POLISH ACADEMY OF SCIENCES
MEDICAL RESEARCH CENTRE

ANNUAL REPORT
2000

WARSZAWA - 2001

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CONTINUATION OF THE STUDIES ON THE MECHANISM AND FUNCTION OF THE CAROTID BODY

Project leader: Mieczysław Pokorski
Contributors: Lidia Faff, Robert Strosznajder, Magdalena Marczak, Ahlam Ramadan, Lidia Zarębska, Jacek Kurnicki

Several threads of the studies concerning the mechanisms and function of the carotid body have been continued. The following aspects were tackled.

Protein kinase C (PKC) in the carotid body. The presence and translocation in response to hypoxia of the classical PKC isoforms alpha, beta I, beta II, and gamma have been systematically studied in the cat carotid body. The enzymes have been identified using immunofluorescent and immunohistochemical techniques. The PKC alpha and gamma have been positively identified in the chemoreceptor cells and their translocation, albeit subtle, towards the periphery of the perikarya shown in hypoxia. No interspecies differences between the cat and rat carotid bodies were seen.

NO synthase (NOS) in the carotid body. NOS has been identified at the ultrastructural level using the histochemical method of NADPH-diaphorase. NOS has been by far described in the autonomic nerve endings and the vascular bed of the carotid body at the level of light microscopy. Our study was the first to show NOS directly in the receptor cells of the organ, which was possible due to the use of electron microscopy. The finding of NOS in these

cells sheds new light on the possible role of NO not only in the vascular regulation but also directly in the process of respiratory chemoreception.

Ascorbate 6-palmitate (AP) in respiratory regulation. This is a newly started avenue of research into respiratory regulation that has to do with the previously described presence of vitamin C in the carotid body. Vitamin C, as a water-soluble compound, is rather unstable and cannot act in the lipid bilayer of the cell membrane, the target area of hypoxia. Therefore, we used AP, a lipophilic derivative of vitamin C, to study the role of reducing compounds, in respiratory responses to hypoxia. We found that AP enhances these responses. This study has clinical implications in light of the lack of pharmacological agents that would be able to invigorate carotid body function and increase its responses to hypoxia. Such agents are highly desirable in the pathological states underlain by hypoxemia. This study was extended by the use of biochemical methods (HPLC) to trace the penetration of AP to both central (brain stem) and peripheral (carotid body) neural tissues. It was found that AP could be recovered from both tissues after its oral administration in the cat.

All these research threads will be continued in following studies.

CONTINUATION OF STUDIES ON THE CONTROL MECHANISMS OF NEURAL AND MUSCLE RESPIRATORY ACTIVITY

Project leader: Krystyna Budzińska
Contributor: Beata Sokołowska

Studies continued on respiratory compensation of the diaphragm's failure. The aim was to assess the dependence between the tidal and frequency components of ventilation during respiration with and without the functioning diaphragm in the cat. We applied an experimental model of diaphragm paralysis in which the C5-C6 phrenic rootlets were bilaterally cut. Experimental data were analyzed by the "*k* nearest neighbor" rule adopted from the statistical pattern recognition theory. The results demonstrate that the ability of the respiratory system to compensate for the bilateral diaphragm paralysis depended on the breathing pattern that developed after denervation. An increase in frequency ensured approximately sufficient

compensation whereas other compensatory strategies were ineffective, which led to a decline in minute ventilation. The evaluation method applied was useful in distinguishing different ways of breathing, in this case with and without the diaphragm. Furthermore, the two selected features, out of a number of affected parameters, minute ventilation and arterial blood CO_2 tension, sufficed to highly strengthen the power of distinguishing the disordered breathing pattern. Assessment of the strength of the dependence might allow anticipating the kind of compensation that would develop soon after the loss of diaphragmatic function.

In the study of the mechanism of respiratory plasticity, we are continuing research on short-term potentiation of hypoglossal nerve activity due to laryngeal stimulation and modification of this potentiation by ethanol. Ethanol is known to depress breathing. Moreover, it affects differentially the motor activities of the upper airways and the phrenic nerve. The results show that ethanol administration does not significantly influence the mechanism of phrenic and hypoglossal inhibition during stimulation of the superior laryngeal nerve but modifies the short-term potentiation of hypoglossal activity. Such an effect suggests that a depression, of the laryngeal origin, of the short-term potentiation of hypoglossal activity may contribute to respiratory disorders evoked by ethanol.

EFFECT OF AMYLOID β UPON THE BIOCHEMICAL MECHANISM OF DNA INJURY AND NEURON(S) APOPTOSIS

Supported by the State Committee for Scientific Research: grant # 4 P05A 051 12

Research co-ordinator: Robert Strosznajder

Previous studies on the changes of poly (ADP-ribose) polymerase (PARP) activity in aging processes have shown that PARP activity was enhanced in the hippocampus, the cerebral cortex, and the cerebellum of old adult rats (14 months old). However, we observed that in the aged hippocampus (24-27 months old) the enzymes' activity was significantly lower compared with controls (4 months old). These data suggest that the decrease of PARP activity in the aged hippocampus may be responsible for a lower ability of DNA to undergo repair in this part of the brain and for a higher vulnerability of hippocampal neurons to toxic insults.

Continuing these studies, the role of peroxyradical processes in homogenates of hippocampus, cerebral cortex and cerebellum from 4, 14 and 24-27 months old rats, in *in vitro* aging experiments was examined.

The results show a significant increase (2-3 fold) of TBARS accumulation after 60 min incubation at 37^{0}C in the hippocampus comparing to the cerebral cortex and cerebellum in each of the investigated groups (4, 14, 24-27 months old). The most evident results were observed in aged rats where TBARS accumulation was 1.00 nmol/mg protein in the hippocampus, 0.33 nmol/mg protein in the cerebral cortex and 0.41 nmol/mg protein in the cerebellum. We did not observe any noticeable changes of TBARS accumulation in the group in which the variable was the age.

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Publications:

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LABORATORY OF RESPIRATION PHYSIOLOGY

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THE EFFECT OF CHEMOSENSORY C-FIBRES' BLOCKADE ON THE RESPIRATORY PATTERN OF PULMONARY CHEMOREFLEX

Project leader: Małgorzata Szereda-Przestaszewska
Contributors: Katarzyna Kaczyńska, Beata Kopczyńska

Pulmonary chemoreflex was induced in anesthetized cats and rats by an intravenous injection of capsaisin. Capsaicin is a neuroactive extraneous substance, a selective stimulant of nonmyelinated broncho-pulmonary C-fibres of the vagus nerve. Respiratory sequence of post-capsaicin chemoreflex in cats consists of: an expiratory apnoea, followed by resumed breathing of depressed tidal volume and increased respiratory rate. Midcervical vagotomy precludes the response. In the rat, the apnoea triggered by an in-

travenous capsaicin is followed by stimulated breathing of increased tidal volume, which is independent of the lung vagal feedback.

In this study, we used Ruthenium red, a selective antagonist of capsaicin, to assess the effect of vanilloid (capsaicin) receptors blockade on the respiratory constellation of pulmonary chemoreflex. In cats, Ruthenium red affected the timing component of the breathing pattern, eliminating the apnoea and shortening the duration of the frequency response evoked by the capsaicin challenge. In rats the blockade of vanilloid receptors excluded the respiratory response to capsaicin.

The results suggest that effectiveness of the blockade in rats may be due to their high capsaicin sensitivity and rich vanilloid receptors' expression on the vagal ganglia.

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Publication:

Kaczyńska K, Szereda-Przestaszewska M: Respiratory effects of capsaicin occur beyond the lung vagi in anaesthetized rats. *Acta Neurobiol Exp* 2000, 60, 159-165.

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LABORATORY OF PHARMACONEUROCHEMISTRY

Head: Professor Jerzy W. Łazarewicz

CALCIUM AND EXCITATORY AMINO ACIDS IN PHYSIOLOGICAL AND PATHOLOGICAL PLASTICITY IN BRAIN NEURONS

Project leader: Jerzy W. Łazarewicz
Contributors: Wanda Gordon-Krajcer, Dorota Makarewicz, Halina Nowińska, Elżbieta Salińska, Anna Sobczuk, Aleksandra Stafiej, Apolonia Ziembowicz, Elżbieta Ziemińska

The role of metabotropic glutamate receptors (mGluR) in neurodegeneration and neuroprotection was studied in two *in vivo* models of brain ischemia. ABHxD-I, a novel mixed agonist of group I, II and III mGluR, was applied intracerebroventricularly (i.c.v.) or intraperitoneally (i.p.) to adult Mongolian gerbils submitted to 3 min bilateral carotid occlusion, 30 min before the insult. In the other approach, this agonist was tested in a model of rat perinatal asphyxia, in which 7-day old rats were submitted to hypoxia-ischemia, followed by ABHxD-I treatment after 30 min. In gerbils, only i.c.v. injection of ABHxD-I provided partial protection of the pyramidal neurons in the CA1 sector of the hippocampus, detected 14 days after ischemia. However, in this group ABHxD-I was found to induce a postischemic hypothermia, which may be at least partially responsible for the neuroprotection. On the other hand, in the model of perinatal asphyxia in immature rats, ABHxD-I applied i.p. induced significant neuroprotection without symptoms of postischemic hypothermia. It seems that low permeability of the blood-brain barrier for ABHxD-I in adult animals limits its neuroprotective potential.

Studies were initiated concerning the mechanism of a long-term ischemic preconditioning. In these experiments, a gerbil model of global cerebral ischemia evoked by a bilateral common carotid artery ligation was utilised for induction of preconditioning and injurious ischemia. A standard protocol for the induction of preconditioning was developed, comprising 2-min preconditioning, followed after 2 days by 3-min neurodegenerative ischemia. This procedure provided significant protection of CA1 neurons which almost disappeared when the duration of neurodegenerative ischemia was extended to 5 min. Studies on the effects of NMDA receptor antagonists on development of the ischemic preconditioning are in progress.

The role of calcium and NMDA receptors in the mechanism of a short-term adaptation to anoxia (anoxic preconditioning) was studied *in vitro* in superfused slices of rat olphactory cortex. Changes in intracellular concentration of bound and free calcium were detected fluorometrically. Normoglycemic preconditioning and pathological anoxia was applied for 2 min and 10 min, respectively with 90-min interval. This procedure significantly reduced disturbances in intracellular calcium homeostasis (increases in free and bound calcium) induced by 10 min anoxia. The potential of different competitive and non-competitive antagonists of NMDA receptors applied at different periods of incubation to reverse anoxic preconditioning was studied.

The role of mitochondria and disturbances in calcium homeostasis in the mechanism of neuronal damage evoked by perinatal asphyxia was studied in 7-day old rats submitted to hypoxia-ischemia. Ultrastructural examination 30 min and 4 h after the insult demonstrated swelling of neuronal mitochondria with calcium deposits and chromatin condensation in nuclei. After 24 h, advanced injury of the cortex and white matter was noticed. These results point to the role of activation of mitochondrial megachannels and of induction of proapoptotic mechanisms in neuronal damage evoked by perinatal asphyxia.

In the collaborative studies concerning pathogenic mechanisms of the rat brain glucodeprivation induced by sublethal doses of 2-desoxy-D-glucose (2-DG), it was demonstrated that i.p. application of 2-DG (500 mg/kg) leads to increased expression of β-amyloid precursor protein and to increased phosphorylation of τ protein in the rat brain.

NEUROPROTECTIVE EFFECT OF DANTROLENE IN BRAIN HYPOXIA/ISCHEMIA OF NEONATAL RATS

Supported by the State Committee for Scientific Research: grant # 4 P05A 025 16

Project leader: Dorota Makarewicz
Contributor: Anna Sobczuk

The project, which tested a neuroprotective potential of dantrolene, antagonist of the ryanodine channels and inhibitor of intracellular calcium mobilisation, in a model of perinatal asphyxia (hypoxia/ischemia of neonatal rats) has been completed. The rats were treated with dantrolene (2 μM of 4.8 mM applied i.c.v. or 10 and 20 mg/kg i.p.) 30 min after the insult. Animal's rectal temperatures were monitored during the 3 hr recovery interval. Brain injury was evaluated 14 days after the insult by macroscopic examination, by measurement of a deficit of the weight of the ipsilateral versus contralateral hemisphere and by the morphometric evaluation of the volume of lesion. Treatment with dantrolene i.p. significantly reduced the brain damage as compared to the vehicle control, whereas i.c.v. injection of dantrolene even exacerbated the insult. Dantrolene i.p. did not influence body temperature as compared with the vehicle (DMSO). Results of somatosensoric tests in the open field demonstrated that dantrolene reduced the total distance moved and the number of entries to the central zone. The mechanism of dantrolene neuroprotection in the model of perinatal asphyxia will be the subject of a subsequent project.

ROLE OF RYANODINE-SENSITIVE INTRACELLULAR CALCIUM STORES IN MITOCHONDRIAL PERMEABILITY TRANSITION IN RAT CEREBELLAR GRANULE CELLS

Supported by the State Committee for Scientific Research: grant # 4 P05A 031 17

Project co-ordinator: Elżbieta Ziemińska

In this 1-year project the effect of dantrolene on glutamate-induced disturbances in intracellular calcium homeostasis, mitochondrial permeability transition (MPT) and neurodegeneration was studied in cultured granule neurons. The results demonstrate that dantrolene significantly reduces

glutamate-induced release of LDH and decreases the number of death cells. In progress are ultrastructural and immunochemical approaches aimed at demonstration of the effect of dantrolene on glutamate-evoked mitochondrial swelling and cytochrome c release and measurements of dynamics of intra-cellular calcium concentration.

EFFECT OF METABOTROPIC GLUTAMATE RECEPTOR AGONISTS ON PROCESSES OF LEARNING AND MEMORY FORMATION INDUCED BY PASSIVE AVOIDANCE TRAINING IN THE DAY-OLD CHICK

Supported by the State Committee for Scientific Research: grant # 4 P05A 111 19

Project co-ordinator: Elżbieta Salińska

Studies within this project where just initiated by development of the experimental set-up for the one-trial passive avoidance training.

NEUROPROTECTIVE EFFECTS OF CYCLOSPORIN A IN EXCITOTOXIC BRAIN INJURY

Supported by Project Phare SCI-TECH II PL9611, sub-project 03.01:
Preparatory study for "Centres of Excellence",
Centre of Studies on Mechanisms of Neurodegeneration

Project co-ordinator: Jerzy W. Łazarewicz

Studies were continued on the mechanisms of cyclosporin A (CsA) – induced neuroprotection. Previous experiments demonstrated *in vitro* and *in vivo* CsA-evoked inhibition of mitochondrial permeability transition (MPT) in neurons. In the present studies, attention was focused on the ability of CsA to inhibit calcineurin-mediated activation by NMDA of nitric oxide synthase (NOS) and potentiation of oxidative stress in the rabbit hippocampus *in vivo*. In these studies microdialysis technique has been utilised for 1 mM NMDA application for 20 min to the hippocampus and for measurements of nitric oxide (NO) and hydroxyl radicals ($^{\cdot}OH$) production. Oxy-hemoglobine and 4-hydroxybenzoic acid were used as the NO and $^{\cdot}OH$ traps, with spectrophotometric or HPLC detection, respectively. The results indicate that NMDA application results in the L-NAME-sensitive

transient release of NO. Moreover, NMDA induces a prolonged release of ·OH to dialysate. CsA applied to the microdialysis medium inhibits both these effects. These results point to the role of inhibition by CsA of calcineurine in the mechanisms of CsA-evoked neuroprotection in acute excitotoxicity.

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Publications:

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CONTINUATION OF STUDIES ON THE DISTURBANCES OF METABOLISM AND MORPHOLOGY OF THE CENTRAL NERVOUS SYSTEM CAUSED BY ISCHEMIC PATHOLOGY AND TOXICITY OF LEAD

Project leader: Professor Urszula Rafałowska
Contributors: Irena Bubko, Beata Dąbrowska-Bouta, Aleksandra Lenkiewicz, Lidia Strużyńska, Grzegorz Sulkowski, Jolanta Waśkiewicz

Astroglial neuroprotection during acute lead toxicity in the adult rat brain

It was established that the developing nervous system is susceptible to lead (Pb) exposure, but little is known about the effect of this toxic agent in adult rat brain. Astrocytes serve as a cellular Pb deposition site and simulta-

neously play a significant role in numerous functions of the central nervous system. Thus, it was of interest of the present studies to investigate the response of astroglial cells in the adult rat brain after acute lead exposure. The biochemical measurements were performed using brain homogenates and subcellular fraction of astroglial origin (i.e. glial plasmalemmal vesicles – GPV).

Changes in GFAP content, visualized by an increased immunoreactivity of that protein on Western blots, were especially present in GPV fractions and homogenates from hippocampus and cerebral cortex, but not in cerebellum. The features of enhanced astrocytic reactivity (i.e. large accumulation of mitochondria, activated Golgi apparatus and increment of gliofilaments) were also observed in electron microscopic studies. The enhancement of glutathione level both in GPV fraction and in brain homogenates was checked – mostly in cerebellum so as in hippocampus. Results of current studies indicated that acute lead exposure is accompanied by astrocyte activation connected with the presence of the enhanced expression of GFAP, what may be the evidence of the coexisting lead-induced neuronal injury. At the same time the regional enhancement of detoxicative mechanisms (GSH) was noticed, indicating the existence of astrocytes-mediated neuroprotection against toxic Pb action.

