Determination of trace elements in peripheral lymphocytes from tumor-bearing rats

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Summary. Neutron activation analysis of peripheral lymphocytes from healthy and Walker 256 carcinosarcoma-bearing rats resulted in the quantitative determination of Co, Cu, K, Mn, Rb, Se and Zn, and a qualitative determination of Au and Sb. From corresponding plasma samples, Co, Zn, Rb, Se and Cs were analyzed. Differences in trace element concentrations could only be detected in plasma of tumor-bearing animals which showed a decreased Zn and Co content of 50% and 30%, respectively, and a rise of Rb when compared to plasma of control animals.

One of the most extensively studied trace elements in biology is zinc. About 25 zinc-containing enzymes have been isolated to date[1], and the need for zinc in cellular DNA and RNA synthesis has been demonstrated.

Of particular interest to many authors is the role of zinc during transformation of white blood cells. Rühl et al.[2] and also Berger et al.[3] found a stimulating effect of Zn^{2+} on DNA synthesis of cultured lymphocytes. Phillips[4] investigated the different Zn transferrin uptake of normal and leukemic lymphocytes after stimulation with poly-L-ornithine. Zinc content of leucocytes from normal and leukemic subjects was determined by Dennes et al.[5]. Zinc content of granulocytes from normal subjects and patients with collum carcinoma was investigated by Weise et al.[6].

The present work was done in order to investigate additional trace elements other than zinc in peripheral lymphocytes, and to determine whether a fast-growing tumor, which in general is accompanied by an increase of the

