

## ATPases Activity, Lipids and Heat Shock Protein 70 kDa in Spinal Cord in Rats with Experimental Neoplastic Disease

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**Abstract:** Pathology of spinal cord belongs to neurological syndromes observed in the course of the cancer. Due to clinical uncertainties the underlying mechanisms still await elucidation. The aim of this study, involved analysis of lipid pattern, ATPase activities and expression of heat shock protein 70 kDa in spinal cord of tumor bearing rats. The histological abnormalities were also investigated in spinal cords of the rats. Under light microscopy shrinkage of neurones was evident in the anterior and lateral horns of the spinal cord, in its thoracic region. The abnormalities corresponded to morphological changes found in paraneoplastic myelopathy. In biochemical studies a decrease in cholesterol esters, phosphatidylcholine and phosphatidylinositol content were detected in spinal cord of experimental animals. The qualitative abnormalities were expressed also by decreased molar ratios of gangliosides to phospholipids, of cholesterol to galactolipids as well as of cholesterol to phospholipids and an increased ratio of gangliosides to phospholipids. Analysis of lipids peroxidation revealed lowered content of conjugated dienes and of malonyldialdehyde in spinal cord. Content of sulfhydryl groups remained unchanged. Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase activities and expression of heat shock protein 70 were increased. Our data suggested that lipids pattern, ATPase activities and heat shock protein expression in the spinal cord, manifested in the tumor-bearing Buffalo rats, may promote understanding of underlying molecular mechanisms responsible for cancer-associated myelopathy.

**Key words:** Paraneoplastic myelopathy, spinal cord, morris hepatoma, lipids, ATP-ases, heat shock protein

### INTRODUCTION

Paraneoplastic myelopathy, the pathology of spinal cord observed among a variety of neurological manifestations, develops in the course of neoplastic disease and is not related to metastases or infiltrations of the nervous system. In general, the remote effects of cancer when studied in 60 000 patients have shown a low incidence (0.9%) (Pittock *et al.*, 2006) of onconeural antibodies. However, clinical criteria for the diagnosis of neurological paraneoplastic syndromes have not been published until recently (Graus *et al.*, 2004) so not all the previous studies satisfy them. If identified, the antibodies present in patients with paraneoplastic myelopathy are known as anti-Ri or directed against amphiphysin. The clinical observations and follow-up of patients with subacute necrotising myelopathy show that it may precede the diagnosis of cancer (prostate cancer,

lung cancer (Rudnicki and Dalmau, 2000; Ojeda *et al.*, 1984), myeloma (Storey and McKelvie, 1991), but in some cases it develops after the diagnosis of the neoplasm. The clinical signs, which may mimic acute transverse myelitis or spinal cord compression, involve sudden onset, progressing flaccid paraplegia, sphincter disturbances, deep sensation abnormalities and respiratory insufficiency. The spinal cord is damaged particularly in thoracic and lumbar region. Histologic examination of patients presenting subacute necrotic myelopathy reveals nonspecific necrosis, Wallerian degeneration, gliosis and focal meningeal thickening. Inflammatory reaction, if present, is minimal (Vernon, 1974).

Until now, the pathomechanism responsible for development of neurological paraneoplastic syndromes is believed to be immune-mediated.

Despite advanced immunological studies revealing antibodies, which react with structures of the nervous

system, the pathomechanism of neurological paraneoplastic syndromes remains incompletely understood. Recent studies (Pittock *et al.*, 2006) have addressed the onconeural antibodies as coexisting and predicting cancer, rather than inducing the neurological syndrome. Also, immunosuppressive treatment (Keime-Guilbert *et al.*, 1999) as well as intravenous immunoglobulins (Keime-Guilbert *et al.*, 2000) exert no clinical effect, indicating that a different, probably metabolic, pathomechanism is involved. This possibility has been suggested by the clinical observation of coexisting myasthenic Lambert-Eaton syndrome and subacute paraneoplastic degeneration. Grauss (2002) noticed an improvement of myasthenic symptoms as a result of an immunomodulating treatment while the symptoms of coexisting subacute paraneoplastic degeneration persisted. Clinical studies using immunosuppression combined with immunomodulating treatment showed no improvement in the clinical outcome (Gultekin *et al.*, 2000). These clinical observations raise questions as to the mechanisms involved in etiology of neurological paraneoplastic syndromes.

Screening potential experimental approaches to the problem, we have decided to examine the effect of advanced experimental neoplastic disease on lipids pattern in the spinal cord. Three weeks after tumor inoculation in Buffalo rats strain we observed neuropathological abnormalities in the spinal cord which corresponded to morphological changes in paraneoplastic syndromes. Basing on this observation we undertook the biochemical studies.

In contemporary literature of the subject no data have been identified which would concern lipids, the main constituents of structural and functional significance in the spinal cord, in the course of neoplastic disease. Spinal cord belongs to the most heavily myelinated regions of central nervous system and for this reason lipid pattern is particularly significant for its physiology and pathology. Several data indicate the role of lipids in spinal cord disorders. Ischemia produced degradation of phospholipids and was followed by their resynthesis, which did not include phosphatidylinositol and was most pronounced in the dorsal horns and in the white matter of the spinal cord (Lukacova *et al.*, 1998 a). Ischemia-reperfusion studies (Lukacova *et al.*, 1998 b) revealed improved levels of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol after resumption of the circulation. In the course of experimental allergic encephalomyelitis demyelination in the spinal cord was associated with depletion of phospholipids, cerebroside and cholesterol, while cholesterol esters tended to accumulate (Taranova,

1985). Gangliosides, on the other hand, remained unchanged. Plasmalogens, which are recognized to represent potential antioxidants, were found to be decreased following spinal cord trauma due to stimulation of plasmalogen-selective phospholipase A<sub>2</sub> (Farooqui and Horrocks, 2001).

Hydrolysis of the spinal cord lipids seemed to be related to a decreased Na<sup>+</sup>/K<sup>+</sup>-ATPase activity following trauma (Clendenon *et al.*, 1978; Hall and Braughler, 1982). This crucial membrane enzyme is highly affected by lipid pattern changes and/or lipid peroxidation. ATPase activity is manifested by the N-terminal portion of heat shock protein 70 kDa, representing a cellular chaperone expressed in spinal cord in ischemia (Matsumoto *et al.*, 2001), hypothermia (Motoyoshi *et al.*, 2001), experimental autoimmune encephalomyelitis (Aquino *et al.*, 1993) or neurodegeneration (Liu *et al.*, 2005).