Synaptosomal dysfunction during global ischemia caused by cardiac arrest correlated with early and late recirculation

The aim of this part of study was to assess the sensitivity of brain synaptosomes with its mitochondria to effects of global cerebral ischemia caused by temporary cardiac arrest and the early and late consequences of this ischemia. The effects of 10 min of global ischemia were measured immediately and then after 1 h, 24 h and 7 days post-resuscitation. Ischemia and early post-resuscitation caused a decrease of oxygen consumption by synaptosomes, a drop in ATP/ADP and CrP/Cr ratios, reduction of synaptic vesicles and disturbances in mitochondrial structure in isolated synaptosomes and in nerve endings in brain slices. After 7 days post-resuscitation, the observed changes normalized but small numbers of destroyed neurons simultaneously appeared. It is concluded that global ischemia and early postresuscitation after cardiac arrest may lead to damage of synaptosomes and synaptic mito-

chondria that modifies substrate oxidation and synthesis of energetic parameters and affects neurotransmitter function. Observed disturbances are normalized in longer times after resuscitation but ischemic events and reoxygenation caused selective morphological injury of certain neurons and this may form the basis for irreversible future brain damage.

Astrocytic response in cardiac arrest-induced global cerebral ischemia in the rat

In this studies, using cardiac arrest model of global cerebral ischemia we tested the influence of ischemic and postischemic conditions on astrocytic cells. In biochemical and immunochemical procedures, the cellular fraction of astrocytic origin GPV was used. A tendency towards an elevation in immunocontent of glial fibrillary acidic protein (GFAP) was noticed after 24 h post resuscitation whereas a significant increase was observed 7 days post ischemic event. The features of astrocytic stimulation were also observed in electron microscopy studies. An enhanced amount of gliofilaments was noticed in brain sections obtained from rats after 7 days of recovery. Simultaneously, a gradual decrease of the total glutathione level, depending on the duration of reperfusion, was observed in brain homogenates and in fractions of astroglial origin. The most considerable reduction was observed in brain homogenates in day 1 (52%) and day 7 (65%) of reperfusion so as in day 7 (47%) in the case of the GPV fraction. The results indicate the enhanced reactivity of astrocytic cells in ischemic conditions (resulting from cardiac arrest) concomitantly with a long lasting decrease of total glutathione.

Collaborating units:

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Publications:

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Strużyńska L: The protective role of astroglia in the early period of experimental lead toxicity in the rat. *Acta Neurobiol Exp* 2000, 60, 167-173.

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POSTTRANSLATIONAL PROTEIN MODIFICATION IN RESPONSE TO CEREBRAL ISCHEMIA: MITOCHONDRIAL PROTEIN CHANGES CORRELATE WITH CYTOCHROME C RELEASE AND DELAYED NEURONAL DEATH IN GERBIL HIPPOCAMPUS

Project leader: Krystyna Domańska-Janik
Contributors: Agnieszka Bronisz-Kowalczyk, Barbara Zabłocka

Recent findings support the hypothesis which gives mitochondria and the mtPTP (mitochondrial permeability transition pore) a pivotal role for determining the destiny of cells after various lethal injuries. After cerebral ischemia, several signals potentially involved in initiation of apoptosis seem to integrate on mitochondrial membranes resulting in execution of cell death. We have shown that after transient global forebrain ischemia in gerbils, the amount of mitochondria-connected deltaPKC and activated /phosphorylated JNK protein increase steadily starting from 24 hrs after injury. It corresponds to several *in vitro* data showing that these protein kinases, when translocated, promote mitochondrial outer membrane permeabilisation and release of caspases and endonucleases activators. However, the target proteins for their phosphorylating activity are still under investigation. Con-

comitantly, the amount of activated P-Raf1, recently shown to act anti-apoptotically in our *in vitro* study (see grant # 4 P05A 072 18), has decreased in the total homogenate as well as in the mitochondrial fraction at 24 and 48 hrs of reperfusion. It corresponds with decreased phosphorylation of its supposed target protein – BAD as well as with delayed inhibition of ERK activity in hippocampal homogenate demonstrated at 48-96 h after injury. Moreover, at this time point of CA1 neuronal apoptosis, a statistically significant increase of cytochrome C level was detected in cytoplasmic fraction of hippocampus. All these data indicate that ischemia-induced pathological signalling triggers several proapoptotic changes at the level of mitochondrial protein composition which could be a novel, promising target for neuro-protective treatment.

Collaborating unit:
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EFFECT OF CYCLOSPORIN A ON DELAYED NEURONAL DEATH AFTER TRANSIENT, GLOBAL CEREBRAL ISCHEMIA *IN VIVO* AND ON STAUROSPORINE, H_2O_2 OR GLUTAMATE-INDUCED APOPTOSIS IN PRIMARY CORTICAL CULTURES *IN VITRO*

Supported by the State Committee for Scientific Research: grant # 6 P04 101 14 and SciTech II PL961/03.01 program

Project leader: Krystyna Domańska-Janik
Contributors: Leonora Bużańska, Paula Berdowska, Barbara Zabłocka, Małgorzata Ziemka-Nałęcz

During reperfusion after ischemia calcium is taken up by reenergised mitochondria and reactive oxygen species are produced. These events promote reversible opening of a large pore on mitochondrial inner membranes (mtPTP). In certain cellular populations or compartments, however, more severe disruption of mitochondrial function and subsequent osmotic swelling can participate, together with the other pathological signals, to leakage of pro-apoptotic factors from these organelles and to delayed cell death.

In agreement with the other recent data we have confirmed that CsA a compound which besides its immunosuppressive activity specifically blocks mtPTP, can protect CA1 neurons of hippocampus in the model of 5 min cerebral ischemia in gerbils. We have demonstrated that single intra-carotid injection of CsA in a dose of 5-10 mg/kg (but not in doses equal or lower than 2.5 mg/kg) immediately after ischemia significantly increases the average number of surviving pyramidal neurons from 10% to about 50% of control with a relatively high individual dispersion of this reaction. Protection was not observed when CsA was given after 6 to 48 hrs of reperfusion – the time when neurogenic inflammation begins in a similar ischemic model. This suggests that the immunosuppressive effect of CsA was rather not involved in neuronal protection. The above observation is in accordance with data showing ineffectiveness of FK506 (a specific cyclofillin A ligand and exclusive calcineurin inhibitor) in similar global ischemia model in rats (Friberg et al., *J Neurosci* 1998, 18, 5151-5159). To get more insight into the mechanism of CsA protection we have established a new model of primary cortical culture *in vitro*. By differential staining of living cells with Hoechst 33258 and immunochemical markers for neuronal and glial populations we have shown that these cells respond with apoptosis to glutamate (0.5 mM) , H_2O_2 (25 μM) or staurosporine (25 nM) treatments. This response was more pronounced in neurons (up to 40% of apoptotic cells) than in astrocytes (only in the case of staurosporine the reaction was significant) after 6 hrs of the lag period. Neuroprotection was achieved with relatively low doses (0.5-1 μM) of CsA. In concentration higher than 5 μM CsA showed strong cytotoxic effect toward the whole cell population. In conclusion, our results confirmed the neuroprotective effect of CsA in global cerebral ischemia *in vivo* and neuronal apoptosis *in vitro*.

THE ROLE OF RAF1 PHOSPHORYLATION IN N2A NEUROBLASTOMA CELLS APOPTOSIS

Supported by the State Committee for Scientific Research: grant # 4 P05A 072 18

Project leader: Krystyna Domańska-Janik
Performed by Agnieszka Bronisz-Kowalczyk

The N2a cells, when grown in the absence of serum, respond with apoptosis to PKC-specific, low doses of kinase inhibitor, staurosporine (STS 10 nM). Similar response is observed after treatment of N2a cells with an IP3K inhibitor – wortmanine (WM 300 nM). Our previous results showed correlation between cellular survival and activity of several kinases (PKC, Raf1, AKT) affecting phosphorylation of proapoptotic protein – BAD. To prove the crucial role of Raf1 and/or AKT activity for N2a cells survival *in vitro* in the absence of serum, we have transfected the cells with mutated Raf1 and AKT plasmids.

It was demonstrated that transfection of N2a cells with mitochondria-tagged Raf1 dominant negative (DNM) or dominant active (DAM) constructs can significantly alter N2a cells reaction to STS treatment being without effect on the fate of cells treated with wortmanine. After STS application, the extent of apoptosis in the cells transfected with DAM decreased from 30% in control to 8% in the mutants. Concomitantly, transfection with DNM increased apoptosis to above 60% of control. In contrast, transfection of cells with active (phosphorylated) P-AKT plasmid did not influence STS-induced apoptosis but significantly lowered cell death after WM treatment from 35% in control to 13% in the transfectants. The results indicate that in N2a cells, at least two pathways can participate in serum-dependent survival signalling: one activated by STS sensitive (PKC) $\Rightarrow$ P-Raf kinases and the other, dependent on IP3K $\Rightarrow$ P-AKT activity. Moreover, in both types of apoptotic signals induced by above kinases inhibitors, we have observed lowered amounts of P-BAD (Western blot) and higher binding of the proapoptotic BAD protein to mitochondrial membranes (electron microscopy immunochemistry). These confirm the role of BAD protein in mitochondrial integration of serum-dependent death signalling.

THE ROLE OF PROTEOLYSIS IN NEURODEGENERATION

Project leader: Teresa Zalewska
Contributors: Krystyna Domańska-Janik, Małgorzata Ziemka-Nałęcz

It has long been evident that extracellular matrix provides signals that control cell viability. The disruption of interaction between the cells and matrix *in vitro*, leads to programmed cell death. One of the factors respon-

sible for cell detachment-induced apoptosis may be proteolysis of extracellular matrix components. The main goal of this year's activity was to verify the hypothesis that ischemia-induced degradation of extracellular matrix proteins is spatially coincident with brain regions that exhibit postischemic neuronal apoptosis.

We sought to clarify this point by measuring the activity of metalloproteinases (gelatinases, MMPs 2 and 9) – the key enzymes, responsible for remodelling of the extracellular matrix. In our study we used model of 5 minutes forebrain ischemia in gerbils, which results in selective neuronal apoptosis, becoming evident 3-5 days of reperfusion in CA1 sector of hippocampus. Enzymatic activity was assessed at various reperfusion times in two parts of hippocampus: dorsal part containing vulnerable CA1 neurons and abdominal part considered as an ischemia-resistant area. Our current results show that a marked increase of proteolytic activity of MMP9, evident particularly in the late phase of reperfusion (72 hours) is observed in hippo-campal region that experienced neuronal degeneration. The results are confirmed by *in situ* zymography, showing extensive proteolytic activity in CA1 region of hippocampus. In contrast, the activity of MMP2 does not show significant changes during reperfusion. This is the first description of the potential role of metalloproteinases in global ischemia.

Since last September, part of this project has been supported by grant # 4 P05A 086 19 from the State Committee for Scientific Research for the project entitled: The effect of ischemia on signal transduction from extracellular matrix – the role of metalloproteinases and calpains (Project leader: Teresa Zalewska).

Collaborating unit:

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NEURAL STEM CELLS FROM HUMAN CORD BLOOD CELL FRACTION *IN VITRO*

Collaborative project

Contributors: Leonora Bużańska, Krystyna Domańska-Janik

Neural progenitor stem cells capable to differentiate along multiple cell-type lineages *in vitro* can be used for replacement or gene therapies in various pathological states of the central nervous system. However, an ethical and legal controversy significantly delays human embryonic or fetal stem cells research. As an alternative source of human neural stem cells we have used umbilical cord blood and have now demonstrated that certain defined populations of these cells can be induced to differentiate into neurons, astrocytes and oligodendrocytes under selected *in vitro* conditions. These cells express nestin as a marker of neural precursor protein and in the differentiated state typical neuronal and glial markers.

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Publications:

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The inauguration of Molecular Biology Unit's activity was in September 2000. The proposed studies which will be undertaken in the new laboratory address questions related to the expression of proteins and genes connected with signal transduction, apoptosis and neuroprotection in the pathology of delayed neuronal cells death after transient brain ischemia. This is a continuation of the work carried on in Laboratory of Molecular Neuropatology, where Dr. B. Zabłocka worked in the previous years. The collaboration between this two units will be continued. Despite of this main topic, the unit is open for other collaborations. The equipment which is gathered in the laboratory is suitable to perform PCR reactions with different variations of this method, various types of electrophoresis and hybridizations: Western, Southern or Northern. In this year the investigation of the role of mitochondria in the initiation and in the execution phase of postischemic degeneration was undertaken in the collaboration with the Laboratory of Molecular Neuropathology. Together with Department of Neurotoxicology the first experiments on the expression of glutamine transporters in C6 glioma cells grown in various conditions were started. The study of gene mutations related to muscular dystrophies and neuropathies were initiated under the supervision of Neuromuscular Unit.

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CHANGES OF TRANSPORT OF NEUROACTIVE AMINO ACIDS IN HYPERAMMONEMIC ENCEPHALOPATHY

Project leader: Jan Albrecht
Contributors: Jan Albrecht, Monika Dolińska, Wojciech Hilgier, Hanna D. Borkowska, Magdalena Zielińska

Lubeluzole is a newly designed neuroprotectant which proved effective in the treatment of experimental stroke in rats, mainly by inhibition of the glutamate-activated NO pathway, but also by counteracting osmotic stress by a mechanism associated with the release of the osmoactive amino acid taurine (Tau). Here we show that lubeluzole administered i.p. decreases by 25% the high (50 mM) K^+-evoked accumulation of Tau in striatal microdialysates of healthy rats and by 34% in rats with thioacetamide-induced hepatic failure, where increased extracellular accumulation of Tau signifies ongoing hepatic encephalopathy. Lubeluzole does not affect nonstimulated accumulation of Tau in either group of rats. The results indicate that lubeluzole may be effective in ameliorating osmotic stress in a range of pathological conditions involving an increase of extracellular K^+ but also in decreasing the vulnerability to the stress in rats with hepatic failure.

Cerebrocortical minislices derived from control rats ("control slices") and rats with thioacetamide (TAA)-induced hepatic failure showing moderate hyperammonemia and symptoms of hepatic encephalopathy (HE) ("HE slices"), were incubated with physiological saline in both the absence or presence of 5 mM ammonium acetate ("ammonia") at potassium ion (K^+) concentrations ranging from 5 to 15 mM. The efflux of endogenous aspartate (Asp), glutamate (Glu) and taurine (Tau) to the incubation medium was assayed by HPLC. At 5 mM K^+, perfusion of control slices with ammonia

did not affect Glu and slightly depressed Asp efflux. Raising K^+ concentrations in the incubation medium to 7.5 mM led to inhibition of Glu and Asp efflux by ammonia and the inhibitory effect was further potentiated at10 mM K^+. The inhibition was also significant at 15 mM K^+. This suggests that depression of excitatory neurotransmission associated with acute hyperammonemia is more pronounced under conditions of intense neuronal activity than in the resting state. HE moderately increased the efflux of Glu and Asp, and the stimulatory effect of HE on Glu and Asp efflux showed virtually no variation upon changing K^+ concentration up to 15 mM. Ammonia (strongly) and HE (moderately) increased Tau efflux at 5 mM K^+. However, both the ammonia- and HE-dependent Tau efflux decreased with increasing K^+ concentration in the medium and was no longer significant at 10 mM concentration, indicating that intense neuronal activity obliterates the neuroprotective functions of this amino acid triggered by hyperammonemia.

Collaborating units:

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MECHANISM AND REGULATION OF CELL MEMBRANE GLUTAMINE TRANSPORT

Supported by the State Committee for Scientific Research: grant # 4 P05A 060 18

Project leader: Jan Albrecht
Contributors: Monika Dolińska, Anna Dybel

Glutamine (Gln) is one of the key metabolites in the CNS (energy metabolite, precursor of neurotransmitter amino acids, end product of ammonia detoxication, osmolyte), and as such is a routine supplement of CNS cell culture media. C6 glioma cells relatively easily adapt to culturing in a Gln-deprived medium. The present study investigated the effects of Gln deprivation on the characteristics of the different systems that mediate Gln cell membrane transport in the cells. In contrast to a variety of CNS and non-CNS

cells, the absence of Gln did not derepress the methyl-amino-isobutyric acid (MeAiB)-sensitive ("system A-dependent") uptake. System ASC became relatively more and system N less active than in cells grown in the presence of Gln but the ion and substrate specificity of the uptake remained unaltered. System ASC in C6 cells grown in a Gln-supplemented medium shows two features distinct from most other cell types: a) strong pH sensitivity and b) partial tolerance of lithium substitution (Dolińska et al., *Neurochem Int*, 2000, 37, 139-146), pointing to domination of system ASCT2 - an ASC variant strongly expressed in cultured astrocytes (Broer et al., *J Neurochem*, 1999, 73, 2184-2194). Cells grown in Gln-deprived medium lost lithium tolerance, but not pH-dependence of the uptake, their properties thus resembling system GlnT (SAT1), a neuron-specific variant of system A (Varoqui et al., *J Biol Chem*, 2000, 275, 4049-4054). By contrast, transport of threonine, a standard ASC system substrate, was not affected by Gln deprivation and showed neither pH dependence nor lithium tolerance, which is typical of an ASC in all the non-CNS tissues.

Collaborating unit:
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HE AND DOPAMINERGIC SYSTEM

Project leader: Jan Albrecht
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Studies were continued regarding the role of dopaminergic system in the pathomechanism of encephalopathy associated with acute liver failure. The binding of a D2 receptor ligand, [^{3}H] spiperone, was measured in striatal membranes derived from rats in which acute hepatic failure induced with thioacetamide (TAA) was associated with symptoms of hepatic encephalopathy (HE), and during recovery from HE. A 28% decrease of Bmax for the binding was measured in a symptomatic stage of HE, 1 day after TAA administration. The Bmax for [^{3}H] spiperone binding was not longer different from control 7 days after TAA administration, when blood and brain biochemical correlates of HE were already absent. At 21 days after TAA administration, the Bmax was increased by 31% above the control

level, consistent with other aspects of metabolic activation of the brain characteristic of the late recovery period from acute HE.

