see plasma triglycerides in SHR/N-cp lean males and SHR/N-cp obese females) and where the correlation between pre-treatent values and the terguride effect expressed in percent does not attain statistical significance. Some words to insulinemia in the normotensive rats of both sexes.It is apparent that terguride is potent to increase plasma insulin very profoundly in the mentioned type of rats. When we omit the fact that this finding is in line with the ambivalent princip of the terguride treatment, then the insuline increase suggest possible relation to the parallel increase in plasma triglycerides in the mentioned type of animals.

It is accepted that insulin increases triglyceride stores. Increased entry of glucose into adipose tissue facilitates fatty acid and glycerophosphate synthesis, which, by mass action, drives triglyceride synthesis. In addition, insulin inhibits the enzyme which catalyses triglyceride break down (6).

In Table 2 we have demonstrated in the normotensive rats of both sexes that the long lasting terguride treatment induced increase of insulin (+39% in males and 38% in females) and at the same time this drug increases in normotensive rats the plasmatic triglycerides (+l7% in males and 30% in the females).The same can be found in SHR/N-cp males, i.e.,there the terguride increases insulinemia (+30%) and plasma triglycerides (+30%) as well. On the other hand, terguride does not change insulinemia in SHR/N-cp lean females and in SHR/N-cp obese females which is accompanied by decrease of plasma triglycerides in the former (-28%) as well as in the later (-57%) group of animals. In

SHR/N-cp obese males tergutide decreases insulinemia (-26%) and decreases plasma triglycerides (-17%). The mentioned data are in agreement with the ambivalent princip of the terguride effect. Moreover, these data suggest a possible causal relationship between ambivalent effect of terguride on insulinemia on one side, and on plasma triglycerides on the other side. Thus, under the long lasting terguride treatment the changes of triglycerides can be considered as a consequence of the primary changes of insulinemia.This working hypothesis is in accordance with a recent conception of the role of insulin in the increase of triglyceride stores. But the causal relationship between elevation of insulinemia and increases of plasma triglycerides must be controlled in the father series of experiments. Nevertheless, data obtained in the normotensive rats of both sexes and in SHR/N-cp lean males suggest that long lasting terguride treatment is potent to increase insulinemia.To what extent this reality can be utilized in clinical practice it ramains to be solved.

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References

l. Dixon WJ in Wissenschaftliche Tabellen, Ciba-Geigy, AG, Basel l97l:53.

2. Golda V, Petr R. Dopaminergic mechanism and genetic factor in blood pressure regulation. Sb Ved Pr Lek Fak Karlovy Universisty Hradec Králové l982;25:419-27.

3. Golda V, Cvak L. Terguride but not bromocriptine alleviated glucose tolerance abnormalities and hyperlipemia in obese and lean genetically hypertensive Koletsky rats. Physiol Res l994;43:299-305.

4. Golda V, Cvak L. Ambivalent effect of terguride on glucose tolerance. Physiol Res l995;44:l4P.

5. Koletsky S. Pathologic findings and laboratory data in a new strain of hypertensive rats. Am J Pathol l975;80:129-40.

6. Vander AJ, Sherman JH, Luciano DS Human Physiology, The mechanism of body function, McGraw-Hill, Inc., New York, l975:399.

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ORIGINAL ARTICLE

HYPOCHOLESTEROLEMIC EFFECT OF PRAVASTATIN IS ASSOCIATED WITH INCREASED CONTENT OF ANTIOXIDANT VITAMIN-E IN CHOLESTEROL FRACTIONS

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Summary: Metabolic studies support the findings that antioxidants inhibit atherosclerosis. Treatment with vitamin E reduced both the susceptibility of low density lipoprotein cholesterol (LDL-C) to in vivo lipid peroxidation and atherosclerosis and smooth muscle proliferation. Thus the aim of present study was to examine metabolic consequences of reduced plasma LDL-C during hypolipidemic therapy and the distribution of antioxidant vitamin E. A group of l0 patients (4 men, 6 women, age 35 - 65y) with familial hypercholesterolaemia was treated using pravastatin (Lipostat® Bristol Myers Squibb, 40mg daily at 6.00 PM). Blood samples were examined before treatment, after 4 and 8 weeks of therapy. After ultracentrifugation, samples were analyzed for lipoprotein fractions and the content of vitamin E and cholesterol. Pravastatin reduced both total cholesterol (9.85±0.74 vs. 6.81±0.51 mmol/l; $p<0.01$), LDL-C (6.42±0.45 vs. 4.51±0.45 mmol/l; $p<0.01$), light LDL1-C (4.56±0.50 vs. 3.11±0.34 mmol/l; $p<0.05$) and dense LDL2-C (1.86±0.27 vs. 1.42±0.17 mmol/l; ns). Serum vitamin E was reduced during hypolipidemic therapy in the fraction of total, LDL1, LDL2 and VLDL-cholesterol. However, the ratio of serum vitamin E/total serum cholesterol (4.57±0.32 vs. 5.12±0.37 mmol/l/mmol/l; $p<0.05$) and ratio of LDL2-C vitamin E/LDL2-C (3.92±0.07 vs. 4.64±0.37 mmol/l/mmol/l; $p=0.08$) increased in comparison to pre-treatment values. We conclude that pravastatin therapy may possess anti-atherogenic properties which involve not only its hypocholesterolemic effect, but also its favorable effects on the distribution of LDL subclasses and the content of antioxidant vitamin E in atherogenic lipoproteins.

Key words: *Antioxidants; Vitamin E; Atherosclerosis; Lipoprotein fractions; Lipid peroxidation*

Introduction

Oxidative modification of low density lipoprotein (LDL) is the key event in the initiation and development of atherosclerosis (21). Macrophages, the precursors of most foam cells in early stages of atherosclerotic lesions, cannot take up native LDL quickly enough to cause lipid loading (19). However, macrophages take up considerable quantities of oxidatively modified LDL, leading to their loading with cholesterol and cholesteryl esters (17). Oxidized LDL possesses additional atherogenic properties, because it is highly toxic to cells and may be responsible for further damage to the endothelial layer and destruction of smooth muscle cells (8).