Following those previous observations and experimental data we undertook this study to test the hypothesis if abnormalities in lipid pattern, ATPase activities and expression of heat shock protein 70kDa in spinal cords of rats bearing transplantable Morris hepatoma 5123 are involved in pathomechanisms of malignancy associated myelopathy.

The transplantable Morris hepatoma 5123 used in this study originated from the cell line obtained after the treatment of Buffalo-strain rats with N-(2-fluorenyl)-phtalamic acid. The observations made by Morris *et al.* (1960) and other authors revealed lung metastases 6 weeks after tumor inoculation. Microscopically, the tumor present morphological features of an hepatocellular carcinoma (Morris *et al.*, 1960). This experimental neoplastic disease was previously used in studies of the effect of cancer on muscle metabolism and erythrocytes (Michalak, 1996; Torlinski *et al.*, 1991).

We used transplantable Morris hepatoma bearing rats, because no data are available on immune-mediated paraneoplastic syndromes involving spinal cord in the course of this type of neoplasm. No data indicate also the presence of onconeural antibodies in the course of the tumor and, thus, we hoped to avoid possible immunologically mediated mechanisms.

The study was conducted in Department of Neurochemistry and Neuropathology at Poznan University of Medical Sciences (Poland) in a period of 2006-2007.

## MATERIALS AND METHODS

**Experimental animals:** Adult, male Buffalo-strain rats, 3½ months of age (300-350g) were used for our experiments. The homogenate of Morris hepatoma 5123 was inoculated

intramuscularly (0.5 mL) into the left hind limbs of the animals. Twenty one days after the tumour transplantation rats were sacrificed using halothane anesthesia and perfused with a 4% neutral formalin solution. At that time the animals presented clinical signs of cachexia and their body mass was reduced by nearly 30%. Previous observations on the model demonstrated that at 21<sup>st</sup> day there were no metastases, the first of which appeared in lungs after 6 weeks. The spinal cords were removed and fixed by immersion in neutral formalin. The tumor was dissected and used for morphological examination. Hematoxylin-eosin, Nissl and Klüver-Barrera staining of the spinal cord was performed. Ten control Buffalo rats and ten Morris hepatoma bearing males were used for the experiment.

The lyophilized samples of spinal cord were used for lipid analysis while fresh tissue was tested for enzymes activity and lipid peroxidation.

**Microscopical examination:** A light microscope examination was performed using the JENAVAL (Carl Zeiss, Jena) instrument, a color Video Camera (CCD, Sony) and, for the image saving, MultiScan software (Computer Scanning System 2, Poland). The quantitative examination was performed using ImageJ v1.32 software (NIH, <http://rsb.info.nih.gov/ij/>). The results on cell density were tested with Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilk tests for normality and, then, significance of the respective differences was tested using the non-parametric Mann-Whitney test.

**The activities of ATP-ases:** Activities of Na<sup>+</sup>/K<sup>+</sup>-ATPase, Ca<sup>2+</sup>-ATPase and Mg<sup>2+</sup>-ATPase were estimated by means of a modified method described by Samson and Quinn (1967), using adequate concentrations of sodium, potassium, magnesium and calcium ions and ouabain inhibition. The activity was estimated basing on the amount of released inorganic phosphorus, using the technique of Bartlett (1959) and expressed in  $\mu$ moles per minute per miligram of protein.

**Lipids:** The spinal cord lipids were extracted from spinal cord using Folch-Pi method (Folch-Pi *et al.*, 1957) with chlorophorm-methanol (2:1, vol vol<sup>-1</sup>). The extract was washed with 0.05 N NaCl and left for 24 h. After this time the upper (methanol-aqueous) phase was used for ganglioside analysis, the lower one (chlorophorm-methanol) for column chromatography of the lipids. Three main classes of lipids: Neutral lipids, glycerophospholipids and galactolipids were eluted from Florisil column (100-200 mesh, FLUKA). Neutral lipids were eluted with hexane-ethyl ether (8:2, vol vol<sup>-1</sup>)

according to Kishimoto *et al.* (1965) and galactolipids with chlorophorm-methanol (2:1, vol vol<sup>-1</sup>) according to Svennerholm (1964). Thin-layer chromatography was carried out according to Svennerholm (1964) and Sperry and Webb (1950) method was used for quantitative analysis of cholesterol and cholesterol esters. Following thin-layer chromatography according to Svennerholm (1964), galactolipids were analyzed qualitatively using orcinol technique of Svennerholm (1956). Glycerophospholipids were separated using two-dimensional thin-layer chromatography according to Singh *et al.* (1971) and were quantitated basing on phosphorus estimation by the Bartlett (1959) technique.

High-Performance Thin-Layer Chromatography (HPTLC) (Silica Gel 60, 0.2 mm, Merck) according to the technique of Yu and Ando (1980) and the resorcinol technique of Svennerholm (1957) was used for ganglioside analysis.

The standards used for all the analysed lipids originated from SIGMA.

**Lipid peroxidation:** Lipid peroxidation was estimated with the use of Recknagel and Glende (1984) procedure for conjugated dienes and Ohkawa technique (Morris *et al.*, 1960) for malonyldialdehyde content. The content of each of them was expressed per miligram of protein, estimated according to Lowry (1951). Sulfhydryl groups content was analyzed basing on spectrophotometric technique with 5,5'-Dithiobis-2-Nitrobenzoic Acid (DTNB) according to Ellman (1959) and expressed per miligram of protein.

**Heat shock protein 70 kDa:** Expression of heat shock protein 70 kDa (Hsp70) in spinal cord was estimated using 12% Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli (1970). Each sample contained the same amount of protein (45  $\mu$ g) estimated using the technique of Lowry *et al.* (1951). One of the obtained gels was stained with 0.025 % solution of Coomassie blue in 25% isopropanol in 10% glacial acetate acid. The second gel was used for transfer of the proteins onto nitrocellulose membrane (SIGMA) by means of Western-blotting (Towbin *et al.*, 1979). Hsp 70 was identified with mouse anti-rat-Hsp70 (SIGMA) antibodies and secondary, peroxidase conjugated goat anti-mouse IgG's (SIGMA). The semiquantitative analysis was performed using GS-710 densitometer (Bio-Rad) and the "Quantity One" software. Density of bands corresponding to Hsp 70 was normalized to relating it to density of actin bands. The molecular standard produced by SIGMA was used. The expression of Hsp 70 in the

spinal cord of Morris hepatoma bearing rats was expressed as percentage of controls.