Collaborating unit:

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MOLECULAR MECHANISM OF BRAIN AGING AND POSTISCHEMIC ENCEPHALOPATHY; EFFECTS OF AMYLOID BETA AND ETHANOL

Project leader: Joanna B. Strosznajder
Contributors: Małgorzata Chalimoniuk, Grzegorz Czapski, Bronisław Głód, Henryk Jęśko, Agata Zambrzycka

The studies carried out in Department of Cellular Signalling concerned molecular mechanisms of brain aging, and amyloid beta neurotoxicity, postischemic encephalopathy and the alteration in neurotransmission evoked by ethanol.

MOLECULAR MECHANISM OF BRAIN AGING

Effect of aging on choline acetyltransferase (CAT)

Regulation of CAT activity in different parts of adult, elderly and old rats (4, 14, 24 months, respectively) was investigated. The highest activity of CAT was found in striatum, lower in cerebral cortex and the lowest in hippocampus. Our data indicate that this enzyme is Ca-independent and is not regulated by phosphorylation-dephosphorylation processes. Arachidonic acid and free radicals significantly decreased CAT activity, but exclusively in striatum. Aging decreased CAT activity in brain cortex and striatum but had no effect in hippocampus.

Poly(ADP-ribose)polymerase (PARP) preparation and purification for the studies of phosphorylation-dephosphorylation regulation in brain aging

The studies on regulation of PARP activity were continued. Transfection of *Escherichia coli* with cDNA for PARP was performed using vector

containing coding sequences of His-tag. Enzyme purified on Ni-NTA column was used for studies on the regulation of PARP activity by DNA-kinase from adult and aged brain. The results indicated significant alterations of PARP activity during brain aging. However, further studies must be done to elucidate the mechanism of PARP activity alterations in the aged brain.

MOLECULAR PROCESSES OF NEURONAL DEGENERATION

PARP in transient forebrain ischemia

Studies on the role of PARP during reperfusion after forebrain ischemia in gerbils using PARP inhibitor were carried out. The results indicate that after short-term forebrain ischemia (3 min) about 26% of neurons survive in CA1 layer of hippocampus. PARP inhibitor 3-aminobenzamide (3-AB) injected intravenously 10 min before or directly after ischemia in a dose of 30 mg/kg b.w. significantly ameliorated brain edema and enhanced survival of 58-64% of neurons in CA1 layer of hippocampus. 3-AB removed hydroxy radicals but was not able to protect the membrane lipids from peroxidation. The results suggest that PARP inhibitors together with a potent free radicals scavenger may offer a good therapeutic approach for short term brain ischemia.

Effect of nitric oxide (NO) on protein oxidation in endothelial cells in culture

The aim of this study was to elucidate the role of NO in the endothelial cells during reperfusion after ischemia. The concentration-dependent effect of NO on tyrosine oxidation of endothelial proteins was investigated under different experimental conditions.

The results indicated that depending on the type and duration of oxidative stress, extracelullar or intracellular proteins are oxidized. Exogenous donor of NO diethylamine nitric oxide (DEANO) activates dityrosine formation and significantly decreases protein oxidation in endothelial cell in culture. NO liberated in appropriate amount inactivates reactive oxygen radicals and protects endothelial cells against protein oxidation. The results indicate that depending on the conditions, NO might act as a pro or antioxidant.

Phosphatidylinositol transfer proteins (PI-TP) in brain ischemia-reperfusion injury and in PC-12 cells in culture

Studies on PI-TP α and β isoforms during ischemia/reperfusion in hippocampus and brain cortex were carried out. The results indicate that stimulation of NMDA receptor during ischemia and reperfusion is responsible for the allosteric modification of cytosolic and membrane bound PI-TPα and their protein level exclusively in the hippocampus. Stimulation of NMDA receptor in PC-12 cells in culture enhanced PI-TPs protein level in a time-dependent manner. These results suggest that protein-protein interaction evoked by NMDA receptor stimulation is responsible for the alteration of PI-TPs proteins involved not only in PI transport but also in the transport of other macromolecules in vesicle traffic and in regulation of cytoskeleton dynamics.

Mechanism of spinal cord neurons degeneration

The mechanism of arachidonic acid (AA) evoked neurotoxicity in the spinal cord neurons in culture was investigated. It has been found that AA induces release of cytokine MIF (macrophage migration inhibitory factor) and that this cytokine is involved in the activation of oxidative stress and neuronal cell death.

Moreover, the role of cGMP and cGMP mediated processes in spinal cord neuronal degeneration was determined. Midthoracic spinal cord constriction caused a significant decrease of cGMP level in the ventral column of Th5-Th6 segments and a significant increase in the lateral column of Th8-Th9 segments. The level of cGMP in the dorsal column, located either rostrally or caudally to the site of the spinal cord injury remained unchanged.

EFFECT OF ETHANOL ON SIGNAL TRANSDUCTION PROCESSES IN THE BRAIN

Time-dependent effects of different ethanol concentrations on free radical processes and NMDA receptor mediated signal transduction pathway were investigated. Preincubation of hippocampal slices with ethanol for 90 min at 37°C in a concentration range of 50-200 mM decreased NMDA-dependent NO synthase activity and NO-mediated processes. However, after 30 min of preincubation, ethanol had no effect on NMDA receptor mediated signal-

ling. Ethanol in a concentration range of 10-200 mM had no effect on lipid and protein oxidation. During the short time of action (2 min) ethanol inactivated hydroxy radicals.

THE ROLE OF AMYLOID BETA (Aβ) PEPTIDE IN THE MODULATION OF CAT ACTIVITY, PHOSPHOINOSITIDES KINASE AND PHOSPHOLIPASE C IN ADULT AND AGED BRAIN

Supported by the State Committee for Scientific Research: grant # 4 PO5A 072 19

Project leader: Joanna B. Strosznajder
Contributor: Agata Zambrzycka

The effects of Aβ peptides on the activity of CAT and on free radical dependent processes were investigated. The study was carried out using neurotoxic fragments of amyloid beta, $A\beta_{25-35}$ and full length $A\beta_{1-40}$ in 1-100 mM concentration range. The effect of non aggregated and aggregated form of Aβ peptides on CAT in different parts of the adult and aged brain was studied (brain cortex, striatum and hippocampus). It has been found that only the aggregated form of Aβ affected CAT activity in aged striatum but had no effects in other parts of the brain in spite of the activation of free radicals.

CHRONIC ALCOHOL ON CHOLINERGIC SIGNALLING PATHWAY AND NOS

Supported by the National Institute of Health,USA:
Fogarty International Research Collaboration Award (FIRCA) # PA-95-011

Project leader: Grace Y. Sun*
Contributors: Joanna B. Strosznajder, Agata Zambrzycka

The effect of ethanol on CAT and cholinergic receptor-mediated signal transduction processes in normal and ischemic brain was investigated. It was found that ethanol *in vitro* has no effect on CAT and cholinergic receptor--dependent NO cyclic cGMP signalling; however, it modulates cyclooxygenase activity.

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Publications:

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PATHOGENIC MECHANISMS OF POSTISCHEMIC ENCEPHALOPATHY

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Studies were continued on the regulation of cerebral microcirculation in the rat: 1) following transient focal ischemia/reperfusion and 2) during chronic hyponatremia.

In the rat model of transient suture occlusion of the middle cerebral artery, the participation of endothelin in early hypoperfusion and the response of microflow (LDF) at different time points of early reperfusion to increasing doses of intravenously administered exogenous endothelin were studied. Blockade of ETA receptors for endothelin (BQ123) did not affect early hypoperfusion suggesting that in this model of ischemia, endothelins are not involved in the decrease of microflow observed at early stages of reperfusion. Intravenous administration of increasing doses of endothelin resulted in progressive similar decreases of LDF both in sham-operated rats and in the rats with ischemia/reperfusion. That does not support the hypothesis that there is an exaggerated response of cerebral microcirculation to vasoconstrictors during early reperfusion following focal cerebral ischemia.

The experiments on the effect of chronic hyponatremia on cerebral circulation in the rat were conducted in collaboration with the Laboratory of the Cell Ultrastructure in order to study the impact of chronic plasma sodium decrease on the neuronal and endothelial nitric oxide synthase (NOS). Using immunogold technique, an increased concentration of the endothelial isoform of the enzyme was found both in the endothelium as well as in neurons. The results of these and previously performed studies suggest the induction of NOS in both types of cells in the course of hyponatremia.

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Publications:

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POSTISCHEMIC CHANGES IN THE CENTRAL NERVOUS SYSTEM IN DIFFERENT EXPERIMENTAL MODELS

Project leader: Janina Rafałowska
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Focal cerebral ischemia may be one of the etiologic factors in Alzheimer's disease. Immunization of rats after cardiac arrest with human β-amyloid peptide caused the appearance of Alzheimer's diffuse plaques, probably connected with phagocytosis or reverse transport of β-amyloid peptide across the blood-brain barrier. Anti-oxidative therapy of cerebral amyloidosis following ischemia-reperfusion brain injury was not effective.

In the focal brain compression model alterations in capillary ultrastructure in the rat cortex and neurohypophysis were observed. Clogging of capillaries, accumulation of collagen fibrils and necrosis or apoptosis of endothelial cells were noted.

In the model of photochemically-induced microvascular injury in rat brain, microthrombi and alterations of endothelial cells were reduced after administration of 20 mg/kg GM1 ganglioside before the photochemical reaction.

Comparative studies on ET-1 and ET-3 vasoconstrictor properties showed that ET-1 produced more severe neuronal injures in the hippocampal CA1-CA3 zones and evoked stronger oxidative stress. Coenzyme Q(10) diminished the morphological and biochemical changes indicating its neuroprotective effect.

Organotypic cultures of rat hippocampus were treated with subtoxic concentration of GLU and $AlCl_3$ added to growth medium separately or in com-

bination. The exposure of cultures to GLU in the presence of Al^{3+} ions for up to 24 hours resulted in the development of typical excitotoxic neuronal changes, whereas separate GLU or Al application did not produce any apparent tissue damage. The result supports the view a possible role of Al in the development of glutamate-mediated excitotoxic neuronal injury.

Investigations of the relation between Apolipoprotein E, amyloid beta peptide and ceroidolipofuscinosis are being continued.

Collaborating units:

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Institute of the Brain, Russian Academy of Medical Sciences, Moscow, Russia (I. Barskov)

Medical Research Centre, PASci, Warsaw, Poland:

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- Neuropeptide Laboratory (A. Lipkowski)
- Department of Neurodegenerative Disorders (M. Barcikowska)

AIDS IN CNS

Project leader: Irmina B. Zelman
Contributor: Mirosław J. Mossakowski

Progressive Multifocal Leucoencephalopathy occurred in 20 cases (10%) of all AIDS cases, collected in the period from 1987 to 1999. Due to relatively high frequency of cases of isolated or strongly predominant involvement of cerebellum, separation of the cerebellar form of the disease has been suggested.

NEOPLASMS OF THE NERVOUS SYSTEM

Project leader: Ewa Matyja
Contributors: Anna Taraszewska, Ewa Nagańska, Dorota Dziewulska

Three cases of extremely rare epithelioid schwannomas originating from the acoustic nerves in the cerebello-pontine angles were presented. Both the

spindle and epithelioid tumor cells showed strong positivity for S-100 protein and vimentin but were negative for EMA, cytokeratin and HMB45. Differential diagnosis of this unusual variant of schwannoma in relation to malignant transformation of the epithelioid component was discussed.

Clinicopathological study of the disseminated spinal and cerebral ependymoma with unusual histological pattern and with retrograde tumor spread (*via* the cerebrospinal fluid) was reported.

A study of metaplastic xantomatous changes in association with atypical and anaplastic meningiomas was performed in 5 surgical cases. Mixed meningeal/macrophagic nature of xantomatous cells was shown.

Light and ultrastructural examination of nerve fibers in two cases of painful glomus tumor revealed thin nonmyelinated axons within the tumor, presumably contributing to the complex mechanism of severe paroxysmal pain caused by these tumors.

Histological and immunohistochemical evaluation of neuroectodermal elements in ovarian teratomas revealed divergent morphological and immunophenotypic differentiation of the neuroectodermal cell lines in the immature teratomas and some abnormality in their maturation in the mature teratomas.

In paraneoplastic syndrome in the course of lung adenocarcinoma inflammatory infiltrates were composed of cytotoxic T lymphocytes. The expression of platelet-endothelial cell adhesion molecule 1 (PECAM-1) was increased in comparison with control material, which may facilitate transendothelial lymphocyte migration, triggering a cascade of biochemical and morphological reactions observed in paraneoplastic syndrome.

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BRAIN GLUCODEPRIVATION BY 2-DEOXYGLUCOSE LOADING: A POTENTIAL ANIMAL MODEL OF ALZHEIMER'S DISEASE

Supported by the State Committee for Scientific Research: grant # 4 P05A 041 17

Project leader: Mirosław J. Mossakowski
Contributors: Paweł Grieb (Laboratory of Experimental Pharmacology, MRC), Wanda Gordon-Krajcer (Department of Neurochemistry, MRC)

Antibodies detecting chosen epitopes of beta-amyloid precursor protein (β-APP) and phosphorylated and unphosphorylated tau protein and Western blotting were used to determine the kinetics of changes in these proteins in the rat brain following single intraperitoneal injection of 2-deoxyglucose, 500 mg/kg. Highly significant changes have been observed, including quick, marked (approx. 2x) and sustained increase in the C-terminal and middle part, the delayed increase in the M-terminal of (β-APP). The quick, marked and sustained increase in tau phosphorylation was also found. The results are in accordance with the hypothesis that a decrease od glucose turnover in brain may be a primary cause of β-APP overexpression and tau protein hyperphosphorylation in Alzheimer's disease.

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GENETIC AND ENVIRONMENTAL RISK FACTORS OF DEMENTIA

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Alzheimer's disease (AD) is a degenerative disease of insidious onset, characterized by memory loss, a variety of cognitive dysfunctions and behavioral disturbances. The aim of our study was: 1) to estimate the occurrence and intensity of some psychopathological symptoms in the course of AD and 2) to examine whether the occurrence of behavioral and psychological symptoms increases with the deepening of dementia among persons with AD living in their homes with outpatient treatment. The study was conducted among 94 persons (38 men and 56 women) aged from 52 to 86 years (mean 72.4). In the estimation of occurrence of behavioral and psychological disturbances Alzheimer's Disease Assessment Scale - non-cognitive behavior (ADAS-noncog) and subscale "Change in Personality, Interests, Drive" of Blessed Dementia Scale were used. The results have shown that with the progress of dementia the occurrence of the following psychopathological symptoms such as hallucinations, intensive motor activity, purposeless hyperactivity, pacing and rigidity increase and there is a relinquishment of hobbies.

Recent studies suggest that α2-macroglobulin (α2m) may play a role in the pathogenesis of AD. The presence of α2m G/G genotype is thought to increase the risk of AD. However, in the cohort of our AD patients, the lack of statistically significant difference between G/G frequences in both groups (AD *vs* control cases) may suggest that α2m G/G genotype is not a risk factor for AD.

The evaluation of interaction between the occurrence of APOE ε4 alleles and hippocampal atrophy was a subject of our other study. The degree of

atrophy of medial parts of temporal lobes was evaluated by computed tomography in 60 patients with Alzheimer type dementia. At least one APOE ε4 allele was identified in genotype of 30 patients, the other 30 patients formed the control group. Any statistically significant correlation between APOE genotype and the results of the *in vivo* measurements of temporal lobe atrophy in examined patients was shown which was probably due to the completely different nature and application of both tests. We report also a case of GSS disease with a new mutation at the codon 232 (Met to Thr) of the PRPN gene . This mutation was absent in 40 healthy Polish controls and in 16 other Polish CJD cases. Therefore, we believe that 232 Thr is a new pathogenic mutation, not a benign polymorphism.

In recent years, evidence is increasing that vascular disease is associated with cognitive impairment and dementia. Moreover, the presence of cerebrovascular disease may intensify the clinical symptoms of AD. The aim of the study was to determine the incidence of vascular risk factors in age and sex matched patients with dementia. We included 109 patients with AD and 37 patients with vascular dementia (VD). DSM-III-R test for dementia, NINCDS-ADRDA guidelines for AD and NINDS-ARIEN for VD were applied. Arterial hypertension was associated in 51.3% with VD and in 30.3% with AD ($p<0.05$), hypotension in 11.1% with VD and in 23.6% with AD ($p<0.05$), the incidence of atrial fibrillation was similar in AD and VD, coronary artery disease was associated with 64.8% of AD and 46.8 % of VD ($p>0.05$) and type 2 diabetes with 21.6% and 10.1% ($p>0.05$), respectively. Vascular disease and AD have to some extent a common etiology and risk factors they have in common increase the risk of both disorders independently and vascular etiology may be involved in AD.

To asses long term prognosis in young adults with cerebral ischemia, the follow up study was performed. Eighty-four patients aged 18 to 45 years with the diagnosis of the first-ever ischemic stroke or transient ischemia attack (TIA) were observed. Among the patients with the first-ever ischemic stroke, calculated incidence of vascular death or recurrent stroke was 5.6% per year, and after 24 months - 10.9%. Twenty-eight day mortality rate in this group was 5.6%.

Paradoxical embolism is considered a cause of cerebral ischemia in young patients with patent foramen ovale (PFO). We intended to establish the prevalence of paradoxical embolism in these patients. In eighty-four patients

aged 18-45 years with the diagnosis of first-ever ischemic stroke or TIA Doppler ultrasonography of extracranial arteries, transthoracic and transesophageal echocardiography and ultrasonography of the veins of lower extremities (patients with PFO) were performed and information was obtained at the onset of stroke. In 34 (40.5%) patients cerebral ischemia of known origin was diagnosed. In 8 patients with cerebral ischemia of unknown origin the diagnosis of probable (in 1) or possible (in 7) paradoxical embolism was made. Paradoxical embolism could be the cause of cerebral ischemia in 9.5% of young patients with ischemic stroke or TIA - in 38% of patients with PFO and cerebral ischemia of undetermined cause.