Circulating LDL particles in plasma are protected from the effects of lipid peroxidation by cellular and extracellular antioxidant mechanisms which serve to trap reactive oxygen species close to their site of formation and which also function to inhibit the chain reaction of free radical formation. LDL-particles contain fat-soluble endogenous antioxidants that can also prevent or limit the chain reaction of lipid peroxyl radical formation. These natural antioxidants, which include ubiquinol, vitamin E, lycopene and β-carotene, are preferentially oxidized before oxidation of LDL polyunsaturated fatty acids (7,22). Vitamin E is quantitatively the most abundant endogenous antioxidant present in LDL, and in vitro studies suggest that α-tocopherol slows down the oxidation of LDL and prevents the cytotoxic action of this event on the cellular processes (6).

In addition to their LDL-lowering effect, statins may have further beneficial effects on LDL metabolism and LDL composition. Statins increase LDL receptor activity, mainly in the liver, and thus contribute to removal of aged LDL particles from the circulation (20). Studies have shown greater resistance to in vitro oxidation of LDL from subjects treated with statins (13). Such effects may correlate with the concentration of endogenous α-tocopherol in isolated LDL.

The aim of present study was to evaluate the effects of pravastatin on the content of endogenous vitamin E in lipoprotein subfractions and on the distribution of LDL subclasses during such hypolipidemic intervention.

Material and Methods

Study design

This protocol comprises an open, single-center study. Ten patients (4 men, 6 women, age 35 - 65y) with familial hypercholesterolaemia were treated using pravastatin (Pravastatin®, Bristol-Myers-Squibb, 40mg daily with the evening meal). The subjects were not taking vitamin supplements before the study. Approximately 20% of the subjects were treated by other hypolipidemic drugs before the study, and had stopped such treatment at least 6 weeks before the entry into the study. Upon entry into the 6-week baseline phase and further on, patients were counseled to follow the National Institute of Health (NIH) Cholesterol Education Program (NCEP) Step I diet which limits dietary cholesterol to <300mg/day, saturated fats <10% of total calories, and total fats to <30% of total calories (9).

The baseline biochemical characteristic of the patient group was as follows: total cholesterol (TC) 9.85±0.74 mmol/l, low density cholesterol (LDL-C) 6.42±0.45 mmol/l, and triacylglyceroles 2.91±0.42 mmol/l.

The study protocol had been accepted by local ethical committee, Charles University, Faculty Hospital, Hradec Králové, Czech Republic.

Methods

Blood samples were examined at the beginning of the study, after 4 and 8 weeks of therapy after twelve hour overnight fasting.

Lipoprotein fractions were detected by density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA) (18). The lipoprotein fractions were distinguished in the following density ranges: VLDL <1.006 g/ml; LDL1 <1.019 g/ml; LDL2 <1.063 g/ml; HDL >1.063 g/ml. The fractions were analyzed for content of vitamin A, E and C by HPLC (Hewlett Packard 1084 A, Palo Alto, CA) and fluorescence detector (Perkin Elmer MPF-3, Norwalk, CT) (1,5). Total concentration and/or lipoprotein fraction concentration of cholesterol (3) and triacylglyceroles (4) were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Statistical analysis

Values are reported as means ± SEM. Data were analyzed by paired t-test using BMDP software (Solo 4.0, Charles University Medical Faculty, Hradec Králové, Czech Republic). A value of $p < 0.05$ and less was taken as criterion of significance.

Results

Total cholesterol.

Pravastatin was an effective hypolipidemic drug, and significantly reduced total plasma cholesterol after 8 weeks of therapy (9.85±0.74 vs. 7.92±0.68 mmol/l; p<0.01; not shown in Figures).

LDL, LDL1, LDL2 and HDL-cholesterol.

The treatment with pravastatin significnatly reduced plasma LDL-C (6.42±0.45 vs. 4.51±0.45 mmol/l; p<0.01), light LDL1-C (4.56±0.23 vs. 3.36±0.45 mmol/l; p<0.01) and heavy LDL2-C (1.86±0.50 vs. 1.26±0.11 mmol/l; p<0.05) after 8 weeks of therapy (Figure 1). HDL-C did not change significantly after 8 weeks of therapy (1.22±0.15 vs. 1.03±0.08 mmol/l; n.s.; not shown in Figures).

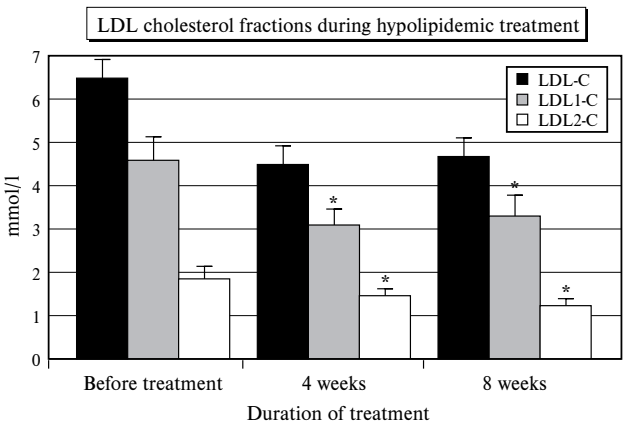

Fig. 1: The changes in serum LDL, LDL1 and LDL2 cholesterol in patients with familial hypercholesterolaemia treated using pravastatin (Bristol-Myers-Squibb, 40mg daily with the evening meal). Blood samples were examined at the beginning of the study, and after 4 and 8 weeks of therapy. The results are reported as means ± SEM. Statistical significance vs. values before treatment is indicated by asterisk above particular bar.

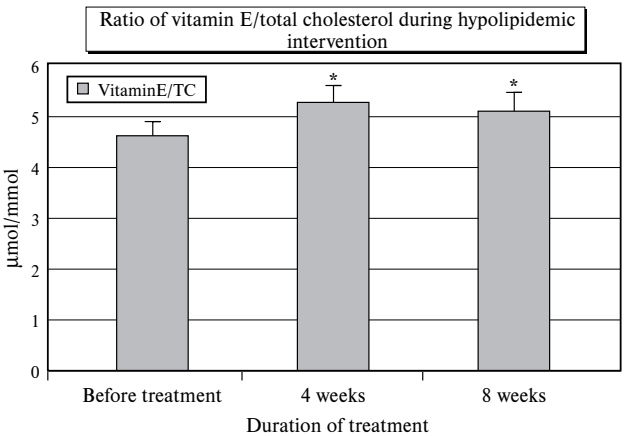

Fig. 2: Ratio of serum vitamin E in total cholesterol/total cholesterol in patients with familial hypercholesterolaemia treated using pravastatin (Bristol-Myers-Squibb, 40mg daily with the evening meal). Blood samples were examined at the beginning of the study, and after 4 and 8 weeks of therapy. The results are reported as means ± SEM. Statistical significance vs. values before treatment is indicated by asterisk above particular bar.