**Statistics:** Statistical analyses were performed using Statistica 5.0 (StatSoft Inc.) software. Distribution of results was tested with Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilk tests for normality and respective differences were tested for significance using the non-parametric Mann-Whitney test.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

### RESULTS

After 21 days following Morris hepatoma 5123 transplantation the body mass of the experimental animals, after tumor mass correction, was  $67 \pm 7\%$  ( $x \pm SD$ ) of the baseline. Tumor bearing animals were cachectic and presented reduced movement activity, paresis of limbs and balance disturbances. Microscopical

examination of the transplantable tumour revealed an active neoplastic process with numerous atypical cells and necrosis (Fig. 1 and 2). Spinal cord examination showed predominant abnormalities in the thoracic region. Hematoxylin-eosin and Klüver-Barrera staining

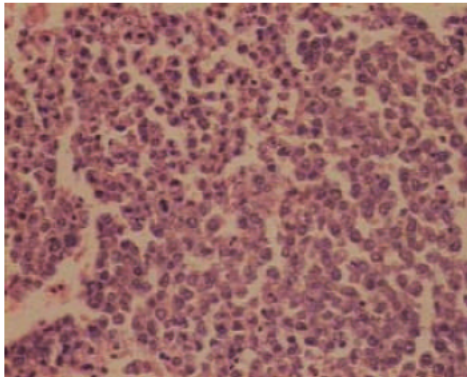


Fig. 1: Transplantable Morris hepatoma (H+E) with numerous atypical cells

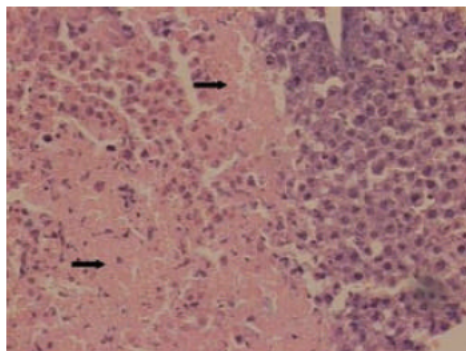


Fig. 2: Transplantable Morris hepatoma (H+E). → necrosis within the tumor

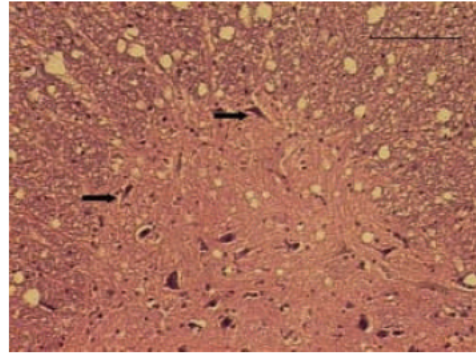


Fig. 3: Spinal cord of Morris hepatoma bearing rats, thoracic region (H+E), anterior horn. → shrinkage of neurons. Scale bar-100  $\mu$ m

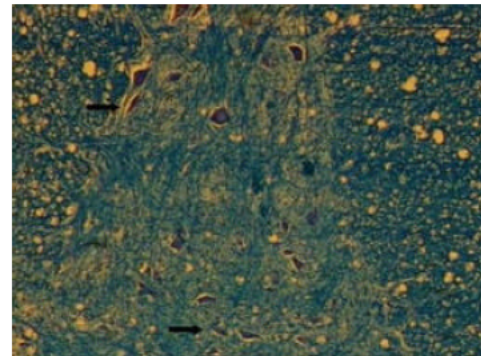


Fig. 4: Spinal cord of Morris hepatoma bearing rats, thoracic region (Klüver-Barrera staining), → shrinkage of neurons in the anterior horn. Scale bar-100  $\mu$ m

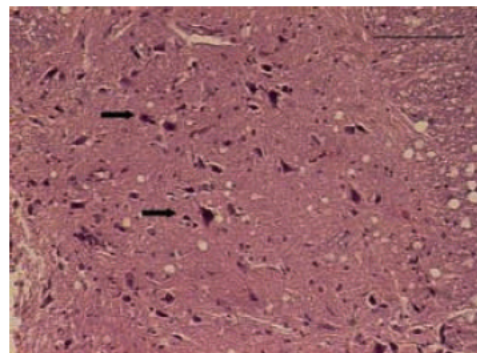


Fig. 5: Spinal cord of Morris hepatoma bearing rats, thoracic region (H+E staining), → shrinkage of neurons in the lateral horn. Scale bar-100  $\mu$ m

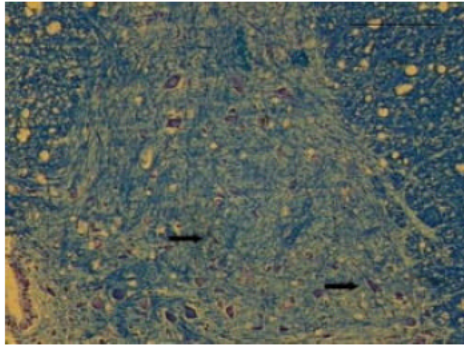


Fig. 6: Spinal cord of Morris hepatoma bearing rats, thoracic region (Klüver-Barrera staining), →-shrinkage of neurons in the lateral horn. Scale bar-100 µm

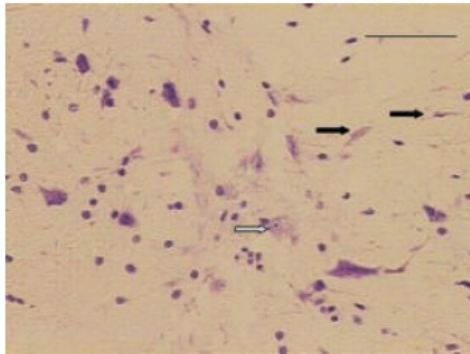


Fig. 7: Spinal cord of Morris hepatoma bearing rats, thoracic region (Nissl staining) →-edema of neurons, →-shrinkage of neurons. Scale bar-100 µm

revealed shrinkage of neurons in the anterior (Fig. 3 and 4) and lateral horn (Fig. 5 and 6), whereas Nissl staining (Fig. 7) showed an edema of the cells and shrinkage of some of them. The results on cell density showed a significant ( $p < 0.05$ ) reduction ( $35 \pm 9 \%$ ) in tumour bearing animals, when compared to the controls.