The aim of our last project was to collect information on environmental determinants of healthy ageing of Polish centenarians and to acquire material to study several aspects of longevity, including the search for genetic determinants. The social aim of the project is to attract public attention to both the ageing of the population and to the individual living conditions of old people. Our intentions was to present the Polish Centenarians Program, its methods, scope and aims as well as to inform the possible additional participants about the availability of the collected information and material to study different aspects of longevity and ageing.

INFLUENCE OF INCREASED LEVEL OF BLOOD HOMOCYSTEINE ON THE COURSE OF CEREBRAL ISCHEMIA AND ON THE DEVELOPMENT OF POST STROKE DEMENTIA

Supported by the State Committee for Scientific Research: grant # 4 P05B 025 18

Project leader: Jarosław Pniewski
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It is known, that a mild increase of blood homocysteine level has an impact on the development of coronary atherosclerosis as well as atherosclerosis of cerebral arteries. Increased blood homocysteine level could be the effect of inherited metabolic disturbances or deficiency of some vitamins, but in many cases remains unexplained. Therefore, homocysteine seems to be a risk factor not only for coronary heart disease but for ischemic stroke as well, although the mechanism of this action is not fully explained.

It is also suggested, that homocysteine could be not only regarded as a

risk factor for stroke but may influence the course of the stroke by promoting free radical generation during an acute phase of ischemic stroke. It could also have an impact on post stroke dementia.

In this work we decided to investigate the influence of homocysteine level on the course of stroke and development of post stroke dementia. The study was performed among patients treated in the Department of Neurology CCH MIA with the diagnosis of cerebral ischemia. Hundred patients with the diagnosis of first-ever ischemic stroke without aphasia were involved in the study. In all cases the diagnosis of ischemic stroke was put according to clinical criteria and proved by computed tomography (CT). In the search for the cause of cerebral ischemia the following studies were performed: Doppler-Duplex of the carotid arteries, lipid fractions, WBC, RBC, PLT, blood sugar, BUN, creatinine level, PTT, INR, fibrinogen level. In all the patients homocysteine level as well as the level of B12 vitamin were assessed. Detailed information concerning other risk factors of the stroke (TIA, arterial embolism, heart rhythm disturbances, diabetes mellitus, hypertension, cigarette smoking, alcohol abuse) were also obtained from the patients and their families. We also assessed (using Rankin Scale) general pre-stroke condition of the patients. We defined the stroke origin using TOAST criteria for probable stroke cause. After the stroke, all the patients received the best medical treatment as far as secondary prevention and management of other conditions are concerned. Before discharge from the hospital, all the patients were assessed using Barthel Index and Scandinavian Scale. Three months after the onset of stroke patients were examined and assessed using Rankin Scale, Barthe Index, Scandinavian Scale, Geriatric Depression Scale. In all of them MMSE is performed in order to asses dementia. After finishing the study we intend to find out the impact of increased level of homocysteine on the development of dementia in our patients and on the course of the stroke.

The study is in progress - till now there is no preliminary data as the level of homocysteine and vitamin B12 will be assessed after collecting the blood samples from all the patients and after the 3 month observation period is finished.

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IMMUNOCYTOCHEMICAL, ULTRASTRUCTURAL AND BIOCHEMICAL STUDIES ON APOPTOTIC CELLS AND INVESTIGATIONS ON THE ULTRASTRUCTURE OF THE VESSELS AND PERIVASCULAR SPACE IN THE CENTRAL NERVOUS SYSTEM IN DIFFERENT PATHOLOGICAL MODELS

Project leader: Barbara Gajkowska
Contributors: Michał Walski, Małgorzata Frontczak-Baniewicz, Hanna Olszewska-Bądarczuk

In 1999 studies on the molecular mechanisms of apoptosis in physiological conditions (involution of lactiferous glands *in vivo*) and in pathological states (*in vitro* studies on camptothecin-induced apoptosis in cancer cell lines) were initiated. In 2000 these studies were continued, mainly on the role of the proapoptotic protein BAX, a member of the bcl-2 protein family.

The ultrastructural studies comparised investigations of the intracellular redistribution of BAX early during induction of apoptosis. Several cell lines, COLO 205, PA-1, U 373 MG were used and treated with camptothecin, a well defined inducer of apoptosis *via* inhibition of topoisomerase I. A novel technique of embedment-free electron microscopy (EFEM) combined with post-embedding immunogold allowed to follow BAX distribution in the nuclear matrix and in the cytoskeleton. We showed that camptothecin induces an early (15-30 min) BAX redistribution from the cytosol to the mitochondria and Golgi and then to the nucleus (60-120 min). It should be underlined that this phenomenon preceded the development of the morphological features of apoptosis. EFEM allowed to demonstrate for the first time the association of BAX with the nuclear matrix: a filamentous, nonchromatin nuclear meshwork associated with intermediate filaments in the cytoplasm. Moreover, BAX was also found to be associated with the filaments of the nuclear pores. These findings are novel and indicate the role of BAX not only in mitochondria but also in the nucleus where it is associated with the matrix.

The finding of the nuclear localization of BAX was also confirmed by quantitative immunofluorescence studies employing laser scanning cytometry. An involution of the lactiferous gland *in vivo* was employed as a model for the morphological and ultrastructural studies on the mechanisms of apoptosis. Apoptotic cells were detected not only in the involuting tissue but also in alveolar milk suggesting an additional to phagocytosis pathway of the elimination of apoptotic cells. BAX redistribution took place from the cytosol to the membranes of different organelles (mitochondria, endoplasmic reticulum, Golgi) and to the nucleus *via* nuclear pores.

The main achievement was the finding of the physical interactions of BAX with nuclear matrix. Nuclear matrix contains several enzymatic proteins such as DNA polymerases and topoisomerases, that play a role in DNA replication, gene expression and DNA repair. Presence of BAX in the nuclear matrix suggests its physiological role in the metabolism of nucleic acids.

The other subject was the ultrastructural investigation on the changes associated with the ischemic and traumatic damage of the central nervous system in rats. Focal cerebral ischemia was achieved in the model of photochemical brain injury. Early platelet aggregates in the capillaries were observed, followed by the ultrastructural changes of the cerebrovascular junction and angiogenesis, even in the remote brain areas such as the hypophysis. Of interest were the results on the post-traumatic alterations in the cerebrovascular junction and other brain areas suggesting that some leukocytes (e.g. monocytes) may function as brain macrophages. In this model we described an activation of a heterogeneous population of brain macrophages.

THE ROLE OF IONIZED CALCIUM AND CALCIUM CHANNELS IN APOPTOSIS IN NORMAL AND CANCER CELLS. EXPERIMENTS USING FLUORESCENT CALCIUM DETERMINATION IN LIVING CELLS AND ELECTRON MICROSCOPY

Supported by the State Committee for Scientific Research: grant # 4 P05A 045 15

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According to the research plan, the aim of the studies in 2000 was the correlation between the ultrastructural changes in the nucleus in differentiat-

ing and apoptotic cells with selected biochemical processes of signal transduction and histone metabolism. Two major techniques were employed: the embedment-free electron microscopy that is a powerful tool revealing the ultrastructure of the nuclear matrix and laser scanning microscopy that quantitatively measures the accessibility of DNA to different fluorescent markers. By choosing the fluorochromes which bind to DNA by different mechanisms it is possible to determine the accessibility of DNA to exogenous factors, a feature that reflects the state of chromatin "openess" and activity.

We showed that proliferating cells contain unstainable DNA, the amount of which increases during differentiation. We hypothesized that strong interactions between DNA and histones are responsible for attenuation of DNA staining. This hypothesis was confirmed by studies showing that removal of histones increases DNA-specific fluorescence. We now determine the effect of histone deacetylase inhibitors, such as trichostatin and butyrate, on DNA availability and nuclear ultrastructure.

Calcium signalling plays a role in chromatin remodelling. Cells exposed to high calcium concentration mobilize this ion and present with hypochromic chromatin. Thapsigargin (inducer of calcium release from intracellular stores) had a similar effect that could be used by calcium chelators (BAPTA).

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ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL STUDIES OF THE BRAIN DEVELOPMENTAL DISORDERS. STUDY OF INFLAMMATORY PROCESS

Project leader: Danuta Maślińska
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Studies on the expression of IL-15 mRNA revealed the presence of its two spliced isoforms in the human fetal brains, but only one of these isoforms in the adult brain. The expression of IL-15 protein was confined to neurons, and was absent in the resting/residual microglial and astroglial cells. However, IL-15 protein was present in activated/hypertrophic glial cells participating in the inflammatory process.

Numerous cytokines may be released by activated mast cells (MC), that contribute to the local inflammatory response of different organs. Since, data concerning the association of mast cells with inflammatory response of the brain parenchyma are not available, the aim of our study was to determine the distribution and phenotypes of mast cells in human brain infested by cysticerci.

In control brains, mast cells were very few, sparsely distributed and contained tryptase and chymase, thus they were tryptase-chymase phenotype (MC_{TC}). In brain sections with neurocysticercosis, mast cells accumulated only in the brain regions containing parasites. A striking features of identified mast cells was their phenotype heterogeneity. The tryptase mast cells (MC_{T}) phenotype dominated over the tryptase-chymase (MC_{TC}) phenotype. Summarising, it is the first report, which documents the accumulation and phenotype heterogeneity of mast cells in human brains with neurocysticercosis. Our findings suggest that the effector mechanism responsible for the host

responses to the parasitic infection that involves in the human brain numerous mast cells may be very important for the pathomechanism of this disease.

Common infections and associated systemic inflammation seldom cause pathological changes in the brain, and numerous studies have denied the entry of microorganisms or their toxins across the blood-brain barrier (BBB). Nevertheless the mortality and morbidity of children and newborns caused by meningo-encephalities have remained significant despite advances in antimicrobial chemotherapy and supportive care. In this study, the effect of *Staphylococcus* α-toxin (one of the major virulence factors), on the brain blood vessels was examined. The results show that α-toxin is a potent membrane-damaging factor, which induces expression of COX-2 mRNA and causes ultrastructural changes (degeneration and disruption of basal lamina) in the wall of some brain blood vessels. These changes may facilitate penetration into the brain parenchyma of blood cells and presumably toxic substances concentrated in plasma.

DEVELOPMENTAL ASPECTS OF CNS MALFORMATIONS

Project leader: Maria Dąmbska
Contributor: Milena Laure-Kamionowska

The studies focused on the malformations of the CNS. Four cases presenting the coincidence of prosencephalic commissures anomalies and dysraphic changes expressed by meningomyeloceles were examined. We concluded that they present an intermediate syndrome combining the elements of two different ones which were formed during a long teratogenic period.

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CONTINUATION OF STUDIES CONCERNING DISTURBANCES OF INTRACRANIAL HOMEOSTASIS

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Studies concerning intracranial hypertension and CSF circulation were continued. New projects were also started.

A new method of arachnoid cyst treatment by the non-valve connection between the cyst and subarachnoid space at the lumbar level was introduced in the clinical practice.

Using TCD sonography, cerebral blood flow changes in the middle cerebral artery were studied in 7 patients subjected to the infusion test leading to the ICP increase. Preliminary analysis shows that all values of blood flow velocity - especially the diastolic one - decrease right after starting the intrathecal saline infusion at the lumbar level. The decrease associated with a rise in the peripheral resistance probably due to the ICP increase.

Disturbances of CSF circulation, which underlie the normal pressure hydrocephalus (NPH), tend to cause a specific picture of cognitive disturbances. Based on the multi-level method for NPH diagnosis developed in the Department of Neurosurgery, 46 patients with diagnosed NPH were studied. Apart from disturbed visual-spatial processes pronounced disturbances in the acquisition of spoken information are observed during neurophysiological tests. This kind of neuropsychological deficit - along with the results of

neurophysiological testing – may be also helpful in the qualification of NPH patients for operative treatment.

Cerebrovascular reactivity investigated by means of TCD rabbit and Doppler laser device (rat) was subjected of experimental studies. Subarachnoid hemorrhage (SAH) was produced in rats and rabbits and cerebrovascular reactivity in response to pCO_2 and MAP changes was investigated. Initial analysis shows an abrupt decrease in blood flow velocity both in small, capillary vessels and in basilar artery directly following SAH. In the next minutes after SAH onset disturbances of cerebrovascular reactivity to CO_2 were observed.

Differences of incidence and intensity of SAH-related vasospasm between patients treated by clipping (25) and by coiling (25) were studied by means of TCD monitoring of blood flow velocity. Higher maximum values of mean blood flow velocity, longer periods of persistence of pathological values and higher incidence of symptomatic vasospasm were observed in the group of patients treated by clipping.

Studies concerning cerebral blood flow in left-handed individuals were continued. Correlations between different types of cognitive activity and indices of cerebral blood flow increase in the middle cerebral artery were studied in 43 of such persons. No hemispheric asymmetry was found in genetically predisposed left-handed persons.

EEG and evoked potentials (EP) including mapping techniques of the analyzed data were used in the electrophysiological studies. Disturbances of the neurophysiological balance between inhibitory and stimulating processes reflected by changes of the EP picture in patients with cerebellar lesions were found and analyzed. Decreased amplitudes of N20 complexes were observed on the side of the disturbed cerebellocortical transmission. Recordings of the P25, N35 and late complexes also showed a considerable decrease in their amplitudes on the side of the disturbed transmission without any change in their latencies.

EEG and EP data in patients with epilepsy were collected. EP studies in patients with cervical and lumbar discopathy were continued.

Two project were started in cooperation with the Department of Neurosurgery in Bonn. The first one was a retrospective analysis of the results of surgical treatment of temporal lobe epilepsy (TLE) in children and adolescents. The success rates in terms of seizure relief were found to be higher

compared to adult population suggesting a beneficial effect of early surgical treatment in TLE. Application of motor evoked potentials (MEP) in the intraoperative neurophysiological monitoring during operations of spinal and posterior fossa lesions was the subject of the other project. Initial results show a prognostic value of MEP applied in the intraoperative monitoring.

CLINICAL DIAGNOSTIC APPLICATION OF IN VITRO MR SPECTROSCOPY OF BRAIN TUMOR TISSUES AND CEREBROSPINAL FLUID

Supported by the State Committee for Scientific Research: grant # 4P05B 05414p

Project leader: Zbigniew Czernicki
Contributors: Paweł Grieb (Laboratory of Experimental Pharmacology), Dariusz Horsztyński, Jerzy Walecki

A simple method of analysis of proton resonance spectra of tissue extracts from intracranial tumor biopsies has been developed which may prove useful for objective tumor diagnosis. The method is based on the determination of the maximal height of eleven resonance lines assigned to: alanine, choline, kreatine compounds, glutamate, glutamine, inositol, N-acetylaspartate, acetate, phospho- and glycerophosphocholine phosphoetanolamine and taurine. These heights are then normalized to the sum of heights of the signals considered. This approach has been applied to the analysis of data collected for 16 meningiomas, 11 glioblastomas multiforme, 4 low-grade gliomas and 7 metastatic tumors. Statistical evaluation of these data with the use of one-way ANOVA and post-hoc tests revealed significant differences between the aforementioned groups of tumors, but none of these differences enabled us to classify tumors with high accuracy. However, when a pattern recognition technique (a two-stage nearest neighbors-type classifier) was used, it was possible to classify tumors on the basis of H-MRS *in vitro* data with high accuracy (total error 2.6%, only one case classified incorrectly).

EXPRESSION OF NUCLEAR T3 AND RETINOID RECEPTORS IN GLIOMAS

Supported by the State Committee for Scientific Research: grant # 4 P05C 005 15

Project leader: Zbigniew Czernicki
Contributor: Paweł Nauman

Experimental investigations were completed. The expression of nuclear receptors for TRα and TRβ on the level of nuclear proteins and mRNA in tumors of glial origin were evaluated. Changes in receptor-related DNA binding were also measured.

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PHARMACOLOGICAL NEUROPROTECTION IN CENTRAL NERVOUS SYSTEM PATHOLOGIES

Project leader: Paweł Grieb
Contributors: Stanisław Chrapusta, Bronisław Głód, Tomasz Kryczka, Mirosław Ryba

Effects of chronic treatment with a non-metabolizable glucose analog 2-deoxyglucose (150 mg/kg) on the long-term epileptic tolerance (ET) evoked by 30-min bilateral carotid artery clamping (BCCA) and the involvement of protein synthesis in the development and maintenance of ET were studied in mice. Seizures were induced 14 days after BCCA with CD97 dose of bicuculline. BCCA resulted in decreased mortality, prolonged latency to the onset of generalized convulsions and decreased overall seizure score. Protein synthesis inhibitor cycloheximide (CHX) given after BCCA did not influence ET, given either prior to or after BCCA. 2-DG treatment resulted in a moderate but significant decrease in mortality and a tendency towards a lower seizure score. BCCA combined with 2-DG resulted in a marked decrease in mortality and a reduction of all indicators of tissue susceptibility compared to BCCA alone. CHX abolished the antiepileptic effects of BCCA alone as well, while it did not influence the 2-DG-related decrease in mortality. The results indicate that the development of BCCA-induced ET, as well as unmasking antiepileptic effects of 2-DG by BCCA is dependent on protein synthesis.

Collaborating unit:
Departments of Neurology and Hygiene, Medical Academy, Lublin, Poland (Z. Stelmasiak, M. Sieklucka-Dziuba)

Two methods of quantification of free radical scavenging properties of antioxidants have been developed and implemented. The first is based on chromatographic assay of the reaction products of dihydrobenzoic acid with hydroxyl radical. The second is based on the measurement of kinetics of reaction of 2,7-dihydrofluorescein diacetate (DFC) with peroxyl radicals generated by AAPH solution in the presence of sample. The AAPH/DFC method was used to assay total radical antioxidative capacity (TRAP) in blood plasma samples taken from patients with heart infarct, who have been subjected to thrombolytic therapy. An increase in plasma TRAP was found. This result casts doubts on the concept that ischemic tissues may effectively utilise plasma antioxidants to counteract intracellular oxidative stress during postischemic reperfusion.