Vitamin E in lipoprotein fractions.

The serum vitamin E was reduced after 8 weeks of hypolipidemic therapy in the fractions of total cholesterol (44.11±3.36 vs. 39.51±3.42 μmol/l; n.s.; not shown in Figures), LDL1-cholesterol (16.34±1.86 vs. 11.29±01.35 μmol/l; $p<0.05$; not shown in Figures), LDL2-cholesterol (7.27±1.01 vs. 7.04±1.37 μmol/l; n.s.; not shown in Figures), and VLDL-cholesterol (15.79±2.70 vs. 13.94±3.49 μmol/l; n.s.; not shown in Figures). However, the ratio of serum vitamin E/total serum cholesterol (4.57±0.32 vs. 5.12±0.37 μmol/mmol; $p<0.05$) (Figure 2) and ratio of LDL2-C vitamin E/LDL2-C (3.92±0.07 vs. 4.64±0.37 μmol/mmol; $p=0.08$) (Figure 3) increased in comparison to the pre-treatment values after 8 weeks of therapy.

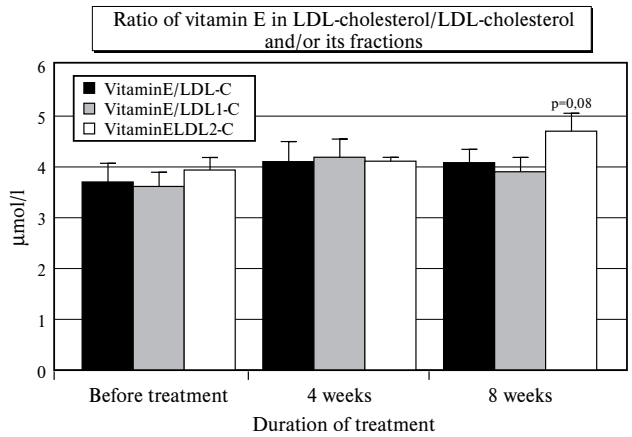

Fig. 3: Ratio of serum vitamin E in LDL, LDL1 and LDL2 cholesterol / LDL, LDL1 and LDL2 cholesterol in patients with familial hypercholesterolaemia treated using pravastatin (Bristol-Myers-Squibb, 40mg daily with the evening meal). Blood samples were examined at the beginning of the study, and after 4 and 8 weeks of therapy. The results are reported as means ± SEM. Statistical significance vs. values before treatment is indicated above particular bar.

Vitamin A, vitamin C.

The serum content of vitamin A (1.87±0.11 vs. 2.27±0.17 μmol/l; n.s.; not shown in Figures) and vitamin C (48.50±6.98 vs. 47.35±8.10 μmol/l; n.s.; not shown in Figures) did not change significantly during hypolipidemic therapy.

Discussion

Intervention that may reduce LDL oxidation is considered to be antiatherogenic. The present study demonstrated that pravastatin therapy, in addition to its hypocholesterolemic effects on plasma LDL, significantly decreased the small, dense LDL2, increased the content of vitamin E in total cholesterol and non-significantly in dense LDL2-cholesterol fractions. Subjects in this study were not vitamin-E deficient (15) and had concentrations of plasma vitamin E slightly above the range observed by others (16). It is of interest, that the serum vitamin A and vitamin C did not change significantly during hypolipidemic therapy. We suggest that the substantial increment of vitamin E in cholesterol and LDL subclasses, despite its total concentration decreased or did not change, may be the result of the hypocholesterolemic effect of pravastatin.

Human LDL contains a variety of antioxidants that can inhibit lipid peroxidation, including α-tocopherol (biologically the most active form of vitamin E). α-Tocopherol is the most abundant antioxidant in LDL and many clinical studies aimed at increasing resistance of LDL to oxidation have used supplementation with vitamin E, either singly (6), or in combination with β-carotene and/or vitamin C (2). The LDL-protective effects were connected mostly with vitamin E. There is a great variation in the ability of LDLs isolated from different donors to resist oxidation, and therefore is not clear whether such response could be a function of the amount of antioxidant present in these samples. A varying correlation exists between the concentration of endogenous α-tocopherol in isolated LDLs and their susceptibility to oxidation (6). However, addition of α-tocopherol in isolated LDL or ingestion of large doses of vitamin E increases the lag time for initiation of oxidation (6).

In addition to their LDL-lowering effect, statins may have further beneficial effects on LDL metabolism and LDL composition. We present that pravastatin therapy significantly increased the content of vitamin E in total cholesterol and non-significantly in dense LDL2 despite decrease of total cholesterol and LDL2 plasma concentration as a lipid carrier. What are the mechanism of such effects is unknown. It may be as a result of the shorter time for which LDL particle circulates. LDL, while circulating, is exposed to time-dependent modifications, such as oxidative stress, depletion of anti-oxidant tocopherols, and non-enzymatic glycation. Agents which stimulate LDL receptor activity are likely to promote the clearance of LDL1 and LDL2, as has been observed (11) and as such shorten the time-dependent consumption of LDL bound vitamin E. There are another possible mechanisms. The α-tocopherol content of lipoproteins is determined to a large extent by factors other than the plasma α-tocopherol concentration (23). If the rate of LDL precursors clearance under pravastatin therapy increases, secretion of α-tocopherol in VLDL from the liver can lead to the enrichment of circulating lipoproteins with α-tocopherol (14). As regards LDL subfraction distribution, pravastatin in our study with familial hypercholesterolemic patients decreased the relative concentration of small, dense LDL2, which is believed to be atherogenic. Others were unable to find such effects of pravastatin therapy in the patients with familial combined hyperlipidemia (10). However, increased VLDL and elevated plasma triglyceride levels in combined hyperlipidemia are emerging as the most important determinant of the

LDL subfraction profile and form pattern-B of LDL profile (LDL2 in excess). Because pravastatin could affect such pathways of lipoprotein metabolism, it is conceivable, but still needs to be proven, that all or some of the above factors may influence the concentration and functional properties of vitamin E in lipoprotein fractions during hypolipidemic therapy.

We conclude that pravastatin therapy may possess antiatherogenic properties which involve not only its hypocholesterolemic effect, but also its favorable effects on the distribution of LDL subclasses and the content of antioxidant vitamin E in atherogenic lipoproteins.