**Lipids:** In Morris hepatoma bearing rats the cholesterol esters content was lowered in spinal cord ( $p < 0.05$ ). Also the levels of phosphatidylcholine and phosphatidylinositol were decreased significantly ( $p < 0.05$ ) (Table 1). Galactolipids and gangliosides remained, however, unchanged (Table 1 and 2).

**Molar ratios:** The calculated molar ratios showed two parallel tendencies: on the one hand Cholesterol to Galactolipid (Ch / GL), cholesterol to glycerophospholipid (Ch / PL) and ganglioside to glycerophospholipid (G/PL)

Table 1: The content of neutral lipids, galactolipids and glycerophospholipids in spinal cord of Morris hepatoma 5123 bearing rats and controls (mmol 100 g<sup>-1</sup> dry tissue)

	Control	Morris hepatoma bearing rats
Neutral lipids:		
Cholesterol	27.01±4.59	24.38±6.30
Cholesterol esters	0.78±0.42	0.55±0.11*
Galactolipids		
Cerebrosides	7.07±3.65	9.70±1.83
Sulfatides	2.54±1.01	2.69±0.96
Glycerophospholipids		
Sphingomyelin	1.83±0.43	2.29±0.48
Phosphatidylcholine	6.80±0.96	5.38±1.07*
Lysophosphatidylcholine	0.39±0.08	0.39±0.11
Phosphatidylserine	2.25±0.94	3.01±0.62
Phosphatidylinositol	0.96±0.28	0.61±0.25*
Phosphatidylethanolamine	7.54±4.03	9.11±1.69
Phosphatidylcholine plasmalogen	2.58±0.40	2.96±0.64
Phosphatidylethanolamine plasmalogen	10.09± 1.18	8.50±2.48

Mean±SD. Number of animals = 10, \* difference between the control and the tumor-bearing rats significant at  $p < 0.05$

Table 2: The content of gangliosides in spinal cord in Morris hepatoma 5123 bearing rats and controls (mmol 100 g<sup>-1</sup> dry tissue)

	Controls	Morris hepatoma bearing rats
GM <sub>3</sub>	0.82±0.19	0.77±0.23
GM <sub>2</sub>	0.10±0.02	0.09±0.02
GM <sub>1</sub>	0.05±0.01	0.04±0.01
GD <sub>3</sub>	12.19±2.51	11.34±1.70
GD <sub>1a</sub>	6.76±1.14	6.19±0.98
GD <sub>2</sub>	4.50±0.80	4.20±0.60
GD <sub>1b</sub>	0.08±0.03	0.07±0.02
GT <sub>1b</sub>	1.86±0.09	1.66±0.14
GG <sub>1b</sub>	0.05±0.02	0.03±0.02

Mean±SD. Number of animals = 10, No significant differences

Table 3: Molar ratios in spinal cord in Morris hepatoma 5123 bearing rats and controls

	Controls	Morris hepatoma bearing rats
Ch:GL	2.81	1.97
Ch:PL	0.84	0.75
GL:PL	0.31	0.38
G : PL	0.84	0.75

Ch-Cholesterol, GL-Galactolipids, PL-Phospholipids, G-Gangliosides, Number of animals = 10

ratios were decreased while, on the other, galactolipid to phospholipid (GL/PL) ratio was increased in spinal cord (Table 3).

**Lipids peroxidation:** Our studies showed lowered content of conjugated dienes in spinal cord ( $p < 0.05$ ) in Morris hepatoma bearing rats and also the malonyldialdehyde level was decreased ( $p < 0.0001$ ). The level of sulfhydryl groups remained unchanged (Table 4).

**ATPases:** A modest increase ( $p < 0.0001$ ) was noted in Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase activities in rats with the experimental neoplastic disease, but activity

Table 4: The content of conjugated dienes, Malonyldialdehyde (MDA) and sulfhydryl groups in spinal cord in Morris hepatoma 5123 bearing rats and controls ( $\mu\text{mol mg}^{-1}$  protein)

	Controls	Morris hepatoma bearing rats
Conjugated diens	2.42±1.14	0.96±0.6 <sup>*</sup>
MDA	2.92 (2.74 ÷ 3.21)	0.63 <sup>xx</sup> (0.36 ÷ 0.97)
Sulfhydryl groups	48.0±10.76	48.73±6.96

Mean±SD for conjugated dienes and sulfhydryl groups, median and upper and lower quartile (25 ÷ 75) for MDA, Number of animals = 10, Difference between the control and the tumor-bearing rats significant at \* p<0.05, xx p<0.0001

Table 5: ATP-ases activities in spinal cord in Morris hepatoma 5123 bearing rats and controls ( $\mu\text{mol P/min/mg protein}$ )

	Controls	Morris hepatoma bearing rats
Na <sup>+</sup> /K <sup>+</sup> -ATP-ase	66.38 ±12.9	452.0±36.3 <sup>xx</sup>
Ca <sup>2+</sup> -ATP-ase	87.25±10.5	376.6±53.6 <sup>xx</sup>
Mg <sup>2+</sup> -ATP-ase	118.9±19.1	137.7±68.7

Mean±SD. Number of animals = 10, xx difference between the control and the tumor-bearing rats significant at p<0.0001. Titles and legends to figures

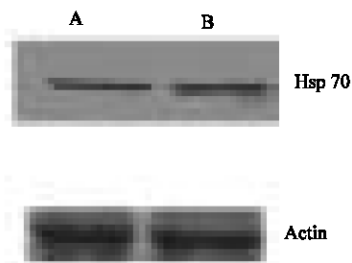


Fig. 8: Western blotting of heat shock protein 70 kDa in tumor bearing rats (B) and control (A)

of Mg<sup>2+</sup>-ATPase showed no significant changes when compared to controls (Table 5).

**Heat shock protein 70 kDa:** Expression of heat shock protein 70 kDa in spinal cords of Morris hepatoma bearing rats reached 130±9% of the control value (p<0.05) (Fig. 8).