BIODEGRADABLE POLYMERS CONTAINING NUCLEOSIDES FOR TREATMENT OF BRAIN TUMORS

Supported by the State Committee for Scientific Research: grant # 405F 0242

Project leader: Paweł Grieb
Contributor: Tomasz Kryczka

The investigations concerning the kinetics of degradation of various biodegradable poly-lactide and lactide-caprolactone polymers containing nucleosides and the kinetics of release of nucleosides from these polymers have been continued in the *in vitro* model developed previously. The relationship between the kinetics of release of a nucleoside and the chemical composition of polymers and the method of their impregnation with a nucleoside were found. Three nucleoside-containing polymers displaying desired kinetic properties have been selected for further development. It has been determined that sterilization of the polymers with ethylene oxide changes their kinetic properties. Therefore, an alternative method of their sterilization shall be sought.

5'-O-ESTERS OF DEOXYADENOSINE AND CLADRIBINE

Project leader: Paweł Grieb
Contributor: Tomasz Kryczka

Enzymatic hydrolysability and biological effects on leukemia lines *in vitro* of newly synthesized 5'-O-esters of 2-deoxyadenosine and 2-chloro-2'-deoxyadenosine (cladribine) with inhibitors of cholesterol synthesis phenyloacetate and phenylobutyrate, differentiating agents (retinoic acids) and biomimetic polymer poly-(hydroxybutyrate) (PHB) have been investigated. Esters with phenyloacetate and phenylobutyrate were resistant to hydrolysis by esterase and devoid of biological activity. The other esters were hydrolysed by esterase. The esters of deoxyadenosine and retinoils displayed differentiating effects in HL-60 human promyelocytic leukemia line, and the esters of cladribine and retinoils displayed both cytotoxic and differentiating effects. The esters of cladribine and PHB also displayed cytotoxicity, but it was developing at much slower rate than that of free cladribine.

Collaborating units:

Department of Chemistry, Agricultural University, Warsaw, Poland (Z. Kazimierczuk)

Institute of Polymer Chemistry, PASci, Zabrze, Poland (M. Bero)

Medical Centre of Postgraduate Education, Warsaw, Poland (J. Kawiak)

TELOMERIC LENGTH HOMEOSTASIS IN TESTICULAR GERM CELL TUMORS

Project co-ordinator: Stanisław Chrapusta

Telomeric restriction fragment (TRF, an index of telomeric length) and telomerase activity were compared between the two main histologic types of human testicular germ cell tumors, seminomas and nonseminomas. All nonseminoma samples studied (n=13, taken from 12 patients and representing a variety of nonseminoma subtypes) showed TRF of $\geq$ 23 kb length, i.e. similar to that in normal human sperm. Fifteen out of 16 seminoma samples examined (taken from 15 seminoma patients) showed TRF of $<$ 13 kb; the remaining sample showed the presence of a small fraction of $\geq$ 23 kb TRF length in addition to the major peak of 18 kb length. There was no correlation between relative telomerase activity and TRF length. These results indicate that a germline-like homeostasis of telomeric length is preserved in human nonseminomas, and suggest seminomas and nonseminomas derive independently from their presumed common precursor, the *carcinoma-in-situ*.

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MOLECULAR, MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL STUDIES ON MUSCLE AND NERVE IN GENETIC AND ACQUIRED MUSCLE DISORDERS

Project leader: Irena Hausmanowa-Petrusewicz
Contributors: Andrzej Kochański, Hanna Jędrzejowska, Hanna Drac

Molecular genetical analysis was performed in twenty Charcot-Marie-Tooth (Ch-M-Th) families. The analysis included the 17p11.2-p12 region and, in addition, three genes: Cx32, MPZ and PMP22. Ten novel 17p11.2-p12 duplications, two 17p11.2-p12 deletions, and a novel E (208) G mutation in Cx32 were detected. Clinical, electrophysiological and morphological data were collected and correlated with genotype. The Unit has become included in the European program on Ch-M-Th studies and started collaboration with the Ch-M-Th World Association.

Collaborating units:
Department of Neurology, Medical School, Warsaw (B. Ryniewicz)
Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus (K. Christodoulou, D.M. Georgiou, T. Kyriakides, E. Zamba)

Contributors: Anna Fidziańska, Katarzyna Rowińska-Marcińska, Małgorzata Dorobek, Irena Niebrój-Dobosz

Studies were performed on Emery-Dreifuss dystrophy, particularly a relatively recently identified autosomal dominant (and recessive) form associated with gene mutation on chromosome 1q32. The clinical phenotype of

this variant was described and compared with the X-linked variant. The type of laminopathy responsible for this variant was identified. Cardiological examinations of E-D patients were aimed at analyzing similarities (or differences) of cardiac involvement in both: X-linked and autosomaly transmitted forms of E-D dystrophy.

Collaborating units:
Department of Cardiology, Medical School, Warsaw, Poland
Cardiomyologic Center, Naple, Italy
Institute of Genetics, Pavia, Italy
Department of Molecular Biology, University of Durham, England

Contributor: Katarzyna Rowińska-Marcińska

Electrophysiologic studies of motor unit (MU) hyperexcitability in peripheral motor neuron lesion were continued. Concentric needle EMG data obtained in the patients with different types of peripheral neuron lesion and widespread spectrum of hyperexcitability phenomena were analysed. Repetitive motor unit discharges (RDs) in a muscle at rest, in the activated state or in response to a single nerve stimulus were considered a sign of motor unit hyperexcitability.

Our own program was a new topic based on modelling of EMG record with a special emphasis on assessment of irregularity.

Collaborating unit:
Institute of Biocybernetics, Warsaw, Poland
Department of Neurology, Medical School, Warsaw, Poland (E. Zalewska)

Contributors: Irena Niebrój-Dobosz, Małgorzata Dorobek, Anna Fidziańska

A further study on a group of patients with limb-girdle muscular dystrophies (LGMD) has resulted in the identification of families with sarcoglycanopathies. The study included immunocytochemical analysis with the use of antibodies to sarcoglycans and phenotype description. This is the

first report in which sarcoglycanopathy was diagnosed in Polish families. Studies of dystrophinopathies were continued. Duchenne and Becker dystrophy patients and mdx skeletal muscles and diaphragm were examined. The results indicate that branched-chain amino acids in the muscle may offer protection against dystrophic devastating processes. Various laboratory tests (clinical, electrophysiological, histopathological, immunohistochemical, immunochemical, serum creatine kinase and genetic studies) in familial and sporadic carriers of Duchenne and Becker dystrophy were performed. Abnormalities in the different tests were more expressed in sporadic cases. Detection of sporadic carriers permits correction of diagnosis in cases previously diagnosed as limb girdle dystrophy.

Contributors: Irena Hausmanowa-Petrusewicz, Maria Jędrzejowska, Anna Fidziańska

Genotype-phenotype correlations in the population of Polish SMA patients were investigated. Up to now, molecular analysis of the presence of deletion in SMA region was performed in a group of 289 patients and 143 patients' relatives. Homozygous absence of exon 7 SMNt was detected in 91% SMA patients, hence, the deletion appears more frequent in Polish SMA patients than in other countries. A novel method is being tested to allow detection of heterozygous SMN1 deletions in SMA carriers and SMA patients without homozygous SMNt deletions. We are examining SMN protein level in fibroblasts.

Collaborating units:
Department of Neurology, Medical School, Warsaw, Poland
Genetical Laboratory, Institute of Psychiatry and Neurology, Warsaw, Poland
Genetical Laboratory, Mother and Child Institute, Warsaw, Poland
Institute of Human Genetics, Aachen, Germany

THE ROLE OF EMERIN IN THE PATHOGENESIS OF THE CLINICAL PHENOTYPE OF EMERY-DREIFUSS DYSTROPHY WITH SPECIAL ATTENTION TO CARDIAC PATHOLOGY

Supported by the State Committee for Scientific Research: grant # 4P05B 061 81

Project leader: Irena Hausmanowa-Petrusewicz
Contributors: Anna Fidziańska, Katarzyna Rowińska-Marcińska, Małgorzata Dorobek, Irena Niebrój-Dobosz

Beside the X-linked form of Emery-Dreifuss dystrophy, one other variant of this disease was genetically identified (autosomal dominant transmission associated with gene mutation on chromosome 1q32). The first case of recessive autosomally transmitted E-D dystrophy was described. In autosomal variants of E-D disease laminopathies were described with preserved emerin. Cardiologic findings assessed by electrocardiography, echocardiography, heart muscle contractility were compared in both X-linked and autosomal types. Genetic heterogeneity of E-D disease was confirmed in local patients.

Collaborating units:
Cardiomyologic Center, Naple, Italy
Institute of Genetics, Pavia, Italy
Department of Molecular Biology, University of Durham, England

SUICIDE MUSCLE CELL PROGRAM - APOPTOSIS IN SPINAL MUSCLE ATROPHY (SMA) TYPE I

Supported by the State Committee for Scientific Research: grant # 4P05E 057 18

Project co-ordinator: Anna Fidziańska

Ultrastructural criteria of suicide muscle cell program - apoptosis were established on the basis of structural changes found in 3 infants with acute, fatal form of SMA characterized by 8 exons deletion. The coexistence of different stages of apoptosis in the same tissue is the most specific feature of suicide muscle cells program in this form of SMA.

Ultrastructural and immunohistochemical analysis of vascular basement membrane (VBM) in developing spinal cord showed two steps in VBM ma-

turation. Fibronectin and laminin are the first components appearing in immature VBM. Collagen IV and merosine, which occur later in mature VBM, are responsible for mature blood barrier stability.

Collaborating units:
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Publications:

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CARDIOVASCULAR, METABOLIC AND NEUROHORMONAL RESPONSES TO SELECTED PHYSIOLOGICAL STIMULI IN HUMAN SUBJECTS OF DIFFERENT AGE AND PHYSICAL ACTIVITY

Project leader: Krystyna Nazar

Effect of static hand-grip on plasma concentration of adrenomedullin (ADM) in patients with heart failure (HF) and in healthy subjects

Contributors: Krzysztof Krzemiński, Barbara Kruk, Gerard Cybulski, Hanna Kaciuba-Uściłko, Krystyna Nazar

The aim of the study was to find out whether ADM release is related to activation of the sympathoadrenal system and hemodynamic changes during static exercise in patients with HF (n=8) and normal subjects (n=8) of matching age. Resting concentrations of ADM and noradrenaline (NA) were significantly higher in HF patients than in normal subjects but exercise-induced increases in ADM and catecholamines were similar in these groups. Plasma ADM was significantly correlated with plasma NA ($r=0.43$, $p<0.01$) and SV ($r=-0.42$, $p<0.01$). The data suggest that an increase in ADM during static exercise may result both from increased sympathetic activity and hemodynamic changes.

Collaborating unit:
Outpatient Cardiac Unit for Diagnosis and Therapy, MRC, PASci, Warsaw (E. Wójcik-Ziółkowska)

Physical activity, menstrual cycle abnormalities and sex hormonal profile in girls 3-5 years after menarche

Contributors: Renata Zabielska, Andrzej W. Ziemba, Tomasz Mikulski

Twenty girls of low physical activity were submitted to a 4-month program of regular recreational exercise training. After the training, the number of regular menstrual cycles was increased and the occurrence and intensity of ailments associated with menstruation decreased. Plasma concentrations of prolactin and progesterone increased whereas testosterone level decreased. The data showed a beneficial effect of recreational training on psychosomatic state and menstrual cycle disturbances in young girls.

Contribution of coordination pattern to sex- and puberty-related differences in walking and running economy

Contributor: Andrzej W. Ziemba

The ratio of oxygen uptake per kg body mass to the speed of walking or running was determined and movement of upper and lower extremities was recorded in pre- and post-pubertal boys and girls. More efficient economy of locomotion and more stable pattern of movement coordination was found in the girls than in the boys of the same age and in the subjects of both sexes after puberty as compared to those before puberty. The study indicates an important role of movement coordination in the sex and age-related differences in walking and running economy.

Collaborating unit:

Department of Human Movement Sciences, Free University of Amsterdam, Netherlands (E. Donkervliet, T. Smits, H.C.G. Kemper, R.C. Wagenaar)

Metabolic and hormonal responses to oral glucose load in middle aged and elderly women

Contributor: Andrzej W. Ziemba

Resting metabolic rate (RMR), thermogenic effect of glucose ingestion (TEG) as well as blood glucose (BG), plasma insulin (IRI) and catecholamine (CA) responses to glucose load (75g) were determined in sedentary, middle aged (47 ± 1.3 yrs) and elderly (72.5 ± 1.4 yrs) physically active women. The elderly women had lower RMR than the middle aged ones and did not show any post-glucose increases in the metabolic rate. However, there were no differences in BG, IRI and CA responses to glucose load between the groups. Thus, it appears that the decrease in RMR and TEG are attributable to aging, whereas high physical activity attenuates the age-induced diminution in glucose tolerance and insulin resistance. The lack of TEG with normal CA response to glucose ingestion suggests a decrease in CA thermogenic action with age.

Effect of training on hemodynamic parameters during Valsalva maneuver

Contributors: Alicja Kodrzycka, Gerard Cybulski, Wiktor Niewiadomski

Continuing the study on the effect of training on circulatory system regulation, changes in cardiac output, stroke volume (SV) and systolic time intervals during Valsalva maneuver in the supine position were compared in 11 well trained swimmers and 17 sedentary students using the impedance cardiography ambulatory device designed and constructed in this Laboratory. Swimmers had lower basal HR and greater SV than sedentary subjects but there were no differences between these groups in the course and magnitude of hemodynamic responses to Valsalva maneuver.

Effect of the whole body criotherapy on physiological responses to exercise in athletes

Contributor: Jolanta Chwalbińska-Moneta

Before and after 23 exposures to -150°C (3 min , twice daily) circulatory, metabolic, hormonal and thermoregulatory responses to graded incremental exercise until volitional exhaustion were determined in 6 elite rowers. It was found that cryotherapy delays the development of muscle fatigue assessed by EMG, decreases blood lactate concentration and shifts the anaerobic threshold towards higher exercise loads, attenuates increases in core body temperature, blood growth hormone, cortisol and testosterone. The data confirmed the beneficial influence of the whole body cryotherapy on exercise performance.

Effect of low carbohydrate diet (LCHO) on exercise thermoregulation

Contributor: Ryszard Grucza

In order to find out whether increased utilization of lipids, induced by LCHO diet, results in elevation of core body temperature, changes in tympanic temperature (Tty) and sweating secretion dynamics were recorded during graded incremental exercise after 3 days of controlled mixed (M) diet and 3 days of LCHO diet (<5% of CHO) in 7 young men. It was found that Tty reaches similar values after both diets, but after LCHO diet the delay of sweating response at the beginning of exercise is shortened.

Collaborating unit:
Department of Physiology, Academy of Physical Education, Katowice, Poland (I. Pokora)

EFFECT OF DIETARY CREATINE SUPPLEMENTATION ON CARDIAC MUSCLE METABOLISM IN RATS AND THE EFFECT OF BRANCHED CHAIN AMINO ACID (BCAA) SUPPLEMENTATION ON EXERCISE TOLERANCE IN MEN

Project leader: Hanna Kaciuba-Uściłko

Effect of dietary creatine supplementation on high energy phosphates and mitochondrial enzymes in the myocardium of rats with cardiac hypertrophy

Contributors: Hanna Kaciuba-Uściłko, Krystyna Nazar, Zofia Brzezińska, Józef Langfort, Ilona Falęcka-Wieczorek

Both human and animal studies have demonstrated that myocardial energy reserves are substantially reduced in chronic heart failure. Thus, the ability of dietary creatine (Cr) to increase myocardial high energy phosphate content and oxidative potential, as it was demonstrated in our previous study in rats, may be of interest from the clinical point of view. The aim of this investigation was to find out whether Cr supplementation influences myocardial energy metabolism in rats with cardiac hypertrophy. In 11 adult Wistar rats cardiac hypertrophy was induced *via* constriction of the descending aorta below renal arteries. Five weeks after surgery, 6 rats received Cr supplementation in the diet (500mg daily) for 7 days, whilst 5 rats were fed commercial rat chow. Fifteen unoperated rats served as controls, 7 of them were supplemented with CR and 8 were fed normal diet. At the end of the supplementation period, all rats were anesthetized with pentobarbital sodium, samples of the cardiac muscle from the left and right ventricle were excised, deep frozen within 15 s in liquid nitrogen, and then stored at -80^{0}C until assayed. In the muscle specimens, total creatine (TCr), phosphocreatine (PCr), Cr, adenylate nucleotide (ATP, ADP and AMP) contents as well as citrate synthase (CS) and β-hydroxy-acyl-CoA-dehydrogenase (HAD) activities were determined. The PCr content in the myocardium of rats with cardiac hypertrophy was diminished, while ATP content was similar to that in controls. Activities of CS and HAD were higher in hypertrophied than in normal

hearts. Creatine supplementation significantly increased TCr (by approx. 15%) and PCr (by approx. 25%) contents in the myocardium in both groups of rats, but ATP content was elevated only in the intact animals. Changes in high energy phosphate contents after Cr supplementation were similar in the right and left ventricles. Supplementation with Cr increased activities of CS and HAD in cardiac muscle of the control rats, while in the rats with cardiac hypertrophy this effect occurred only in the right ventricle. The data obtained so far indicate that hypertrophied cardiac muscle is able to take up Cr supplied in the diet and produce PCr. However, ATP content is not increased and the influence of energy flux by creatine kinase system on the contractile performance of the heart is still uncertain.