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References

1. Aaran RK, Nikkari T. HPLC method for the simultaneous determination of beta-carotene, retinol and alpha-tocopherol in serum. J Pharmacol Biomed Analysis 1988;7:853-7.
2. Abbey M, Nestel PJ, Baghurst PA. Antioxidant vitamins and low density lipoprotein oxidation. Am J Clin Nutr 1993;58:255.
3. Allain CC, Poon LC, Chan CSG, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20:470.
4. Bucolo G, David H. Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem 1973;19:476.
5. De Leenheer AP, De Bevere VORC, De Ruyter MGM, Clayes AE. Simultaneous determination of retinol and (-tocopherol in human serum by HPLC. J Chromatogr 1979;162:402-13.
6. Dieber-Rotheneder M, Puhl H, Waeg G et al. Effect of oral supplementation with D-alpha-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. J Lipid Res 1991;32:1325-32.
7. Esterbauer H, Rotheneder M, Striegl G et al. Vitamin E and other lipophilic antioxidants protect LDL against oxidation. Fat Sci Technol 1989;91:316.
8. Esterbauer H, Dieber-Rotheneder M, Waeg G, Striegl G, Jurgens G. Biochemical, structural and functional properties of oxidized low-density lipoprotein. Chem Res Toxicol 1990;3:77-92.
9. Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults. Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). JAMA 1993;269:3015-23.
10. Franceschini G, Cassinotti M, Vecchio G et al. Pravastatin effectively lowers LDL cholesterol in familial combined hyperlipidemia without changing LDL subclass pattern. Arterioscler Thromb 1994;14:1569-75.
11. Gaw A, Packard CJ, Murray EF et al. Effects of simvastatin on apoB metabolism and LDL subfraction distribution. Arterioscler Thromb 1993;13:170-89.
12. Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. Ann Rev Biochem 1977;46:897.
13. Hoffman R, Brooks GJ, Aviram M. Hypolipidemic drugs reduce lipoprotein susceptibility to undergo lipid peroxidation: in vitro and ex vivo studies. Atherosclerosis 1992;93:105-13.
14. Kayden HJ, Traber MG. Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. J Lipid Res 1993;34:343-58.
15. Maes M, Weeckx S, Wauters A et al. Biological variability in serum vitamin E concentrations: relations to serum lipids. Clin Chem 1996;42:1824-31.
16. Miller NJ, Paganga G, Wiseman S et al. Total antioxidant activity of low density lipoproteins and the relationship with (-tocopherol status. FEBS Lett 1995;365:164-6.
17. Mitchinson MJ, Ball RY, Carpenter KH, Enright JH, Brabbs CE. Ceroid, macrophages and atherosclerosis. Biochem Soc Transact 1990;18:1066-9.
18. Naito HK. Lipoprotein separations using the TL-100 tabletop ultracentrifuge. Application Note Beckman DS-693.
19. Palinsky W, Rosenfeld ME, Herttuala SY et al. Low-density lipoprotein undergoes oxidative modification in vivo. Proc Natl Acad Sci 1989;86:1372-6.
20. Shepherd J. Fibrates and statins in the treatment of hyperlipidemia: in appraisal of their efficacy and safety. Eur Heart J 1995;16:5-13.
21. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: Modifications of low density lipoprotein that increase its atherogenecity. N Engl J Med 1989;320:915.
22. Stocker R, Bowry VW, Frei B. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. Proc Natl Acad Sci USA 1991;88:1646.
23. Ziouzenkova O, Winklhofer-Roob BM, Puhl H, Roob JM, Esterbauer H. Lack of correlation between (-tocopherol content of plasma and LDL, but high correlations for (-tocopherol and caroteinoids. J Lipid Res 1996;37:1936-46.

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ORIGINAL ARTICLE

HOW LONG CAN THE PREVIOUSLY ASSEMBLED CARDIOPULMONARY BYPASS CIRCUIT STAY STERILE?

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Summary: The sterility of previously assembled cardiopulmonary bypass circuits was investigated for 100 extracorporeal circuits. The closed circuits were assembled using aseptic technique and remained in the pump room until time of use. The mean time from point of setup to point of priming for the 100 consecutive circuits was 32 hours, with a range of 19 to 89 hours. Circuits were primed with the calculated volume of priming solution, circulated for 5 minutes and tested for microbial contamination by withdrawing 20 ml of the priming solution and 10 days incubated in Thioglycolate and Sabouraud culture mediums. All were found to be free of microbial comtamination. The results of this investigation demonstrate that the sterility of the extracorporeal circuit, pre-assembled in advance of actual priming, can be maintained over an extended interval when standard aseptic technique is used. This allows the utilization of a pre-assembled circuit for emergency cardiopulmonary support.

Key words: *Open heart surgery; Cardiopulmonary bypass; Cardiopulmonary bypass circuit; Microbial contamination; Pre-assembling of circuit*

Introduction

The initiation of immediate cardiopulmonary bypass in emergency situations is life-saving. However, before emergency cardiopulmonary bypass can begin, the perfusionist must aseptically assemble, prime and debbuble the extracorporeal circuit as quickly as possible. The availability of previously assembled, sterile extracorporeal circuit in these instances can save 20-40 critical minutes.

Although a number of investigations (3,6,8) of the causes of infection in open heart surgery patients have considered cardiopulmonary bypass apparatus, we have found only two reports examining the sterility of assembled extracorporeal circuits. The first one is from 1990. Chorak (2) examined 26 circuits preassembled 24-96 hours prior to priming. He tested the sterility with Bactec NR 660 nonradiometric system and he found all circuits to be sterile. The second one is from 1993. Homishak (5) tested 17 circuits preassembled 13-60 hours with Addi-Chek Quality Control System. He also found all of them to be free of microbial contamination. Because of a such poor information about so important topic, which sterility of extracorporeal circuits certainly is, we have decided to perform our own investigation using larger ammount of tested circuits.

Material and methods

We have investigated one hundred consecutive previously assembled cardiopulmonary bypass circuits.

Our extracorporeal circuits tested consisted of POLYSTAN'S SAFE I closed system (POLYSTAN A/S, Vaerlose, Denmark). A large portion of the circuit was preassembled in a Polystan#s custompack with 7 connections required to complete the set-up.