## DISCUSSION

Even if rare, the paraneoplastic myelopathy remains to represent an obscure complication of cancer and a clinical need can be noted for studies attempting to clarify its pathomechanism. The till now conducted studies on neurological paraneoplastic syndromes have been focused on the immunological pathomechanism affecting brain and peripheral nerves. The experimental attention limited to immunology might represent, according to Darnell and Posner (2003) the reason for the inability to elucidate the background of those neurological paraneoplastic syndromes that are not associated with immune reactions.

No data are available on antibodies reactive with the onconeurological antigens in hepatocarcinoma in humans.

For this reason, the transplantable Morris hepatoma 5123 in the rats was expected to be useful for studies on non-immune-mediated neurological paraneoplastic syndromes. It is noteworthy, that we have noticed no lymphocytic infiltrations in the brain and spinal cord in the Morris hepatoma bearing rats. Therefore, an immune reaction could have been concluded to be less probable in present study. The shrinkage of neurons in the spinal cord of Morris hepatoma bearing rats has been comparable to morphological data seen in humans with neurological paraneoplastic syndromes.

Potential explanation of our findings might involve interaction of neuropathological abnormalities and cytokines, secreted during the growth of tumors. Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) causes oxidative damage to oligodendrocytes by increasing the products of lipid peroxidation and generation of radical oxygen species (Cammer, 2002). The cytotoxic effect of lymphotoxin and, to a lesser extent, TNF- $\alpha$  was shown to be associated in cultured oligodendrocytes with early retraction of cell processes, depolymerization of F-actin and subsequent nuclear degeneration (Selmaj *et al.*, 1991). When injected into spinal cord of experimental animals, Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein 1 $\alpha$  (MIP 1 $\alpha$ ) and interleukin 1 $\beta$  (IL-1 $\beta$ ) lead to expression of chemokines and cytokines and to activation of macrophages and microglia (Perrin *et al.*, 2005). On the other hand, TNF- $\alpha$  stimulates the activity and expression of manganese superoxide dismutase, a scavenger enzyme, via induction of transcription factor NF $\kappa$ B in the spinal cord (Yune *et al.*, 2004). We have found (Michalak *et al.*, 2006) that in Morris hepatoma bearing Buffalo rats serum concentration of TNF- $\alpha$  is decreased and associated with increased level of Monocyte Chemoattractant Protein-1 (MCP-1). Such milieu of factors may create conditions predisposing for the development of myelopathy in the course of neoplastic disease.

The spinal cord ependyma reactions remain under control of factors like epidermal growth factor (O'Hara and Chernoff, 1994) and fibroblast growth factor 2 (Kojima and Tator 2002) which have been assessed as crucial. Ependymal cells themselves may synthesise tumor necrosis factor- $\alpha$  (Liu *et al.*, 1996) and interleukin-1 (Hagan *et al.*, 1996). On the other hand, a peripheral administration of lipopolysaccharide induced in the ependyma the inhibitory factor  $\kappa$ B, which is involved in the regulation of gene expression (Quan *et al.*, 1997).

Our interest, however, was focused on lipid studies in the course of experimental neoplastic disease in the rat. Even if no such studies could have been identified in contemporary literature, some analogies may be found in studies of peripheral tissues.

The lymphocytes originating from tumor bearing rats showed (Zakaryan *et al.*, 2001) decreased phosphatidylinositol and phosphatidylcholine levels and increase in lysophosphatidylcholine level at all stages of maturation. In thymocytes and mature lymphocytes an increased content of sphingomyelin was noticed, while phosphatidylserine was increased in immature cells but reduced in mature ones. Decreased phosphatidylethanolamine was observed only in mature cells and increased cardiolipin level was seen in thymocytes. The authors suggested the increase in phospholipase A<sub>2</sub> activity as an explanation for decrease in phosphatidylcholine and increase in lysophosphatidylcholine, while increased activity of phospholipase C was suggested to be responsible for decrease in phosphatidylinositol and phosphatidylcholine levels. Further, erythrocytes of women with breast cancer (Kaczmarek *et al.*, 2002) manifested decreased phosphatidylserine, sphingomyelin and phosphatidylinositol contents, with simultaneous increase in phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate contents. The decrease in phospholipase C activity in erythrocytes membrane accompanied an abnormal lipid pattern.

Because of the intimate interconnections of lipid metabolism pathways, many of the enzymes that regulate bioactive lipids levels also function as switches by regulating the levels of bioactive substrates and products. We may, thus, suggest that an increased phospholipase A<sub>2</sub> activity and a decreased phospholipase C activity have been responsible for the decreased contents of phosphatidylcholine and phosphatidylinositol in our study on spinal cords of Morris hepatoma bearing rats, respectively.

Batko *et al.* (1992) showed reduction in cholesterol content and cholesterol: Phospholipids molar ratio in erythrocyte membranes in rats bearing Morris hepatoma 5123. These findings are in concordance with that of ours, i.e., with the lowered molar ratios of gangliosides: Phospholipids in all structures and lowered cholesterol: Galactolipid and cholesterol:Phospholipid ratios. The changes observed in molar ratios indicate the possibility of exchange of lipids between membranes in the central nervous system in course of neoplastic disease. The changes in cholesterol ester content in central nervous system were observed in variety of disorders, with subcellular fraction of multiple sclerosis plaque, cerebral infarction, oligodendroglioma and acoustic neurinoma showing its increase (Ramsey and Davison, 1974). In our experiments the content of cholesterol esters in spinal cords has been decreased. Because the only substrates

required for cholesterol ester generation are free fatty acids and cholesterol we may suppose that at the normal cholesterol level the free fatty acids depletion has been responsible for our findings. Lecithin: Cholesterol acyltransferase was suggested as the operating enzyme in cholesterol estrification in the central nervous system (Eto and Suzuki, 1973). Another possible target might involve acyl-CoA: Cholesterol Acyltransferase (ACAT), the activity of which was also detected in the brain (Puglielli *et al.*, 2001). Cytokines regulate the activity of the latter in peripheral tissues, with TNF (Chatterjee, 1994), interleukin 1 $\beta$  (Maziere *et al.*, 1996) and interferon  $\gamma$  (Yang *et al.*, 2001) causing its stimulation. Decreased serum TNF concentration found in our previous study or depletion of fatty acids may be considered to be involved in lowering the level of cholesterol esters in tumor bearing rats. Free fatty acids may origin from the activity of phospholipase A<sub>2</sub> or Lipoprotein Lipase (LPL). Activity of the latter has been shown to be most pronounced in spinal cord when compared to other central nervous system structures (Bessesen *et al.*, 1993). Hydrolysis of exogenous triacylglycerides mediated by LPL is recognized as the main source of free fatty acids for the Schwann cells and myelin biosynthesis in the peripheral nervous system (Huey *et al.*, 1998). Inhibition of LPL activity reduces the incorporation of <sup>14</sup>C into diacylglycerol and cholesterol esters in Schwann cells (Huey *et al.*, 1998).