Effect of supplementation with branched chain amino acids (BCAA) on psychomotor performance during graded exercise

Contributors: Andrzej W. Ziemba, Tomasz Mikulski

It has been suggested that brain serotonin plays an important role in central fatigue phenomenon. Synthesis of this neurotransmitter is stimulated by increased availability of its precursor - tryptophan. During exercise, the plasma level of free tryptophan (fTR) increases whilst that of BCAA decreases. Since BCAA inhibit competitively fTR transport through the blood-brain barrier, attempts have been made to attenuate central fatigue by administration of exogenous BCAA before or during exercise. The aim of this study was to find out whether BCAA supplementation influences psychomotor performance, determined on the basis of changes in multiple reaction time (MRT) during exercise. The investigation was carried out in 16 male soccer players performing twice multistage incremental exercise until volitional exhaustion with BCAA or placebo treatment. During both exercise tests, oxygen uptake (VO_2) and heart rate (HR) were continuously recorded. Reaction time (RT) was measured at rest and during each exercise load. One hour before exercise, the subjects received either 7g of BCAA or placebo in a double blind manner. BCAA ingestion significantly ($p<0.05$) shortened RT at rest. During exercise both after placebo and BCAA, the RT was shortened until work load of approx. 70% of maximal load and then it increased rapidly. In athletes treated with BCAA, the shortest RT occurred at 253±8.5W

and after placebo at 218±10W, $p<0.001$. There was no effect of BCAA on the subjects' working capacity (VO_2max). The study indicates that supplementation with BCAA enables the athletes to maintain the psychomotor performance on increased level at the higher exercise intensities.

Collaborating unit:
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PHYSIOLOGICAL FACTORS MODIFYING LIPID METABOLISM IN RAT SKELETAL MUSCLES

Project leader: Hanna Kaciuba-Uściłko

Effect of fasting on fatty acid uptake by skeletal muscles

Contributors: Marcin Synak, Ryszard Zarzeczny, Monika Górecka, Ewa Żernicka

It is commonly accepted that fatty acid (FA) uptake by skeletal muscles occurs not only by passive diffusion but also by protein facilitated mechanism. One of the proteins participating in this process is fatty acid binding protein in plasma membrane (FABPpm). It has been recently reported that in rats fasted for 48 h the content of FABPpm in the myocyte membrane increases by approx. 68%. These data inclined us to get an extended insight into this problem by finding out whether 48 h fasting influences FA uptake and incorporation of palmitic acid (PA) into the muscle acylglycerols in various muscle types of the rat. A hindlimb perfusion technique with labeled [^{3}H] PA in the perfusate was used. It was found that in fasting rats, whose liver and muscle glycogen contents were markedly depleted (from 330 to 50 $\mu mol \cdot g^{-1}$, and from 130 to 60 $\mu mol \cdot g^{-1}$, respectively), the rate of [^{3}H] PA incorporation into acylglycerols was considerably enhanced in skeletal muscles, regardless of muscle fiber composition. The results obtained so far indicate that fasting up to 48 h, resulting in decreased carbohydrate stores, leads to an elevation of FA incorporation into the skeletal muscle acylglycerols. Basing on literature data, it can be suggested that the facilitated transport of

FA by FABPpm may play an important role in this process. Further studies on this problem are in progress.

Effect of cold exposure on skeletal muscle lipid metabolism

Contributors: Ewa Żernicka, Monika Górecka, Marcin Synak

Investigations on the role of skeletal muscles in thermogenesis of rats exposed to cold were continued. The results previously obtained have shown a marked increase in the muscle potential for lipid utilization as an energy substrate after 24 h of cold exposure (6^{0}C). The aim of the present study was to follow up the dynamics of changes in the indices of lipid metabolism at 3, 6, 12, 18 and 24 h of cold exposure (6^{0}C). Apart from determinations of two fractions (extra- and intracellular) of muscle lipoprotein lipase (LPL) activity, muscle TG content, the plasma free fatty acid and TG concentrations also mRNA for the uncoupling proteins (UCP 2 and 3) in muscle were estimated. Biochemical analyses are in progress.

Cooperating units:
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EFFECT OF OCCUPATIONAL WORK ON CARDIOVASCULAR, HORMONAL AND METABOLIC INDICES IN PATIENTS WITH CHRONIC CARDIOVASCULAR AND METABOLIC DISEASES

Supported by the Governmental Program:
"Labor safety and health protection in work environment", grant no 04.10.6

Project leader: Krystyna Nazar
Contributors: Hanna Kaciuba-Uściłko, Barbara Kruk, Hubert Krysztofiak, Wiktor Niewiadomski, Gerard Cybulski, Alicja Kodrzycka, Andrzej W. Ziemba, Barbara Bicz

The aims of this project were (1) to assess the effects of occupational stress on cardiovascular and metabolic indices in patients with coronary heart

disease (CHD), hypertension (HT), diabetes (D) and obesity (O), (2) to adapt a model of laboratory tests for prediction of the patients' responses to real life stress occurring in occupational work, (3) to elaborate a system of evaluation of health risk connected with occupational work in patients with chronic cardiovascular and metabolic diseases. A group of 204 men aged 35-65 years was examined. The group included 55 patients with CHD, 53 patients with HT, 26 patients with D, 48 patients with O and 18 healthy persons. All of them were white collar workers, engaged in jobs with high mental demands and extensive responsibility. The type of patient behavior (type A and B) and conditions of their work were assessed by using appropriate questionnaires. During 4 separate week days, ambulatory blood pressure and ECG were recorded for 24 hours using Holter methods. The patients with D checked their blood glucose (BG) concentration with a portable device. The subjects were asked to choose two "normal" and two "stressful" days, and to make notes of their activities and events during each of these days. Their mood status during work time was evaluated on the basis of *ad hoc* mood questionnaire. The medication of patients was continued throughout the study.

For assessment of stress reactivity, 75 subjects (15 persons from each group) were submitted to a complex laboratory test. The test included 4 stimuli: exposure to noise (100 dB, industrial noise), active orthostatic maneuver, mental stress (arithmetic Kreapelin test), and static hand-grip separated by 30 min rest intervals. During the whole testing session, ECG and stroke volume were recorded. Blood pressure, plasma catecholamines, ACTH, cortisol, growth hormone and BG were determined before and after each stimulus.

It was found, that (1) circulatory disturbances such as large increases in blood pressure, ischemic changes in ECG, cardiac arrhythmias occurred in approx. 30% of regularly treated patients with chronic cardiovascular and metabolic diseases; (2) approx. 20% of diabetic patients showed high levels of BG during the most active part of working day long time after meals (>2h); (3) in the majority of cases, greater increases in blood pressure occurred in "stressful" rather than in "normal" days along with higher scores of mood characteristics, such as anxiety, anger and depression; (4) approx. 40% of subjects represented type A of behavior (typical for persons susceptible to stress), but no relationship was ascertained between the indices of

cardiovascular disturbances during occupational work and the type of behavior.

The complex test of stress reactivity applied in this study containing psychological and physical stress elements was well tolerated by the patients and showed individual differences in cardiovascular and neurohormonal responses to applied stimuli. Comparison of the results of 24-hour blood pressure registration with the results of the laboratory tests demonstrated that the subjects with exaggerated blood pressure and plasma catecholamine responses to the laboratory stimuli, particularly to the arithmetic test, show also greater variability and higher values of blood pressure under real life conditions. Results of laboratory testing showed, therefore, that the complex laboratory tests may be useful for identification of persons with high reactivity to stress. However, the test applied in this study did not fully accomplish the practical purpose. In order to increase its predicting value, it seems necessary to modify the procedure, namely to replace exposure to noise, which in most cases did not evoke either cardiovascular or neurohormonal responses, by another more stressful psychological stimulus.

In conclusion, the study indicated, that in spite of regular pharmacological treatment, in some patients with chronic cardiovascular and metabolic diseases, psychological stress in occupational work evokes disturbances which may negatively influence their state of health. It is suggested, that frequent ambulatory monitoring of ECG and blood pressure, and in the case of diabetic patients measurements of BG along with evaluation of stress reactivity under laboratory conditions may help to plan individual strategies of stress reduction and/or stress management for these patients.

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THE DEVELOPMENT AND EVALUATION OF A NEW NONINVASIVE METHOD OF RENAL DENERVATION IN THE RAT: APPLICATION FOR A STUDY OF EARLY DENERVATION EFFECTS

Project leader: Janusz Sadowski
Contributors: Elżbieta Kompanowska-Jezierska, Agnieszka Walkowska

The standard technique of renal denervation necessitates considerable dissection around the kidney, cutting all visible nerve fibres of the renal pedicle and stripping the adventitia of the renal artery. Thus, the procedure is extended in time and traumatising and the proper experimental studies can be undertaken no sooner than after 1-hour recovery. Within that time the effects of removal of sympathetic input to the kidney could be compensated for by hormonal and paracrine factors. In the search for a method to enable assessment of the early effects of renal denervation, before potential compensatory mechanisms have come into play, we have developed a relatively non-invasive novel technique. The tissue containing most of nerve fibres entering the renal hilus was surrounded with a thin flexible wire loop with minimal prior dissection. After baseline measurements of renal function, a high frequent current was applied to the wire and the tissue was cut by electrocoagulation. Renal hemodynamic and excretion measurements were started directly after denervation. This approach was used to examine the earliest effects of renal denervation.

The effectiveness of denervation was verified by showing that tissue noradrenaline concentration of the denervated kidney was decreased to about 5% of that measured in the contralateral innervated kidney. Another proof of the effectiveness was the development of typical denervation natriuresis and diuresis: sodium excretion increased significantly, by 54 and 82% in the two parallel groups, respectively, over the first 25-50 min after denervation. This

occurred under constant renal perfusion pressure which was controlled using an adjustable snare on the aorta just above the origin of the renal artery. The glomerular filtration rate did not change after denervation. Renal cortical and medullary blood flows (CBF, MBF) were estimated as laser-Doppler flux and medullary tissue ion concentration was estimated as electrical admittance. Following denervation, in both groups CBF increased significantly, by 8 and 12%, respectively, within the first 25 min, whereas MBF either did not change at all or just decreased slightly. Medullary tissue admittance did not change significantly, indicating stability of medullary ionic hypertonicity.

In summary, with the newly developed method protocols can be designed whereby an experimental intervention and measurement (including suitable controls) can be obtained before and then after denervation, within the same experiment in one animal. The data indicate that the effectiveness of denervation is comparable with that of the standard method which is by far more traumatising. Furthermore, the data document the development of natriuresis within the first 25 min after denervation. The observed increase in cortical blood flow indicated that prior to denervation, the cortical, but not medullary, circulation was under a tonic vasoconstrictor influence of renal nerves. Such a dissociation of neural effects on the renal cortical *vs.* medullary vasculature has not been previously described.

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SPIRO-ERGOMETRIC EXERCISE TEST IN CLASSIFICATION OF HEART FAILURE IN PATIENTS LONG TIME AFTER MYOCARDIAL INFARCTION

Project leader: Ewa Wójcik-Ziółkowska
Contributors: Wiesława Pawłowska-Jenerowicz, Magdalena Płachcińska-Bijak

Chronic heart failure (HF) is a serious outcome of coronary heart disease, negatively affecting life expectancy in patients after myocardial infarction.

From several years exercise testing with the respiratory gas exchange determination has been recommended for objective evaluation of cardiac function deterioration in patients with HF. Measurements of exercise pulmonary ventilation, oxygen uptake (VO_2) adjusted to age and body mass, carbon dioxide elimination (VCO_2) and variables dependent of these parameters enable monitoring of HF in the treated patients. In the course of HF and particularly as a result of pharmacological treatment physiological responses to exercise become modified (nonlinear) increase in heart rate and blood pressure with exercise load). It is commonly accepted that peak VO_2 (ml/kg/min) attained during exercise is the most important index of exercise ability and it closely, negatively correlates with the degree of heart function impairment.

The aims of the present study were: (1) to evaluate a degree of HF at least 10 years after the first myocardial infarction using the spiro-ergometric test, classification of HF in these patients according to Weber comparison of the results with the classic NYHA standards; (2) to find out whether the resting indices of cardiac function e.g. ejection fraction determined by echocardiography, correlate with the results of the spiro-ergometric test. The examinations were performed in 59 patients aged 41-78 years, who had their first myocardial infarction 10 to 28 (17.4±9) years before the study.

Summary of the results:

- In more than 40% patients severe HF (class C and D according to Weber's classification) was found;
- In much as 34% patients HF was mild (class A according to Weber's classification), although clinically they belong to the III class according to NYHA. This questions the value of subjective symptoms and indicates importance of "peripheral factors";
- No significant correlation was found between ejection fraction and the results of the spiro-ergometric test.

Conclusion: The spiro-ergometric test is the only reliable method of monitoring the time-course of HF during several years (stabilization *vs.* progression) and may be used as a basis for qualification of patients to the heart transplantation.

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L-ARGININE SUPPLEMENTATION IN CORONARY ARTERY DISEASE AND IN CONGESTIVE HEART FAILURE

Clinical investigation performed in cooperation with the Department of Cardiology, Postgraduate Medical School, Grochowski Hospital, Warsaw. Head: Prof. L. Ceremużyński

1. In a randomized double blind cross-over study conducted in patients with stable coronary artery disease (n=25), oral supplementation of L-arginine (6g/day for 3 days) significantly increased exercise duration. However, it had no effect on exercise-induced changes in QT interval duration, QT dispersion or the magnitude of ST segment depression. Results suggest that L-arginine does not exert any anti-ischemic (i.e. vasodilatatory) effect on coronary circulation. Increased exercise tolerance in patients on L-arginine is most likely due to improved peripheral vasomotion. The non-stereo-specific effects of L-arginine are the likely underlying mechanism.

Contributors: B. Bednarz, R. Wolk, T. Chamiec, D. Winek.

2. Supplemental oral L-arginine (9g/day for 7 days) significantly prolonged exercise time in patients with chronic heart failure (n=20, functional class NYHA II-IV, ejection fraction below 40%). This was a randomized double-blind cross-over study with a 7 day wash-out period. L-arginine treatment had no effect on the biochemical markers of oxidative stress (serum lipid peroxides and reduced sulphydryl groups, oxygen free radical production by leukocytes).

Contributors: J. Gębalska, B. Bednarz, T. Chamiec.

3. Beside K. Cedro-Ceremużyńska participates in a grant, which encompasses a prospective multicenter randomized placebo-controlled study investigating the effect of L-arginine and antioxidant vitamins E and C on the

clinical course of acute myocardial infarction during hospitalisation and a 6 month follow up period (grant conducted by Tomasz Chamiec)

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IMMUNE CELLS, ANTIBODIES AND CYTOKINES IN HUMAN TISSUES AND BODY FLUIDS

Project leader: Waldemar L. Olszewski
Contributor: Hanna Gałkowska

The physiological and pathological immune events taking place in the interstitial compartment and specifically in its liquid phase (tissue fluid and lymph) were the subject of the year 2000 studies. Insight into the intercellular space allows to continuously follow the processes of release of substances produced and excreted by the parenchymatous cells, as well as those filtered from the blood exchange vessels.

Coagulation factors in human skin tissue fluid (lymph)

The aim of the study was to answer the question of no-coagulation phenomenon of tissue fluid and lymph containing fibrinogen and other coagulation factors upon contact with the ground matrix and in the presence of tissue factor (TF). We found the concentrations of coagulation factors to represent 20% of the plasma level. The tissue fluid/plasma ratios of factors VII, IX and TF inhibitor, obtained from functional testing, were higher than the ratios of their proteins (antigens). The level of the TF inhibitor-factor Xa complex was higher in tissue fluid than in plasma. Interestingly, while the tissue fluid fibrinogen level was lower than in plasma, the D-dimer concentration was 5-times over the plasma values. The obtained results point to the TF inhibitor-factor X complexes and fibrinogen degradation as factors responsible for inhibition of tissue fluid coagulation.

Human tissue fluid (lymph) lipid metabolism

The level of lipids and their metabolism were followed in tissue fluid during daily activities. The question remains open whether the tissue fluid concentration of lipids and apolipoproteins is regulated by their influx from serum or there is a supplementary contribution by the parenchymatous cells. The triglyceride level was found close to zero, whereas the glycerol concentration reached levels higher than plasma. The levels of nonestrified cholesterol, phosphatydilcholine, cholesterol esters and sphingomyelin did not correlate with those of plasma, whereas there was a significantly high level of correlation of HDL, apolipoproteins A-II, IV, CIII and E. The obtained results indicate that tissue fluid cholesterol level depends on both, the capillary transport and reverse traffic from the cells.

Lymph cytokine and cells in limb connective tissue inflammation

Studies carried out on patients with rheumatoid arthritis revealed low levels of lymph TGF beta and INF gamma. This may explain the low local cellular response in inguinal lymph nodes observed in this group of patients. Among the lymph cells an increase in $CD3^+DR^+$ and $CD25^+$ was observed. The entire lymph population showed increased autotranformation level and high responsiveness to mitogens. An evident discrepancy between the clinical evaluation of tissue inflammatory changes and minor alterations in lymph cell composition and activation have been observed.

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TOLERANCE TO ALLOGENEIC TRANSPLANTS AFTER PRETREATMENT WITH DONOR BONE MARROW CELLS

Project leader: Waldemar L. Olszewski
Contributors: Michał Maksymowicz, Bożena Interewicz, Marek Durlik

The main topics were tolerogenic properties of dendritic cells and fast repopulation of bone marrow and lymphoid organs after vascularized bone marrow transplantation.