The extracorporeal setups were assembled and the remaining 7 connections made aseptically with all vents, accesory lines and priming ports remaining capped. The heart-lung machine was covered with a clean sheet and remained in the pump room which is situated between our two operating rooms until next surgery case. At the time of the surgery, the vent caps were removed and remaining connections were completed, the heater-cooler was connected to the oxygenator heat exchanger and circulated. The circuit was then primed with adequate volume of our antibiotic free priming (Tab.1) with recirculation through both reservoirs, the arterial filter, and the oxygenator at a flow of two liters per minute for 5 minutes and system was debbubled.

After recirculation 20 ml of priming was taken into the sterile test-tube for microbiological testing. For our sterility

tests we have chosen the bacteriological methods which are described in detail in the summary of pharmaceutical tests (7), which are used in our country as a quality control system for pharmacists to determine the microbial quality of intravenous solutions. The samples were injected into culture mediums and were incubated for ten days. As the culture mediums to monitor microbial growth there were used Thioglycolate medium for aerobes and anaerobes detection and Sabouraud medium for fungi detection. For a sample to be considered positive the culture mediums must have appeared turbid at some point during the 10 day incubation interval.

Table 1: Oxygenerator prime

Hartmann's solution	750 - 1000 ml (acc. weight)
Rheomacrodex	3% 500 ml
Mannitol	10% 1,0 g/kg
Natrium bicarbonate	8,4% 1 mmol/kg
Magnesium sulphuricum	20% 10 ml
Heparin	2 500 U
Metylprednisolon	5 mg/kg
Ascorbic acid	1000 mg

Results

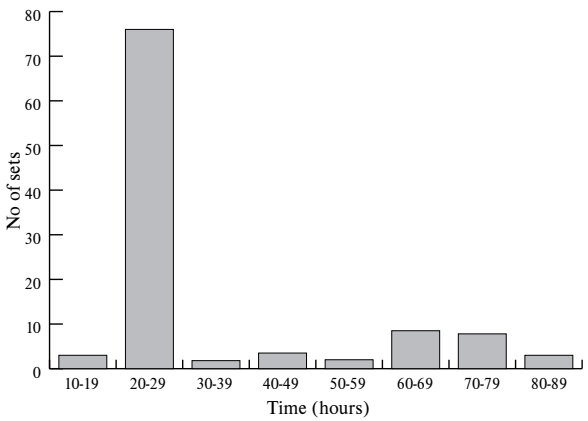
One hundred extracorporeal circuit set-ups were tested for microbial contamination using the above mentioned microbiological methods. Time elapsed from set-up of the circuit to priming ranged from 19 to 89 hours with a mean time of 32 hours (Tab. 2 and Fig. 1). Most of the circuits - 75 - were preassembled twenty to twenty nine hours which is approximately from one day in advance.

All the l00 tested circuits were found to be sterile. No turbidity in the culture mediums was found.

Table 2: Times from setup to priming (hours)

TIME (hours)	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89
NUMBER OF CIRCUITS	3	75	1	3	1	7	7	3

Fig. 1: Times from setup to priming (hours)



Discussion

Mediastinal, intracardiac and wound infections are still very serious complications of open - heart surgery. In spite of widespread use of prophylactic antibiotics, the incidence of these infections is still within the range of 2% in most reported series. Several sources of infection have been suggested.

Staphyloccocus aureus and epidermidis are referred as the most common causes of infection. They are present in the microflora of the human skin and through the process of shedding can these microorganisms be spread into the air of operating theaters. Blakemore, Lathrop (1) and their co-workers suggested that aspiration of such a contaminated air into the extracorporeal circuit can cause postoperative infection. However, contamination of the extracorporeal circuit may also be caused by suction of air-contaminated blood from the wound site (8). Another important source of contamination can be the central venous catheter.

The purpose of our investigation was to determine whether an extracorporeal circuit could be aseptically assembled prior to use and sterility maintained over an extended interval without contamination. The possibility to pre-assemble an extracorporeal circuit in advance of emergency cardiac surgery, such as might occur during evenings, nights, weekends, but also just after the routine procedure due to extended postoperative myocardial ischaemia, affords the opportunity for the perfusionists to start the emergent cardiopulmonary bypass without loosing the time. Our results support the very poor knowledge of this problem (2,5) that the pre-assembled extracorporeal circuit can stay sterile without the risk of contamination more than two days.

It is also necessary to keep in mind that all principles of sterile handling with tubing connections have to be adhered. The pump room must be the integrated part of the operating theatres space and operating theatres air-conditioning system.

The perfusionist has to make sure that all vent caps are in their positions before connecting the remaining lines in the time of use the circuit, otherwise the circuit cannot be used.

Conclusion

The results of our study demonstrate the extracorporeal circuit can be assembled for approximately 2,5-3 days prior of cardiac surgery with no risk of contamination when standard aseptic technique is used. This allows to perfusionists the utilization of a preassembled circuit for emergency cardiac surgery which might occur during evenings, nights and weekends without loosing the time.

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References

1. Blakemore WS, McGarrity GJ, Thurer RJ, et al. Infection by air-borne bacteria with cardiopulmonary bypass. Surgery 1971;70:830.
2. Chorak J, Leader I, Patterson M, Kumar A. Sterility of assembled heart-lung pump beyond 48 hours. Am J Infect Control 1990;18:328-30.
3. Freeman R, Hjersing N. Bacterial culture of perfusion blood after open-heart surgery. Thorax1980;35:754-8.
4. Gee LW, Harvey JMGH, Olson WP, Lee ML. Sterility tests systems for product recovery. J Pharm Sci 1985;74:29-32.
5. Homishak RN, Widmer S, Klementowich M, Cunningham J, Oblack D. Sterility of previously assembled cardiopulmonary bypass circuits. J Extra-Corp Tech 1993;25:84-6.
6. Kluge RM, Calia FM, McLaughlin JS, Hornick RB. Sources of contamination in open heart surgery. JAMA 1974;230:1445-8.
7. Kolektiv autorů. 4.8. Zkoušení mikrobiologické. In: Kol. autorů: Československý lékopis. Praha:Avicenum, 1987: 115-7.
8. VanOevern W, Dankert J, Boonstra PW, Elstrod JM, Wildevuur CR. Airborne contamination during cardiopulmonary bypass: the role of cardiotomy suction. Ann Thorac Surg 1986;41:401-6.