The indicators of lipid peroxidation are significantly lowered in spinal cords of Morris hepatoma bearing rats and may be associated with the mentioned above suggestion of free fatty acids depletion. In our study, conjugated diene and malonyldehyde contents have been reduced in Morris hepatoma bearing rats as a result of limited availability of substrates for peroxidation. This is in concordance with the unchanged sulhydryl groups content in most structures which indicates an unchanged antioxidant status.

The changes in molar ratios of cholesterol to galactolipids, cholesterol to glycerophospholipids, galactolipids to glycerophospholipids and gangliosides to glycerophospholipids indicate the abnormal lipid pattern in biomembranes, which may affect their integrity as well as activity of critical membrane enzymes, including ATPases.

Our study has revealed high activities of Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase in spinal cords in the course of experimental neoplastic disease in the rats. Long chain fatty acids inhibit Na<sup>+</sup>/K<sup>+</sup>-ATPase activity, so the depletion of these compounds may lead to an increased enzyme activity (Ahmed and Thomas, 1971). Stimulation

of ATPase activities was observed in spinal cord disorders including lesions of the myelin sheaths provoked by the spinal fluid exchange (Wender *et al.*, 1977) and calomel intoxication (Kozik and Wygladalska, 1977). Such observations were explained by high enzyme activities in oligo-and astroglia of the spinal cord.

Heat shock protein 70 kDa (Hsp 70), recognized as a "molecular chaperone", is highly expressed in the spinal cord in the course of ischemia and is believed to protect motor neurons (Sakurai *et al.*, 1998). Spinal cord contusion results in co-induction of Hsp70 and heme oxygenase-1 in glia and macrophages, which is believed to exert a protective effect (Mautes and Noble, 2000). The amelioration of the disturbed motor function in the transgenic mouse model of spinal and bulbar muscular atrophy was demonstrated in result of Hsp70 overexpression (Adachi *et al.*, 2003). High expression of Hsp 70 in our study may be regarded as a represent of protective response against remote effects of cancer on spinal cord. Even if Hsp70 exhibits ATPase activity, linked to its N-terminal portion, in our experiment the chances seem low that the chaperone participates significantly in induction of high activities of enzymes in spinal cord.

### CONCLUSION

Our data suggest that lipids of the spinal cord, ATPase activities and Hsp 70 over-expression may be relevant to the paraneoplastic syndromes exhibited by the tumor-bearing Buffalo rats. As an original finding, lipid changes deserve further examination in view of their potential usefulness in diagnosis of patients with paraneoplastic myelopathy by means of magnetic resonance spectroscopy. In this study, we have shown the unique role played by lipids, ATP-ases and Hsp 70 in indirect effects of cancer on spinal cord.

### REFERENCES

- Adachi, H., M. Katsuno, M. Minamiyama, Ch. Sang. G. Pagoulatos, Ch. Angelidis, M. Kusakabe, A. Yoshiki, Y. Kobayashi, M. Doyu and G. Sobue, 2003. Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. *J. Neurosci.*, 23: 2203-2211.
- Ahmed, K. and B.S. Thomas, 1971. Cunningham E. The effects of long chain fatty acids on sodium plus potassium ion-stimulated adenosine triphosphatase of rat brain. *J. Biol. Chem.*, 246: 103-109.
- Aquino, D.A., A.A. Klippel, C.F. Brosnan and W.T. Norton, 1993. The 70kDa Heat Shock Cognate protein (HSC70) is a major constituent of the central nervous system and is up-regulated only at the mRNA level in acute experimental autoimmune encephalomyelitis. *J. Neurochem.*, 61: 1340-1348.
- Bartlett, G., 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.*, 234: 466-468.
- Batko J., B. Plotast-Neèas, T. Warchol and H. Karon, 1992. The effect of an experimental neoplastic disease on the flux of sodium and potassium ions cross red blond cells and on the lipid composition of their membranes. *Acta. Biochim. Pol.*, 39: 317-326.
- Bessesen, D.H., C.L. Richards, J. Etienne, J.W. Goers and R.H. Eckel, 1993. Spinal cord of the rat contains more lipoprotein lipase than other brain regions. *J. Lip. Res.*, 34: 229-238.
- Cammer, W., 2002. Protection of cultured oligodendrocytes against tumor necrosis factor- $\alpha$  by the antioxidants coenzyme Q10 and N-acetyl cysteine. *Brain Res. Bull.*, 58: 587-592.
- Chatterjee, S., 1994. Neutral sphingomyelinase action stimulates signal transduction of tumor necrosis factor- $\alpha$  in the synthesis of cholesteryl esters in human fibroblasts. *J. Biol. Chem.*, 269: 879-882.
- Clendenon, N.R., N. Allen, W.A. Gordon and W.G. Bingham, 1978. Inhibition of Na<sup>+</sup>-K<sup>+</sup>-activated ATPase activity following experimental spinal cord trauma. *J. Neurosurg.*, 49: 563-568.
- Darnell, R.B. and J.B. Posner, 2003. Paraneoplastic syndromes involving the nervous system. *NEJM.*, 349: 1543-1554.
- Ellman, G.L., 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, 82: 70-77.
- Eto, Y. and K. Suzuki, 1973. Cholesterol ester metabolism in rat brain. *J. Biol. Chem.*, 248: 1986-1901.
- Farooqui, A.A. and L.A. Horrocks, 2001. Plasmalogens: Workhorse lipids of membranes in normal and injured neurons and glia. *Neuroscientist*, 7: 232-245.
- Folch-Pi, J., M. Less and G. Sloane-Stanley, 1957. A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.*, 226: 497-511.
- Grauss, F., B. Lang, P. Pozo-Rosich, A. Saiz, R. Casamitjana, A. Vincent, 2002. P/Q type calcium channel antibodies in paraneoplastic cerebellar degeneration with lung cancer. *Neurology*, 59: 764-766.
- Graus, F., J.Y. Delattre, J.C. Antoine, J. Dalmau, B. Giometto, W. Grisold, J. Honnorat, P. Sillevius Smitt, Ch. Vedeler, J.J.G.M. Verschuuren, A. Vincent and R. Voltz, 2004. Recommended diagnostic criteria for paraneoplastic neurological syndromes. *J. Neurol. Neurosurg. Psychiatry*, 75: 1135-1140.