Tolerogenic properties of donor dendritic cells in inducing tolerance to organ transplants

The aim of the project was to investigate the effect of repopulation of the prospective heart graft with the recipient dendritic cells on the survival time after transplantation to the dendritic cells donor. The lethally irradiated heart donor was transplanted with a recipient hind-limb as a source of bone marrow. Within 14 days the lymphoid and non-lymphoid tissues, including heart, became repopulated with limb donor bone marrow cells. Subsequenly heart was harvested from the repopulated donor and transplanted heterotopically into the limb donor strain rat. The repopulating dendritic cells were identified with monoclonal antibodies specific for the bone marrow donor strain. Mixed lymphocyte reaction between the splenocytes of repopulated heart donor and recipient were carried out. Repopulating vascularized bone marrow donor cells initiated an intravascular allogeneic reaction in the prospective heart donor resulting in acute rejection of the heart graft after transplantation to the recipient (donor of repopulating cells). The results indicate that the putative tolerogenic effect of recipient dendritic cells does not operate at the level of organ transplant. Low level of response of heart recipient splenocytes to donor (rat repopulate with recipient bone marrow) splenocytes should be accounted for by low concentration of remaining heart donor cells.

Does irradiation for immunoablation affect the function of stromal cells in the process of repopulation with hematopoietic cells?

Protracted lymphopenia following irradiation of bone marrow recipient points to the possible impairment of stromal cells. Recipients of vascularized bone marrow transplants were irradiated with a dose of 8 and 9Gy. After irradiation syngeneic donor 51 Cr-labelled bone marrow cells were infused i.v. Twenty-four hours later distribution of labelled cells was evaluated in lymphoid organs. There were no differences in the "homing" rate between the two groups irradiated with increasing doses. Although homing kinetics were preserved, this finding does not preclude the damage caused by irradiation at the direct contact between the hematopoietic and stromal cells.

Collaborating unit:
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HUMORAL AND CELLULAR DEFICIT IN PROTRACTED WOUND HEALING PROCESSES IN HUMANS

Project leader: Waldemar L. Olszewski
Contributors: Hanna Gałkowska, Grzegorz Szczęsny

The cellular and humoral aspects of protracted wound healing were studied in humans with venous ulcers, diabetic foot and closed bone wounds. The question remains open which factors are responsible for the deregulation of chemotactic and cytokinemic reaction in chronic wounds. Are these cell expression of growth factors, and keratinocyte and Langerhans cell migration?

Immune events in the healing process of leg venous ulcer

The aim of studies was identification of the phenotypes of infiltrating cells and kinetics of migration of keratinocytes and Langerhans cells in the ulcer edge. The CD68-positive (macrophages) cells were evenly distributed in the dermis and in the ulcer granulation tissue. Granulocytes accumulated on the surface and at the edge of the ulcer. The CD4-positive and CD8-

positive population was found present around the dermal venules. Interestingly, no Langerhans cells could be identified in the epidermis and dermis at the ulcer border. The IL1 alpha, IL1 beta, IL1R-antagonist, IL6 and TNF alpha expression in keratinocytes was rather low, as was that of PDGF alpha, EGF and EGF-R. VEGF was found strongly expressed in migrating but not remote areas keratinocytes. In dermis, cytokines were identified mostly in the areas adjacent to the ulcer. Endothelial and infiltrating mononuclear cells contained IL1 alpha, IL1 beta, IL8 and TNF alpha. The IL1R antagonist and GM CSF were weakly expressed. The VEGF, TGF beta and EGF were present in the granulation tissue infiltrates. The obtained observations indicate that the newly formed epidermis at the edge of a venous ulcer contains only few Langerhans cells, and keratinocytes strongly express VEGF and weakly the proinflammatory cytokines. This points to immaturity of the migrating edge keratinocytes.

The response of regional lymph nodes to closed bone wound

Tissue wound healing without damage to the skin proceeds along different pathways, compared with skin, due to lack of keratinocytes and Langerhans cells. This applies in particular to the bone fractures with activation of endosteum, periosteum and bone marrow cells. Open wounds cause an immediate reaction in the regional lymph nodes. In case of closed wounds no such response has so far been documented. The aim of study was to follow changes in the regional lymphoid tissue in patients with closed injuries of lower extremities with bone fractures. Quantitative lymphoscintigraphy was applied. Dilatation of leg and thigh lymphatics and enlargement of inguinal and iliac lymph nodes was observed. Computer-assisted densitometry revealed an increase in lymphatic diameter by a factor of 4.2±5.6 and of lymph node area by 2.6±3.0. These findings prompted investigations on animals concerning factors responsible for the reaction of the limb lymphatic system. It was found that extravasated blood did not evoke any response, whereas extravasated bone marrow and infection produced changes similar to those observed in clinical conditions. *In vitro* mixed cultures of lymph node cells with bone marrow or bacterial cells revealed stimulatory properties of bone marrow and some strains of *Staphylococci*.

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TOPICAL HOST REACTION TO ALLOGRAFT AND NEOPLASM

Project leader: Waldemar L. Olszewski
Contributors: Urszula Kubicka, Sergiusz Durowicz, Robert Słotwiński

Continuous alloantigen elimination

A concept of "continuous alloantigen elimination" in organ allograft maintenance has been presented, based on studies of distribution of alloantigen and allo-anti-serum against donor lymphocyte antigens. Immediately after organ transplantation, the previously administered alloantibodies against recipient antigens bind to the graft surface and shed soluble antigens. The alloantigen-alloantibody complexes are shed from the graft surface, opsonized and eliminated by macrophages in the liver. Rapid removal of alloantigen prevents activation of recipient antigen presenting cells and lymphocytes and subsequently raising of donor-specific recipient cytotoxic lymphocytes destroying the graft. The organ transplant survives above 100 days. However, the immune reactivity against the donor is maintained as donor lymphocytes administered i.v. to the graft-bearing recipient are rejected within 6 hours. This points to a split reaction to the alloantigen. In case of organ graft, the surface antigens are bound to alloantibodies, shed and removed. The graft survives. In contrast, the transplanted lymphocytes homing to the lymphoid tissue are destroyed there immediately in the same fashion as the alloantigen-alloantibody complexes.

Phenotypes and cytokine production by peritoneal cells in human gastrointestinal cancer

The aim of study was to identify the phenotypes of free peritoneal cells, their cytoplasmic and released cytokines in patients with gastric and colon

cancer in stage T2NOMO. There was a slight increase in the peritoneal cells number in gastric cancer group from 10^6 to $2.6x10^6$. The phenotypic evaluation of this population revealed a rise in numbers of $CD2^+$ and $CD3^+HLADR^+$ and decrease in $CD19^+$, $CD3\ 71^+$, $CD11b^+$, $CD11c^+$, CD545 and $CD29^+$ subsets. In the colon cancer group the increase in free cell total number was insignificant. The dominating populations were CD14 and CD15 and activated $CD3^+DR^+$ as well as $CD19^+$, $CD8^+$ and CD45RO cells. The most expressed cytoplasmatic cytokines were IL1 alpha, IL6, IL8 and TNF alpha. The obtained results indicate that local growth of cancer not penetrating gut serosal membrane does not evoke evident peritoneal cell response. Interestingly, colon cancer was accompanied by significantly elevated CD14 and CD15 (granulocytes) population. We suggest bacterial factors penetrating the tumor infiltrated and permeable colonic wall to be responsible for attraction of the CD14 and CD15 population. A tumor specific reaction expressed by the host is rather limited.

Morphological and functional characteristics of host immune cells infiltrating liver metastatic foci of CC351 colon carcinoma

The study was devoted to defining the mononuclear population accumulating at the site of CC351 liver metastases. These cells logged in sinusoids, around and inside the tumor tissue. They belonged to the monocyte/macrophages (ED1) line, lymphocytes and NK cells. There were phenotypic similarities between these cells and cells washed out from liver sinusoids. The process of selection of specific lines of mononuclear cells takes place during adhesion to the liver endothelial cells. A low cytotoxic activity of mononuclears washed out from liver vasculature compared with normal liver points to the suppressive activity of cancer cells. It can be inferred that certain types of tumors may be antigenic enough to evoke strong local host reaction.

Immune reaction in patients with neoplastic abdominal changes to surgery

The aim of the study was to examine the changes in systemic production of IL-1β, TNFα, IL-6, IL-8, IL-10, IL-1ra and sTNF RI following major elective operative trauma in patients (*n=22*) with colorectal carcinoma.In 13 of these cases the severe postoperative complications occurred. Plasma

cytokines concentrations were measured before surgery and on the days 1, 3, 7, 10 and 14 there after, by ELISA. Preoperative plasma levels of IL-6 (10/3pg/ml p=0.001) and IL-1ra (1350/681pg/ml, p=0.02) were lower in the patients with postoperative complications. Significantly highest levels of sTNF RI were observed in the group of patients with postoperative complications on day 1(p=0.01), 10(p=0.02) and 14(p=0.01) after surgery and on day 7(p=0.04) when IL-6 was measured. Conclusion: IL-6, IL-1ra and sTNF RI concentrations changes are sensitive markers of postoperative complications in patients with colorectal carcinoma.

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SECONDARY LYMPHEDEMA IN VENOUS STASIS, TUMOR SURGERY, TRAUMA AND SKIN INFECTIONS – A ROLE FOR BACTERIA, METHODS OF PREVENTION

Supported by the State Committee for Scientific Research: grant # 4 POC 079 13

Project leader: Waldemar L. Olszewski
Contributor: Hanna Gałkowska

The study completed in year 2000 has shown that irrespective of the etiological factors responsible for development of lymphedema, the so-called "swelling" of the extremity is an agglomeration of protein and water from the exchange vessels, recirculating immune cells, proliferating keratinocytes and fibroblasts, and deposition of ground matrix. Thus, it is not only swelling but increase in cellular and extracellular mass. Lymph stasis is complicated by bacterial infections from the skin resident populations penetrating epidermis. In order to prevent recurrencies of dermatolymphangioadenitis, prophylactic administration of benzathine penicillin was found to be significantly effective.

Collaborating unit:
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POSTTRAUMATIC EDEMA OF LOWER EXTREMITIES – PATHOMECHANISM, NEW DIAGNOSTIC MEASURES, TREATMENT TRIALS

Supported by the State Committee for Scientific Research: grant # 4 PO5C 047 19

Project leader: Waldemar L. Olszewski
Contributor: Grzegorz Szczęsny

Studies were started in July 2000. The first phase was aimed at the investigations of pathomechanism of development of posttraumatic swellings after open and closed injuries of lower extremities, including bone fractures. Activation of cytokines in tissue fluid and serum, identification of cells participating in the local inflammatory process and release of TGF alpha from the damaged bones were investigated. Clinical evaluation of patients based on measuring acute phase protein levels and analysis of lymphoscintigraphic recordings was carried out. The preliminary results confirmed previously observed changes in the local lymphatic system as node enlargement and systemic inflammatory response.

DIABETIC FOOT ULCER – IMMUNOHISTOCHEMICAL ANALYSIS OF INFLAMMATION, ROLE OF BACTERIAL INFECTION, THERAPY WITH ANTIBIOTICS COMBINED WITH CYTOKINES REGULATING WOUND HEALING

Supported by the State Committee for Scientific Research: grant # 4 P05B 023 16

Project leader: Hanna Gałkowska
Contributors: Waldemar L. Olszewski, Joanna Mijal

Studies were continued on the bacterial species cultured from the ulcer surface. Around fifty percent from 30 isolates comprised Gram-positive aerobic bacteria (30% *Staphylococcus aureus*). Mixed flora was present in 65% specimens.Ulcer biopsies were characterized immunohistochemically for the

presence of infiltrating cells (granulocytes, monocytes/ macrophages, lymphocytes), growth factors and their receptors. Evaluation of specimens revealed a low number of CD4+ and CD8+ lymphocytes distributed throughout the dermis. Granulocytes (CD15+) and macrophages (CD68+) demonstrated weak expression. We observed numerous blood capillaries with high endothelium and strong expression of factor VIII. Capillaries were VEGF+, CD54+, CD62E+, but expression of VCAM1, PDGFB and PDGFR-α was weak. Expression of PDGFR-β was reduced and of IL8 R increased, compared to the control tissue. We conclude that in diabetic foot ulcer endothelial cells express molecules responsible for leukocyte extravasation, but leukocytes do not penetrate dermis. Our results suggest that low expression of chemokines as MCP1 could contribute to the low leukocyte number in diabetic foot ulcer.

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GROWTH FACTORS IN LIVER REGENERATION AFTER PARTIAL HEPATECTOMY IN PATIENTS WITH LIVER TUMORS

Supported by the State Committee for Scientific Research: grant # 4 P05C 045 14

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Resection of liver tissue initiates the release of a cascade of local growth factors that results in proliferation of all hepatic elements, leading to regeneration of the liver to its previous size. Various factors produced by tumors may act as a growth stimulators of liver cells in humans. Whether these factors are involved in the post-resection regeneration of liver in patients with metastatic liver tumors remains unknown. In our studies liver volume was measured in 25 patients undergoing partial hepatectomy for colorectal adenocarcinoma metastases (15 cases) and benign liver tumors (10 cases) by using spiral CT, before and 30 days after surgery. Immunohistochemical

examination was carried out on specimens taken 7 days after liver resection for the presence of PCNA, HGF, cMET/HGF-R, TGF, EGF-R/TGF-R, VEGF, flk-1/VEGF-R, TGF-β1, TGFβ-RI and TGFβ-RII was performed on formalin fixed sections. The regeneration rate of liver tissue was higher in patients with colorectal liver metastases (30.8±20.9%) than in patients with benign lesions (11.6±11.9%, $p<0.05$) 30 days after partial hepatectomy. It was correlated with PCNA activity but not with the expression of growth factors and their receptors in liver tissue. Expression of HGF, VEGF and TGF-β1 was detected in colorectal liver metastases specimens. The presence of HGF, VEGF and TGF-β1 in the colorectal liver metastatic cells suggests that these factors derived from microscopic residual tumors remaining in the nonresected liver fragments may influence the regeneration process of the liver in patients with metastatic colon adenocarcinoma.

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FAST HEMATOPOIETIC RECONSTITUTION AFTER VASCULARIZED BONE MARROW TRANSPLANTATION IN LETHALLY IRRADIATED RATS

Supported by the State Committee for Scientific Research : grant # 4 P05A 049 15

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Vascularized bone marrow transplantation (VBMTx) has been shown in experiment on rats as a better source for hematopoietic reconstitution of irradiated animals than bone marrow cells transplanted intravenously (BMCTx). The question arises whether the improved hematopoietic recovery after VBMTx is due to the recruitment of donor-derived stromal cells into the recipient bone cavities. Hind-limbs were transplanted orthotopically into total body irradiated (8 Gy) syngeneic sex-mismatched recipients. In the control group 8x107 BMC were injected i.v. After 10 days BM from recipient tibia was collected and BM stromal cells were obtained after 3 week culture in MEM with 12%FCS, 12% HS, L-glutamine, HC, 2ME and P/S.

Genomic DNA was isolated from BM stromal cells and PCR was performed using specific primers for rat Y chromosome (sex-determining region Y-Sry) to detect male (donor or recipient) cells in sex-mismatched BM graft recipients. DNA isolated from cultured BM stromal cells of VBMTx and BMCTx male recipients of female BM graft demonstrated host-derived Y chromosome fragment. Interestingly, in the male to female BM transplanted rats the donor DNA in BM stromal cells was found after VBMTx. No donor cells were detected in BM stromal cells isolated from female rats repopulated with male BMCTx with the applied methods. It may be suggested that VBMTx enables the donor-derived stromal cells migrating from the hind-limb graft into the host bones to support the engraftment of donor BM hematopoietic progenitors.

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INTERACTION OF OPIOIDS WITH OTHER NEUROPEPTIDE REGULATORY SYSTEMS IN PAIN SIGNAL MODULATION

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Analgesics, like other drugs, are constructed to target particular receptor(s) in particular neurostructures of the human body. Recent studies show that receptors for various analgesics are located not only in structures which regulate nociceptive signals but also in other structures connected with various functions of the organism as a whole. Activation (or inhibition) of such receptors induces side effects, such as respiratory depression, immunosupression, dependency, etc. These negative effects make the discovery of "ideal" drugs unlikely. Recently, popular "multidrug therapies" may provide some resolution to the problem of unwanted side effects. This approach is based on the idea that two drugs with different pharmacological profiles will interact positively (additive or synergic effect) on given pathways. In consequence, the need for each drug will be lowered and unwanted side effects will be less. Nevertheless, multidrug therapy provides number of complications, from simple practical problems with accuracy of daily dosage of several drugs, to pharmacological problems related to different pharmacokinetic and pharmacodynamic profiles of each drug. One of the solutions to this problem has been the development of drugs with broad spectrum of complementary actions (multitarget drugs). Following this idea, the analogues that hybridize substance P and opioid pharmacophore have been synthesized. The ESP7, a first example of such hybride peptide analogue expresses high antinociception combined with spectacular properties of reversing opioid tolerance.

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THE ROLE OF T3 NUCLEAR RECEPTORS, 9-CIS RETINOIC ACID RECEPTORS AND VITAMIN D3 RECEPTORS IN TUMORIGENESIS

Project leader: Janusz Nauman
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Cloning of the mutant thyroid hormone receptors TRα1 and TRβ1 from renal clear cell cancer (RCCC)

3 TRα1, and 7 TRβ1 mutants, all bearing mutations within ligand (T3) binding domains, were cloned from RCCC tissues by RT-PCR method. Sequencing reaction revealed that some of them had additional mutations within other domains. The specificity of the cloning and sequencing reactions was confirmed by analysis of TR receptors cloned from healthy kidneys: all of them were wild type.

Expression of 5'-deiodinase type I (5'-DI) in renal clear cell cancer (RCCC) and in healthy kidney

Northern blots of total RNA isolated from RCCC tissues and from healthy kidneys was performed with specific 5'-DI probe. It has been shown that the amount of specific mRNA was remarkably reduced in RCCC. In addition, the enzymatic activity of the deiodinase was also dramatically reduced in cancer tissues in comparison to healthy kidney.