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THE HISTORY OF PSYCHIATRY IN HRADEC KRÁLOVÉ

Herbert Hanuš, Vladimír Panoušek, Ivan Tůma

*Assoc. Prof. H. Hanuš (*1934)*

*MUDr. I. Tůma (*1952)*

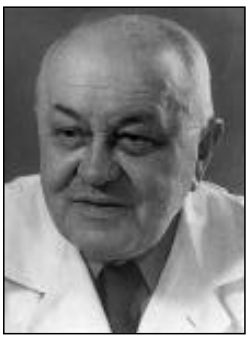
*PhDr. V. Panoušek (*1936)*

Before the establishment of Charles University Faculty of Medicine in Hradec Králové there was no bed ward for psychiatric patients in our town. They were sent to the State Psychiatric Hospital in Bohnice, and since 1928 to the State Psychiatric Hospital in Německý Brod (Havlíčkův Brod now).

In Hradec Králové the well-known physician MUDr. Leopold Batěk was taking care of psychiatric patients from the beginning of the 20 th century to the end of the 1920's. From the notes preserved for his public lectures we can guess that one of the key problems of the psychiatry of that time was the protection of the patient in domestic milieu from an attempt of suicide. In case the mentally ill patient was to be hospitalized, he had to be transferred to a psychiatric hospital. MUDr. Batěk's activity was since 1928 followed up by another outstanding physician MUDr. Stanislav Němeček, a specialist of internal and nervous diseases. He was occupied with the treatment of neuroses and managed the technique of hypnotism. Another neurologist interested in psychiatry before the Second World War was MUDr. Mazačová - Procházková, the widowed wife of Professor of Psychiatry in Brno. Though after coming to Hradec Králové she was engaged rather in organic neurology and the cooperation with the surgeon Bedrna, she was very much interested in psychiatry and proved her unique intuition in the diagnoses of mental disorders later .

The Department of Psychiatry of the Teaching Hospital in Hradec Králové was established on the end of the 1945. On the base of the proposal of the Professors Staff of the Charles University Faculty of Medicine from August 1945 Associate Professor MUDr. Stanislav Krákora, a disciple of Professor Mysliveček, was entrusted. The Department of Psychiatry was built up as a completely new institution. In the meeting of the Professors Staff of Medical Faculty on January 24 th 1946 as a provisional arrangement a building was appropriated to it where the Department of Neurology was placed so far. In February 1946 the lectures on psychiatry were opened with a course for rigorosants, in the summer term 1946 there were regular lectures with demonstration of outpatients and those of the first beds afforded by the Head of the Department of Neurology, Professor MUDr. Václav Piťha. Later on psychiatric patients were admitted to the bed ward established on the ground floor of the building of the Department of Infectious Diseases.

Professor Krákora had been of service in the Army as a medical student in the First World War, since 1919 he was working at the Department of Neurology and Psychiatry built up by Professor Mysliveček in Bratislava. Being obliged to leave Slovakia he was working during the Second World War in an outpatient psychiatric clinic. He came to Hradec Králové in 1945 and was nominated as Professor in 1946. Professor Krákora was the Head of the Department as a Colonel - professional soldier when Faculty of Medicine in Hradec Králové had been appointed as Military Medical Academy in 1951. He was a careful medical doctor, a patient teacher and a unique authority in forensic psychiatry. He concentrated himself to the psychical manifestations of organic and degenerative diseases. He was a pioneer in psychoendocrinology as proved in his habilitation paper „Endocrine Secretion and Mental Diseases" published in 1931 (8). In 1947 Krákora published his paper „General Practitioner and Psychiatry" (9), in 1956 he was the leader of the author's team working out textbook of psychiatry edited by Military Medical Academy in 1956 (10). When the School of Medicine had been changed into a civilian Faculty of Medicine in 1953 again, Professor Krákora was working for a short time only. He died in July 1959.



Professor S. Krákora (1892-1959)

Assoc. Prof. V. Pelikán (1916-1980, sitting) and Assoc. Prof. J. Bílý (1925-1987)

During Professor Krákora's work the Department of Psychiatry had a locked ward for male patients and a unlocked one for female and male patients as well. Beside a comprehensive outpatient services the outpatient clinic for substance - related disorders and children psychiatry clinic were already run.

Associate Professor MUDr. Vilém Pelikán, CSc. became Professor Krákora's successor. He had gotten psychiatric experience at the Department of Neurology in Plzeň as well at the Neuro - psychiatric ward of the Military Hospital in Plzeň which he had established. He passed to the Department of Psychiatry of Military Medical Academy in Hradec Králové in 1954 and was appointed as the Head of the Department in 1959. Assoc. Professor Pelikán was an outstanding specialist in the research and rehabilitation of aphasic disorders. His monograph „The Pathogenesis of Aphasia" in 1970 was dedicated to this problem (11). Pelikán was specially intent on the neurological - psychiatric border, being a much sought for expert in forensic psychiatry and an initiator of Conferences of Forensic Psychiatry in Hradec Králové. At the occasion of this conference on 13.05.1960 the sexuologists led by Associate Professor MUDr. Kurt Freund met the psychiatrists and the lawyers and following a discussion a proposal for the abolition of the punishability of homosexuality was suggested.

Associate Professor Pelikán took part in building-up a network of psychiatric departments in the hospitals in East Bohemia region and thus followed up the heritage of Professor Heveroch, being interested as one of the first Czechoslovak psychiatrists in the relation of cybernetics to neurosciences.

Because of his artless and democratic opinion Associate Professor Pelikán's scientific career was violently interrupted at the beginning of the „normalization" period in 1972. Pelikán passed to the State Psychiatric Hospital in Havlíčkův Brod. He died in 1980. All of us that have known him shall always remember his uncommon and kind character. During Pelikán's activity another provisory annex was added to the low provisory building where a locked female ward and a unlocked one, later on a day hospital were opened. Thus the situation was significantly improved when all agitated or suicidal female patients had not any more to be sent to psychiatric hospitals.

The care of outpatients was developed too. Beside all outpatient clinics mentioned above the activity of the EEG lab was continued. In 1967 a outpatient clinic for affective disorders was established where the lithium thymoprophylaxis was used.

In 1972 Associate Professor, later on Professor MUDr. Miroslav Zapletálek, DrSc., was appointed as a Head of the Department. He passed from the Department of Psychiatry in Olomouc and was considered to be an outstanding specialist in psychopharmacology. He studied the problems of ions in mental disorders and the activity of the vegetative nervous system. In 1976 his monograph was dedicated to the theme mentioned (16).The part in his research, especially in psychopharmacology he developed in Hradec Králové so that the clinic began to play a significant part in the psychopharmacological research in former Czechoslovakia.