- Gultekin, S.H., M.R. Rosenfeld, R. Voltz, J. Eichen, J.B. Posner, J. Dalmau, 2000. Paraneoplastic limbic encephalitis: Neurological symptoms, immunological findings and tumor association in 50 patients. *Brain*, 123: 1481-1494.
- Hagan, P., S. Poole, A.F. Bristow, F. Tilders and F.S. Silverstein, 1996. Intracerebral NMDA injection stimulates production of interleukin-1 beta in perinatal rat brain. *J. Neurochem.*, 67: 2215-2218.
- Hall, E.D. and J.M. Braughler, 1982. Effects of intravenous methylprednisolone on spinal cord lipid peroxidation and Na<sup>+</sup> + K<sup>+</sup>-ATPase activity. Dose-response analysis during 1st hour after contusion injury in the cat. *J. Neurosurg.*, 57: 247-253.
- Huey, P.U., T. Marcell, G.C. Owens, J. Etienne and R.H. Eckel, 1998. Lipoprotein lipase is expressed in cultured Schwann cells and functions in lipid synthesis and utilization. *J. Lip. Res.*, 39: 2135-2142.
- Kaczmarek, J., A. Thieleman, Z. Kopczyński, J. Goslar, K.S. Hoffmann and M. Rybczyńska, 2002. Alterations in skeletal protein, distribution of PKC $\alpha$  and level of phospholipids in erythrocyte membranes of women with primary breast cancer. *Blood Cells Mol. Dis.*, 29: 225-235.
- Keime-Guilbert, F., F. Grauss, P. Broët, R. Rene, J.L. Molinuevo, C. Ascaso, J.Y. Delattre, 1999. Clinical outcome of patients with anti-Hu-associated encephalomyelitis after treatment of the tumor. *Neurology*, 53: 1719-1726.
- Keime-Guilbert, F., F. Graus, A. Fleury, R. René, J. Honnorat, P. Broet and J.Y. Delattre, 2000. Treatment of paraneoplastic neurological syndromes with anti-neuronal antibodies with a combination of immunoglobulins, cyclophosphamide and methylprednisolone. *J. Neurol., Neurosurg. Psychiatry*, 68: 479-482.
- Kishimoto, Y., E.W. Davies and N.S. Radin, 1965. Turnover of the fatty acids of rat brain gangliosides, glycerophosphatides, cerebroside and sulfatides as a function of age. *J. Lipid Res.*, 6: 525-531.
- Kojima, A. and C.H. Tator 2002. Intrathecal administration of epidermal growth factor and fibroblast growth factor 2 promotes ependymal proliferation and functional recovery after spinal cord injury in adult rats. *J. Neurotrauma*, 19: 223-238.
- Kozik M. and H. Wyglądalska, 1977. Enzymatic activity of hydrolases in the spinal cord after an acute and subacute calomel intoxication. *Folia Histochem Cytochem (Krakow)*, 15: 79-85.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- Liu, L., T. Kita, N. Tanaka and Y. Kinoshita, 1996. The expression of tumour necrosis factor in the hypothalamus after treatment with lipopolisaccharide. *Int. J. Exp. Pathol.*, 77: 37-44.
- Liu, J., L.A. Shinobu, C.M. Ward, D. Young and D.W. Cleveland, 2005. Elevation of the Hsp70 chaperone does not effect toxicity in mouse models of familial amyotrophic lateral sclerosis. *J. Neurochem.*, 93: 875-882.
- Lowry, O.H., N.J. Rosenbrough, A.L. Farr, R. Randall, 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Lukacova, N., P. Jalc and J. Marsala, 1998a. Regional changes of membrane phospholipids concentrations in rabbit spinal cord following brief repeated ischemic insults. *Mol. Chem. Neuropathol.*, 35: 61-76.
- Lukacova, N., P. Jalc and J. Marsala, 1998b. Phospholipid composition in spinal cord regions after ischemia/reperfusion. *Neurochem. Res.*, 23: 1069-1077.
- Matsumoto, M., K. Ohtake, H. Wakamatsu, S. Oka, T. Kiyoshima, K. Nakakimura and T. Sakabe, 2001. The time course of acquisition of ischemic tolerance and induction of heat shock protein 70 after brief period of ischemia in the spinal cord in rabbits. *Anesth. Analg.*, 92: 418-423.
- Mautes A.E., L.J. Noble, 2000. Co-induction of HSP 70 and heme oxygenase-1 in macrophages and glia after spinal cord contusion in the rat. *Brain Res.*, 883: 233-237.
- Maziere C., V. Barbu, M. Auclair and J.C. Maziere, 1996. Interleukin 1 stimulates cholesterol esterification and cholesterol deposition in J774 monocytes-macrophages. *Biochim. Biophys. Acta.*, 1300: 30-34.
- Michalak, S., 1996. Posttranslational regulation of the muscle phosphofructokinase-1 activity in rats bearing transplantable Morris hepatoma 5123. University of Medical Sciences, Poznań, Poland. (dissertation).
- Michalak, S., M. Wender and G. Michalowska-Wender, 2006. Cachexia-induced cerebellar degeneration: Involvement of serum TNF and MCP-1 in the course of experimental neoplastic disease. *Acta. Neurobiol. Exp (Wars)*, 66: 113-22.
- Morris, H.P., H. Sidransky, B.P. Wagner and H.M. Dyer, 1960. Some characteristics of transplantable rat hepatoma No 5123 induced by ingestion of N-(2 fluorenyl)-phtalamic acid. *Cancer Res.*, 20: 1252-1254.
- Motoyoshi, N., M. Sakurai, T. Hayashi, M. Aoki, K. Abe, Y. Itoyama and K. Tabayashi, 2001. Establishment of a local cooling model against spinal cord ischemia representing prolonged induction of heat shock protein. *J. Thorac. Cardiovasc. Surg.*, 122: 351-357.