Re-cloning of TRs into eukaryotic expression vector pcDNA3.1(+) and establishment of a transcription activation analysis system in tissue culture

To obtain an expression of recombinant TR proteins in eukaryotic cells, their open reading frames (ORF) were re-cloned into pcDNA3.1(+) expression vector under the control of CMV promoter, that is active in all eukaryotic cells. At the same time conditions for culturing HEK 293 cells (theoretically not expressing endogenous TRs) were established. It has been found that HEK293 cells in fact express endogenous TRs (as confirmed by immunoblot and gel retardation assay with HEK293 nuclear extract), but overexpression of recombinant TRs was high enough to activate reporter vector markedly above background level. Reporter vector containing Firefly luciferase (pGL2-promoter vector containing SV40 promoter, Promega) was modified by addition of enhancer – thyroid response element (TRE, a sequence recognized by TRs) in front of the promoter. Optimal conditions for transactivation activity tests were also established. The characterization of TR mutants cloned from thyroid papillary cancer (15 TRβ1 and 10 TRα1) was performed.

Expression of vitamin D receptor in RCCC

Since multiple mutations within thyroid hormone receptors were found in RCCC, we decided to analyze the expression of other hormonal receptors in this cancer. We selected vitamin D receptor (VDR) since it has been shown previously that expression of VDR can be disturbed in other cancers and active vitamin D metabolites have been already used as a supplementary treatment of other tumors. Twenty six RCCC tissues were divided according to their differentiation status: G1 (well differentiated) – 6; G2 (intermediate level of differentiation) – 10; G3 (poorly differentiated) – 8. Tissue fragments excised from the opposite poles of the same kidneys (with no signs of tumor infiltration) as well as 7 kidneys with no tumor served as two types of controls. No statistically significant differences of VDR mRNA and protein level have been found in RCCC in comparison to healthy kidney. In contrast, abnormally decreased ligand (vitamin D3) binding was found in cancer tissues in all differentiation groups in comparison to their respective controls.

The most pronounced difference was found in G2 group. The fact that the difference of D3 binding in G3 tumors and its control tissues was not even lower, can be explained by very low D3 binding in the tissues surrounding these poorly differentiated RCCCs (paracrine effect of the tumor on surrounding tissues). In addition, in 70% of analyzed cases the VDR binding to DNA was disturbed: in 52% of cases was low or almost undetectable, while in 18% it was higher than in respective control.

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EXPRESSION AND FUNCTION OF THYROID HORMONE RECEPTORS (TR) AND 9-CIS RETINOIC ACID RECEPTORS (RXR) IN THYROID CANCERS (DEPENDENT ON IRRADIATION?)

Supported by the State Committee for Scientific Research: grant # 4 P05B 041 15

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We checked the hypothesis that the function of TRs could be impaired in cancer tissues as a result of disturbed expression and/or somatic mutations. Thyroid hormone receptors (TRs) are ubiquitously distributed transcription factors involved in the regulation of cell proliferation, differentiation and apoptosis. Importantly, TRs are cellular homologs of transcriptionally inactive viral oncogene v-ErbA, indicated in neogenesis of avian erythroblastosis, some sarcomas, liver cancer and thyroid abnormalities. As a model system, we selected human thyroid papillary cancer (PTC), in which, except for RET/PTC rearrangements, none or only a low frequency of abnormalities of different tumor suppressors (e.g.: p53, retinoblastoma), or oncogenes (e.g.: erbB-2, ras, and Fhit) are observed. The expression of TRα and TRβ genes on mRNA level was analyzed by Northern blot. Overall, the amount of specific TRβ mRNA was 3.5 times lower in PTC than in healthy tissue and 3.6 times lower than in adenoma nodules (Mann-Whitney U-test, $p<0.001$). The mean amount of specific TRα mRNA in cancer tissues was 1.77 times lower

than in healthy controls and approximately 2 times lower than in adenomas (Mann-Whitney U-test, $p<0.001$). To evaluate the protein level of TRs, Western blots of nuclear extracts isolated from PTC tumors and their respective controls were performed. The mean amount of TRβ1 protein was 1.82 fold higher in tumor tissues than in healthy controls (Mann-Whitney U-test, $p<0.005$) and 1.27 fold higher than in adenomas (not significant), while mean amount of TRα1 protein was 1.36 fold higher in tumor tissues (not significant) and 1.72 fold higher than in adenomas (Mann-Whitney U-test, $p<0.005$). To look for mutations potentially altering receptor function in cancer tissues, cDNAs of TRβ1 and TRα1 receptors were cloned from 16 PTCs by the RT-PCR method and sequenced. 100% of analyzed PTCs contained mutated TRβ1 gene. In 93.75% of analyzed PTCs mutations resulted in amino acid substitutions 68.75% of PTCs had TRα1 mutated. In 62.25% PTC cases mutations resulted in amino acid substitution. To check transactivation activity of mutant TRs, reporter construct pGL2-TRE containing Firefly luciferase gene driven by SV40 promoter and diTRE enhancer, was activated by recombinant TR receptor proteins (in pcDNA 3.1(+) vector). pRL-CMV vector, containing *Renilla* luciferase gene, served as an internal control. All experiments were performed in HEK293 cultured cells. After transfection and 24 h incubation with 100 nM triiodothyronine, the activity of both luciferases was measured and results obtained for reporter Firefly luciferase activity were normalized against that for *Renilla* luciferase. It was found that two mutants containing silent mutations within their cDNAs activated transcription similarly to their respective wild type receptors. All TRβ1 and TRα1 mutants with amino acid substitutions were defective in transcription activation (2 - 50% of wild type TR activity). The dominant-negative activity (ability to inhibit action of wild type TRs) of the mutants was also assayed. All but two mutants presented such activity. In summary, we conclude that TRs, once mutated, became oncogenes that play an important role in PTC tumorigenesis. It remains to be elucidated, though, if their major role is the initiation of tumorigenesis or if they act as secondary factors, responsible for tumor progression and poor clinical outcome of the disease.

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GANGLIOSIDES OF DIFFERENTIATED THYROID CARCINOMAS AND THYROID BENIGN TUMORS: THEIR ROLE IN THE DEVELOPMENT OF IMMUNE RESPONSE, INFLUENCE OF THYREOMETABOLIC STATUS AND TREATMENT

Supported by the State Committee for Scientific Research: grant # 4 PO5B 042 15

Project leader: Jacek Kiljański
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Expression of sialyltransferases (ST) in benign and malignant thyroid tumors and in thyroid tissue derived from patient with Graves' disease

The presence of sialic acid content glycolypids and glycoproteins seems to be responsible for metastatic behavior of neoplastic cells. We have studied expression of sialyltransferase genes in benign and malignant thyroid tissue and in tissue derived from patient with Graves' disease. We have chosen two types of sialyltransferases - enzymes responsible for sialic acid transfer from citidil-5'-monophospho-N-acetylneuraminic acid to the N-terminal moiety of glycoproteins and glycolipids, namely type I and type IV sialyltransferases (ST-1 and ST-4 respectively). We used Northern blot analysis of ST-1 and ST-4 mRNA content. Interestingly, we found large, exceeding 30-fold differences in ST-1 and ST-4 gene expression between some neoplastic and normal tissue derived from the same patients and marked differences between their expression in the group of patients with Graves' disease. These differences may be explained by altered metabolism of neoplastic cells in some types of benign and malignant tumors and more interestingly, by influence of subpopulation of anti-TSH receptor autoantibodies exerting their influence on metabolic pathways in different patients.

We have also performed lectin-based immunohistochemistry with benign and malignant thyroid tumors. We used MAA (*Maackia amurensis* agglutinin) selective for NeuAca 2-3, SNA (*Sambucus nigra* agglutinin) selective

for NeuNAca 2-6, ConA (*Conavallia ensiformis* agglutinin) selective for a-Man > a-Glc > a-GlcNAc and AAA (*Aleuria aurentia* selective for Fuca 1-6 GlcNAc. SNA staining was found to be positive staining of cytoplasm in 67% of malignant thyroid tumors and only in 30% of normal tissues and 42% of Graves' thyroid. These results may reflect increased sialic acid (NeuNAca 2-3) content in malignant thyroid tumors and some patients with Graves' disease.

THE ROLE OF GENETIC FACTORS IN THE DEVELOPMENT OF GRAVES' DISEASE

Supported by the State Committee for Scientific Research: grant # 4 P05B 131 19

Project leader: Tomasz Bednarczuk
Contributors: Janusz Nauman, Rafał Płoski

The aim of this study is to determine the role of genetic factors in the development of Graves' disease. Genomic DNA was isolated from Polish patients with Graves' disease (n=180) and healthy controls (n=200). We started to investigate the frequencies of polymorphisms of 5'-flanking region of the tumor necrosis factor alpha (TNF-α) gene at positions -1,031 (T to C change, termed as -1,031C), -857 (C to T, -857T), -308 (G to A, -308A) and -238 (G to A, -238A). Preliminary results suggest that polymorphism of the 5'-flanking region of the TNF-α gene is not involved in the susceptibility to Graves' disease in Poland.

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LFA-1 AND PECAM-1 EXPRESSION IN PATIENTS WITH RELAPSING-REMITTING AND CHRONIC PROGRESSIVE MULTIPLE SCLEROSIS (MS)

Project co-ordinator: Jacek Losy

LFA-1 and PECAM-1 are both adhesion molecules - members of the immunoglobulin family - involved in transendothelial migration of leukocytes. LFA-1 is expressed on lymphocytes, monocytes and granulocytes whereas PECAM-1 is also found on endothelial cells and platelets. We present data showing that the expression of cell-bound forms of both molecules is significantly higher on monocytes of patients with chronic progressive compared to relapsing-remitting MS and controls. We also show that cell-bound form of PECAM-1 is significantly over-expressed on lymphocytes in patients with active MRI lesions as compared to those without gadolinium enhancing lesions. Our results suggest that the cell-bound form of PECAM-1 may be regarded as a marker of MS activity and confirm that relapsing-remitting and chronic progressive types of MS are different immunological entities.

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IN VIVO EFFECT OF INTERFERON BETA 1A ON INTERLEUKIN 12 (IL12) IN PATIENTS WITH MULTIPLE SCLEROSIS (MS)

Project leader: Jacek Losy
Contributor: Grażyna Michałowska-Wender

Interleukin 12 is the principal cytokine that regulates the generation of Th1 type effector cells crucial in the development of autoimmune disorders including MS. Our study showed for the first time a significant increase in the serum level of IL-12 in relapsing-remitting MS patients. IL-12 synthesis may be a target for interferon beta action in MS. This *in vivo* study showed after 3 and 6 months of treatment with interferon beta 1a, a trend towards a decrease in IL-12 serum level in MS patients.

The results are consistent with previous *in vitro* studies on INF-beta effect on IL-12.

THE EFFECT OF SHORT TERM TREATMENT WITH INTERFERON BETA 1A ON ACUTE EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE)

Project co-ordinator: Mieczysław Wender

The clinical, histological and immunocytochemical expression of some cytokines on infiltrates in the central nervous system were studied in the course of short-term therapy of acute EAE with interferon beta 1a. Beneficial effect of short-term treatment with interferon beta 1a, applied at the very onset, on the development of the experimental disease was established. The effect of INF beta 1a on EAE may be associated with inhibition of proinflammatory cytokines and stimulation of antiinflammatory ones.

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TUMOR NECROSIS FACTOR-ALPHA (TNF-ALPHA) IS INCREASED IN CEREBROSPINAL FLUID (CSF) AND SERUM OF ISCHEMIC STROKE PATIENTS WITH BRAIN INFARCTION VOLUME

Project co-ordinator: Jacek Losy

A growing body of evidence suggests the involvement of inflammatory mediators, including cytokines, in the development of ischemic brain lesions. The aim of the present study was to investigate whether TNF-alpha, the proinflammatory cytokine, contributes to early pathophysiological mechanisms leading to brain damage as a consequence of acute stroke. We studied TNF-alpha levels in CSF and serum in 23 stroke patients within the first 24 hrs after ischemic stroke, confirmed by computerized tomography (CT) of the brain. The control group consisted of 15 patients with the diagnosis of tension headache and neurasthenia. In the stroke patients, the levels of TNF-alpha both in CSF and serum were significantly higher in comparison to the control group. The positive correlation between the levels of TNF-alpha in CSF and serum of the patients studied was observed. Furthermore, the correlation between TNF-alpha levels both in CSF and serum and the brain infarction volume was shown.

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CD2, CD4 AND CD8 MARKERS AND CYTOKINES: IL-2 AND TNF-α IN PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS (ALS)

Project leader: Jacek Losy
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The cause of amyotrophic lateral sclerosis is still unknown. In this study CD2, CD4 and CD8 markers on mononuclear cells as well as levels of TNF-α and IL-2 in sera from 15 patients with ALS have been evaluated. There was a significant increase of TNF-α in sera of ALS patients in comparison to the

control group. This is a novel observation. It supports the concept that immune mechanisms may play a role in the pathogenesis of ALS.

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METABOLISM OF ADENOSINE IN DIABETES

Project leader: Leszek Kalinowski
Contributors: Mirosława Szczepańska-Konkel, Stefan Angielski

The goal of this study was to investigate changes in the activities of 5'nucleotidase (5'-NT), AMP deaminase, adenosine deaminase (ADA) and adenosine kinase (AK) in kidney, heart and liver of streptozotocin-induced diabetic rats. The activities of 5'-NT, ADA, and AMP deaminase were unchanged in cytosols of diabetic tissues. Our results show that the activity of AK was lovered by as much as 50% in diabetic kidney and by 40% in diabetic liver and heart. Decline in AK activity was associated with a lowered level of its mRNA and protein content. Based on the obtained data we suggest that the turnover of the adenosine-AMP cycle in diabetes might be impaired leading to a rise in adenosine level.

DISSOCIATION OF PARATHYROID HORMONE AND CYCLIC-3'-5' AMP EFFECTS ON NA-PI UPTAKE BY CELLS ISOLATED FROM PROXIMAL STRAIGHT TUBULES OF RAT KIDNEY

Co-ordinator: Stefan Angielski

The aim of the present work was to compare the involvement of the PHT-cAMP signalling system in phosphate transport in cells originating from PST and from PCT portions of rat kidney proximal tubules. We elaborated a method of isolation of alive cells from the same rat kidney representing the PST and PCT cells, which were pure and homogeneous enough to study the regulation of Na-dependent phosphate uptake. We found that PTH inhibits

Na-dependent phosphate uptake in both types of cells to a similar extent, but in contrast to PCT cells, the cells representing the PST segment are insensitive to the inhibitory effect of dibutyryl cAMP and a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX).

CO-OPERATION BETWEEN PARTICULATE AND SOLUBLE GUANYLYL CYCLASE SYSTEMS IN THE RAT RENAL GLOMERULI

Co-ordinator: Stefan Angielski

Atrial natriuretic peptide (ANP) and nitric oxide (NO) are crucial relaxatory factors in the cardiovascular system. In the kidney, besides modulating the blood flow they play an important role in the control of the glomerular filtration rate and tubular transport processes.

ANP and NO act *via* different receptors, although inducing the common intracellular messenger – cyclic GMP. However, interaction betwen both factors remains unclear. Our observations suggest that in the kidney glomeruli, activities of the ANP- and NO-dependent guanylyl cyclase systems may be mutually compensated. To check this, we have tested the effects of ANP and sodium nitroprusside (SNP) on cGMP synthesis and relaxation of glomeruli contracted with angiotensin II. Our results strongly support the hypothesis that both ANP- and NO-dependent systems co-operate in regulating the function of kidney glomeruli.

Collaborating unit:

Medical School, Gdańsk, Poland (A. Hoppe. B. Lewko, R. Kowara, A. Matecki, A. Rybczyńska, J. Stępiński)

Publications:

Dobrucki LW, Kalinowski L, Uracz W, Maliński T: The protective role of nitric oxide in the brain ischemia. *J Physiol Pharmacol* 2000, 51, 695-703.

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Doctors theses

Sergiusz Durowicz:
Liver sinusoidal leukocytes reacting to tumor metastases.

Sława Janczewska:
The mechanism of fast repopulation of bone marrow after vascularized bone marrow transplantation.

Iwona Maszczyńska:
Central, analgesic effect of the peptide AA501, opioid agonist and neurokinin antagonist.

Anna Pfeffer-Baczuk:
Apolipoprotein E genotype and the rate of decline in Alzheimer's disease.

Andrzej Kochański:
Analysis of mutations in Charcot-Marie-Tooth disease.

Organisation of symposia and conferences

Educational Conference **"Alzheimer's disease, dementia and Parkinson's disease"**, Warsaw, May 19, 2000, organized by MRC PASci, Polish Alzheimer's Section of Polish Neurological Association, and Neurological Clinic, Central Clinical Hospital, Ministry of Internal Affairs.

Conference **"Inlflammatory aspects of lower limb atherosclerosis"**, Warsaw, October 25, 2000.

VIth Neurosurgical Meeting **"Advances in hydrocephalus treatment"**, Pułtusk, November 16-18, 2000, organized by Department of Neurosurgery MRC PASci. Topics: "Monitoring in neurosurgery", "Encephalomeningocele". 110 participants including 6 foreign quests. 30 oral presentations including 1 presented by the worker of the Department of Neurosurgery MRC PASci.

Conference **"The role of the lymphatic system in healing traumatic changes in limbs"**, Warsaw, November 29, 2000.

Polish-German Symposium **"Experimental and clinical pathophysiology"**, Warsaw, December 8-9, 2000, organized by MRC PASci and Alexander von Humboldt Foundation. Organizers: Prof. Dr. J. Strosznajder and Assoc. Prof. Dr. E. Koźniewska.

Conference **"Immune factors in tissue fluid and lymph"**, Warsaw, December 13, 2000.

Vth Domestic Neurochemical Conference **"Molecular basis for pathology and therapy of neurological diseases"**, Warsaw, December 14, 2000.

International Symposium **"Molecular mechanisms of neurodegeneration"**, Warsaw, December 15, 2000.

Conference **"Immune response tu gastrointestinal tumor"**. Warsaw, December 19, 2000.