The Department was enlarged with a ward for the treatment of alcohol addiction established in a manor-house in Podlesí during Zapletálek's activity. In the ward containing 30 male beds a psychotherapeutic regime was kept and the biological treatment was made use of, too. The therapeutic results of the above ward were thanks to PhDr. Miloslav Pleskač among the best in the country. The ward was being kept from 1988 to 1991 when it was closed due to restitutional and economic reasons. However, the spirit of the ward went on surviving in the AP-Club which is an association of former abstinent patients of the ward.

At the time when Professor Zapletálek was the Head of the Department a sexuological outpatient clinic was established which has been led by MUDr. Jan Zbytovský for many years. At the period mentioned some significant papers were written. The monograph by MUDr. Hanuš in which the experience of many years with the lithium thymoprophylaxis was elaborated and where he tried to determine the prognostic factors of thymoprophylaxis in 1984 (2) belongs to this papers. In that year the first paper dealing with the antisuicidal influence of lithium was published (3). The authors from department published in 1980 the first paper concerning the problem that clozapine can be an addictive drug (12). In 1983 MUDr. Tůma, CSc. et al. elaborated an original study dealing with the cerebral atrophy in schizophrenic patients (17). Two years later he published a paper concerning the problems of the immunology of schizophrenia (18). In 1990 Associate Professor MUDr. Herbert Hanuš, CSc. was appointed as the Head of the Chair of Psychiatry and the Head of Department of Psychiatry on the base of a competition.

Professor Zapletálek was working at the clinic until 1993 then he passed to private practice.

The Department of Psychiatry carries out the instruction of psychiatry for the students of the fourth and fifth classes of the general course, the instruction of medical psychology

for the third class of the general course and the instruction of psychiatry for the fourth class of the stomatological course. In the last years not compulsory subjects, as medical sexuology, psychotherapy and medical ethics were lectured. In the school -years 1994-1995 an instruction of the foreign students in English was started. The Department of Psychiatry is till working under very unfit space conditions but the number of the teachers is considered to be sufficient so that a practical instruction for small groups of medical students can be carried out with the aid of video-records. We considered the official nation-wide textbooks from the beginning of the eighties to be out of date already. We therefore tried to bridge over that gap by writing the textbook „Chapters of Psychiatry" in 1992 (4), then „Chapters of Medical Psychology in 1994 (5) and „Comprehensive Psychiatry" in 1997 (7). At present textbook concerning clinical psychiatry are being prepared. A special literature in English is at our disposal for the instruction of foreign students.

Five psychiatrists are carrying out postgradual training at the Department. University teachers of our Department are taking part in the instruction of bachelor study of nursing, some of them are lecturing at the Pedagogical Faculty.

The research tasks of our Department are directed at psychopharmacology. Within the frame of postgradual training The Comparative study of Three Antidepressants was conducted with a pharmaco-economic evaluation which was awarded with the Scientific Prize by American Psychiatric Association and of Czech Psychiatric Society at the opportunity of its first congress in 1996 (MUDr. Hosák, MUDr. Tůma, CSc). Another study deals with the effectivity of two antidepressants in obsessive compulsive syndrome (MUDr. Pidrman, MUDr. Tůma, CSc.). MUDr. Libigerová took part in an international study concerning the genetics of bipolar affective disorder with the Professors Grof (Canada), Zvolský (Czech Republic) and others.

In other papers Alzheimer's disease, the psychological problems of patients suffering from addictive disorders and a long-time treatment of sexual paraphilias are under study. A Number of studies deals with the clinical testing of new drugs in mental disorders.

A professional level of medical doctors and clinical psychologists has been developed. They take an active part in clinical seminars three times in a month as well as in the regional ones which take place twice a year. Many of them take a systematical postgradual training in psychotherapy. Within the frame of taking part in conferences and congresses they visited the US, Austria, Denmark, Hungary, Italy and the Netherlands in 1997. A research stay of MUDr. Ladislav Hosák in New York University in 1997 contributed to an increase of the professional skills, too.

There are 66 beds in the Department of Psychiatry of the Teaching Hospital in Hradec Králové. Both the inpatients and the outpatients are treated by the most modern biological methods including the bright light therapy as well as by the psychotherapeutic methods including confined psychotherapeutic groups or the dancing therapy. Not only the work-therapy and art-therapy, but also common walks outside the area of the hospital as well other activities are considered to be very important. Psychoeducation of schizophrenic patients and members of their families is provided within the frame of „Prelapse" project.

On December the 1st, 1996 there was a very significant event for the department : the first inpatient unit for the patients suffering from addictive diseases were opened in Nechanice (15 km from Hradec Králové). In the course of the next months the beds were extended to a number of 29, for the year 1998 is planned 50 beds. Except male patients the female ones are hospitalized. The work of the hospital mentioned takes up -as to the contents and the personal respect - that of the treatment of drug addictions in Podlesí. MUDr. Jiří Čížek is a Chief of this unit. Thus the Teaching Hospital has been at least partially prepared to catch up the proceeding explosion of the abuse of addictive drugs.Their occurrence increased among the hospitalized male patients by 700% by 1986 to 1997. For the part of care as well as for ensuring the intensive care of psychotic patients a unit of intensive psychiatric care and a unit for detoxication of addicted patients would have to be established.. The plan mentioned is unfortunately in project only because of the lack of financial resources.

Six outpatient clinics continue to work - that is a comprehensive outpatient department, a clinic for children and adolescents, an outpatient clinic for the treatment of drug abusers and alcoholics, another one for the treatment of affective disorders as well as one for the University students and a sexuological outpatient clinic.

A total of 13899 patients was examined in all of the outpatient clinics in the last year (1997). 929 patients were hospitalized in the inpatient wards including Nechanice in 1997.

A long time and one of the most important problems of the department is a bad technical condition of the buildings, missing the children s and adolescent inpatient unit and a intensive psychiatric care unit. A certain hope appeared us in 1996 when the hospital for addictive disorders was opened. However, the bad space conditions of the Department of Psychiatry as well as the unfavorable technical facilities have been a considerable obstacle for us to carry out the teaching duties.