- O'Hara, C.M. and E.A. Chernoff, 1994. Growth factor modulation of injury-reactive ependymal cell proliferation and migration. *Tissue Cell.*, 26: 599-611.
- Ohkawa, H., N. Ohishi and K. Yagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, 95: 351-358.
- Ojeda, V.J., 1984. Necrotizing myelopathy associated with malignancy. A clinicopathologic study of two cases and literature review. *Cancer*, 53: 1115-1123.
- Perrin F.E., S. Lacroix, M. Aviles-Trigueros and S. David 2005. Involvement of chemoattractant protein-1, macrophage inflammatory protein-1 alpha and interleukin 1 beta in Wallerian degeneration. *Brain*, 128: 854-866.
- Pittock, S.J., H. Yoshikawa, J.E. Ahlskog, S.H. Tisch, E.E. Benarroch, T.J. Kryzer and V.A. Lennon, Glutamic acid decarboxylase autoimmunity with brainstem, extrapyramidal and spinal cord dysfunction. *Ayo Clin. Proc.*, 81: 1207-1214.
- Puglielli L., G. Konopka, E. Paek-Chung, L.A. Ingano, O. Berezovska, B.T. Hyman, T.Y. Chang, R.E. Tanzi and D.M. Kovacs, 2001. Acyl-coenzyme A: Cholesterol cytransferase modulates the generation of the amyloid beta-peptide. *Nature. Cell. Biol.*, 3: 905-912.
- Quan, N., M. Whiteside, L. Kim and M. Herkenham, 1997. Induction of inhibitory factor  $\kappa B\alpha$  mRNA in the central nervous system after peripheral lipopolisaccharide administration: An in situ hybridization histochemistry study in the rat. *Proc. Natl. Acad. Sci. USA.*, 94: 10985-10990.
- Ramsey, R.B. and A.N. Davison, 1974. Steryl esters and their relationship to normal and diseased human nervous system. *J. Lip. Res.*, 15: 249-255.
- Recknagel, R.O. and E.A. Glende 1984. Spectrophotometric detection of lipid conjugated dienes. *Methods in Enzymol.*, 105: 331-333.
- Rudnicki, S.A. and J. Dalmau, 2000. Paraneoplastic syndromes of the spinal cord, nerve and muscle. *Muscle Nerve*, 23: 1800-1818.
- Sakurai, M., T. Hayashi, A. Koji, M. Aoki, M. Sadahiro and K. Tabayashi, 1998. Enhancement of heat shock protein expression after transient ischemia in the preconditioned spinal cord of rabbits. *J. Vasc. Surg.*, 27: 720-725.
- Samson, F.E. and D.J. Quinn, 1967. Na<sup>+</sup>-K<sup>+</sup>-activated ATPase in rat brain development. *J. Neurochem.*, 14: 421-427.
- Selmaj K., C.S. Raine, M. Farooq, W.T. Norton and C.F. Brosnan, 1991. Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. *J. Immunol.*, 147: 1522-1529.
- Singh, H., N. Spritz and B. Geyer, 1971. Studies of brain myelin in the quaking mouse. *J. Lipid Res.*, 12: 473-481.
- Sperry, W. and M. Webb, 1950. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.*, 187: 97-106.
- Storey, E. and P.A. McKelvie, 1991. Necrotizing myelopathy associated with multiple myeloma. *Acta Neurol. Scand.*, 84: 98-101.
- Svennerholm, L., 1956. The quantitative estimation of cerebroside in nervous tissue. *J. Neurochem.*, 1: 42-53.
- Svennerholm, L., 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta.*, 4: 604-611.
- Svennerholm, L., 1964. The distribution of lipids in the human nervous System. *J. Neurochem.*, 11: 839-859.
- Taranova, N.P., 1985. Disturbance of lipid composition in spinal cord of rabbits with experimental allergic encephalomyelitis. *Neurochem. Res.*, 10: 1483-1497.
- Torliński, L., A. Czech, H. Karoń, T. Torlińska, W. Pawlowski and S. Michalak, 1991. Dissociation and reassociation of phosphofructokinase-1 with erythrocyte membranes as a form of posttranslational regulation of enzyme activity. *Poznańskie Roczniki Medyczne XII/XV*, pp: 31-42.
- Towbin, H., T. Staehelin and J. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA.*, 76: 4350-4354.
- Vernon, J.R., 1974. Acute necrotic myelopathy. *Eur. Neurol.*, 11: 83-87.
- Wender, M., M. Kozik and A. Goncerzewicz, 1977. Histochemical studies in the myelinopathy provoked by the cerebrospinal fluid exchange. *Acta Histochem.*, 58: 17-30.
- Yang J.B., Z.J. Duan, W. Yao, O. Lee, L. Yang, X.Y. Yang, X. Sun, C.C.Y. Chang, T.Y. Chang, B.L. Li, 2001. Synergistic transcriptional activation of human acyl-coenzyme A: cholesterol acyltransferase-1 gene by interferon- $\gamma$  and all-trans-retinoic acid THP-1 cells. *J. Biol. Chem.*, 276: 20989-20998.
- Yu, R.K. and S. ando, 1980. Structures of Some New Complex Gangliosides. In: Svennerholm L., P. Mandel, Dreyfus H, Urban P-H (Eds.), *Structure and function of gangliosides*. Plenum New York.
- Yune, T.Y., S.M. Lee, S.J. Kim, H.K. Park, Y.J. Oh, Y.C. Kim, G.J. Markelonis, T.H. Oh, 2004. Manganese superoxide dismutase induced by TNF-alpha is regulated transcriptionally by NF-kappaB after spinal cord injury in rats. *J. Neurotrauma*, 21: 1778-1794.
- Zakaryan, A., K. Karageuzyan, L. Hovsepian, L. Karabashyan and G. Zakaryan, 2001. Quantitative analysis of phospholipids and gangliosides in bone marrow progenitors of lymphocytes, thymocytes and mature lymphocytes in tumor bearing animals. *Int. Immunol.*, 13: 1141-1145.