We are very pleased to be informed by the management of the Teaching Hospital that before long an actual solution would be possible. Even under present conditions we try to further develop a complex psychiatric care which ought to be full of understanding and humanitary respect. We aim at instructing our students in supporting all of the principles mentioned, too.

For more than fifty years a number of medical doctors have been working at our Department so that the summary would include tens of their names. Let us remember at least those that have played the most significant role in the history of the Department and of Czech psychiatry as well. From Krákora's era they were: MUDr. Oldřich Bureš, later on a Head physician and one of the initiators of the modernization of the State Psychiatric Hospital in Havlíčkův

Brod, MUDr. Svatopluk Stuchlík, another outstanding psychiatrist and significant organizer of forensic psychiatry, later on the head physician of the Department of Psychiatry in Pardubice, and finally MUDr. Vladimír Vojtík, a distinguished Czech childpsychiatrist, later on the Head of Children's Psychiatric Hospital in Opařany. Ineffaceably the name of Associate Professor MUDr. Jiří Bílý, CSc., a precise scientist and a musician and angler in his private life, has been entered in the history of the Department and Czech psychiatry. His scientific work on catatonic stupor in 1963 belonged to his most outstanding papers which Bílý modeled as a paroxysmal inhibition in animals (1). The paper by MUDr. Jiří Rigel, an outstanding psychiatrist, psychotherapist and writer, dealing with a case of Jacob - Creutzfeld presenile dementia, published with MUDr. Stanislav Němeček in 1957 (13) belonged to the most important papers. One of his popularly scientific work - his book „Personality and neurotic difficulties" edited in Slovakia in 1974 was of interest (14). MUDr. Václav Šklíba, a careful psychiatrist, became the first head o f the psychiatric ward in Nové Msto nad Metují. An outstanding work was done at our department by MUDr. Jarmila Poláčková, MUDr. Olga Preiningerová and MUDr. Marie Hametová. We often remember head physician MUDr. Sáva Pazdírek, a prematurely deceased long-time worker of the clinic. The present psychiatrists working in the city outpatient clinics - MUDr. Jaroslava Hrubecká, MUDr. Tomáš Hübsch, MUDr. Helena Pavlová and MUDr. Hana Sádlová are former workers of the Department. Professor of the Third Faculty of Medicine, Charles University in Prague, MUDr. Jan Libiger, CSc., left the Department in 1992.

The magazine SCAN inseparably belongs to the recent history of psychiatry in Hradec Králové, too. Though edited since eight years by the Teaching Hospital and the Charles University Faculty of Medicine it also belongs to our psychiatry at least because of the fact that its leading editor PhDr. Vladimír Panoušek has been a clinical psychologist of the Department of Psychiatry for many years and that a number of our co-workers psychiatrists contribute to it. The present paper is taking up again an analogous communication published in SCAN in 1995 (6).

The Department of Psychiatry is also engaged in the Medical Ethics taught for Czech as well as foreign students. Medical Ethics is also engaged with the lectures for the students of postgradual training and other public lectures and scientific papers, for example by MUDr. Ivan Tůma and MUDr. Herbert Hanuš in 1994 (15). The Head of the department is also a chairman of the Ethics Committee of the Teaching Hospital, of the Charles University Faculty of Medicine and of Military Medical Academy.

Mental diseases are as old as mankind itself. The history of psychiatry as a modern medical branch is old a few tens of years only. Except many whiles of powerlessness and hundreds of questions which often did not let us in sleep we have also remembered a lot of exceedingly joyous hours (whiles) when we were witnesses that a human soul was returning to the reality of life. Psychiatry will always be an everlasting search for the biological determinants of mental disorders as well as for the multiform truths about human soul. Just for this reason one must like psychiatry.

References

1. Bílý J. The problem of pathofysiology of catatonic stupor (in Czech). Sb Ved Pr Lek Fak Karlovy Univerzity Hradec Králové 1963;6(Suppl. 6):225-45.
2. Hanuš H, Zapletálek M. The prophylactic lithium treatment of affective disorders and a possibilities of the outcome prediction. Sb Ved Pr Lek Fak Karlovy Univerzity Hradec Králové 1984;27:3-75.
3. Hanuš H, Zapletálek M. Suicidal activity of patients with affective disorders in the course of lithium prophylaxis (in Czech) Cesk Psychiatr 1984;80:97-100.
4. Hanuš H et al. Chapters of psychiatry (in Czech). Praha:Karolinum, 1992:50.
5. Hanuš H et al. Chapters of medical psychology (in Czech). Praha:Karolinum, 1994:125.
6. Hanuš H, Panoušek V. Departments are introducing. Department of Psychiatry (in Czech). SCAN 1995;5:10-3 .
7. Hanuš H et al. Comprehensive psychiatry (in Czech). Praha:Karolinum, 1997:183.
8. Krákora S. The endocrine secretion and mental diseases (in Czech). Revue v neurologii a psychiatrii 193l;28:152-63.
9. Krákora S. General practitioner and psychiatry. Královéhradecké lékařské spisy 1947;1:91-102.
10. Krákora S. Comprehensive psychiatry (in Czech). Hradec Králové: Vojenská lékařská akademie, 1956:196.
11. Pelikán V. The pathogenesis of aphasia (in Czech). Praha: Avicenum, zdravotnické nakladatelství, 1970:208.
12. Preiningerová O, Hanuš H, Zapletálek M. Is Clozapin an addictive drug ? (in German). Agressologie 1980;21:19-22.
13. Rigel J, Němeček S. A case of Jacob-Creutzfeld presenile dementia (in Czech). Cesk Psychiatr 1957;53:353-8.
14. Rigel J. The personality and the neurotic difficulties (in Slovak). Martin:Osveta, 1974:128.
15. Tůma I, Hanuš H. Ethics in clinical research (in Czech). Lek Zpr LFUK Hradec Králové 1994;39:181-9.
16. Zapletálek M. Disorders of the autonomic nervous system and their examination in psychiatric patients (in Czech). Sb Ved Pr Lek Fak Karlovy Univerzity Hradec Králové 1976;19 (Suppl. 1):123-210.
17. Zapletálek M, Tůma I, Kudrnová K, Černoch Z, Hubert J. Cerebral atrophy in schizophrenic patients - clinical and scannographical study (in Czech). Cesk Psychiatr 1983;81:43-5
18. Zapletálek M, Tůma I, Fixa B, Kudrnová K. Toward the problems of immunology in schizophrenia (in Czech). Cesk Psychiatr 1985;81:43-5.

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