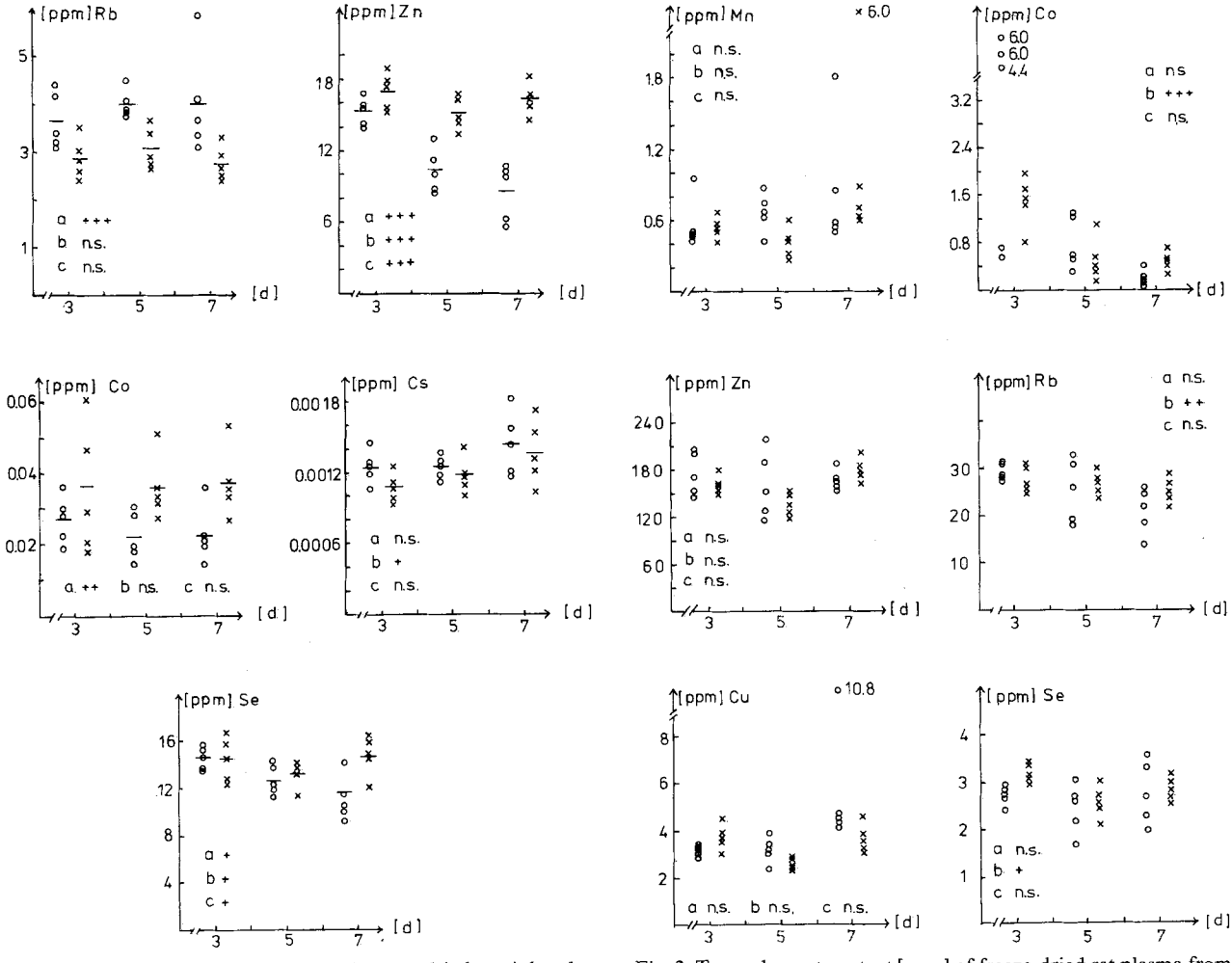

Fig. 1. Trace element content [ppm] of freeze-dried, peripheral rat lymphocytes (male Sprague-Dawley rats 200 g). Abscissa: days after transplantation. ×, Controls; O, tumor-bearing animals. 2-way variance analysis: difference between controls and tumor bearing animals = a, time response = b, difference in time response = c. Degree of significance + + + p = 0.001, + + p = 0,01, + p = 0.05, n.s. = not significant.

Fig. 2. Trace element content [ppm] of freeze-dried rat plasma from male Sprague-Dawley rats 200 g. Abscissa: days after transplantation. ×, Controls, O, tumor-bearing animals. Mean value (for statistical evaluations see figure 1) is designated by horizontal line.

leukocyte number, changes the trace element concentration of lymphocytes of the host animals.

Materials and methods. Male Sprague-Dawley rats (200 g, obtained from Mus Rattus, Brunnthal, Munich) were injected s.c. with 20 million of the Walker 256 carcinosarcoma ascites cells in 0.5 ml Hanks' balanced salt solution. Animals were kept in Macrolon cages and received a standard diet Altromin (Altrogge, Lage/Lippe) and water ad libitum. 3, 5 and 7 days after transplantation 5 tumor-bearing and 5 control animals received 0.2 ml of heparin (1000 I.U.) i.v., were etherized and killed by heart puncture with a plastic needle (Braun, Melsungen). Plasma was isolated by centrifuging at 3000 rpm for 20 min, tumors were removed and weighed, leukocytes from whole blood were counted with a Coulter Counter model ZBI (Coulter Electronics Dunstable, Bedfordshire, GB).

Lymphocytes from heparinized blood were separated by means of a Ficoll-Isopaque gradient during a centrifugation at $400 \times g$ as described by Böyum[7]. The procedure was again repeated to ensure a complete loss of erythrocytes. Cells were then washed 3 times with 10 ml of a 0.9% NaCl solution (NaCl Suprapur® Merck, Darmstadt) to remove platelets. Before the last centrifugation, an aliquot of the cell suspension was taken and the amount of cells per ml was determined with a Coulter Counter. The final cell pellets were freeze-dried, weighed and stored in either quartz ampoules for instrumental neutron activation analysis (NAA), or in polystyrole vials when chemical treatment of the samples followed irradiation. The yield on freeze-dried lymphocytes from 7.5 ml of blood ranged from 0.5 to 3.0 mg; 1 mg freeze-dried material corresponds to 14.9±2.0 million of lymphocytes.

Instrumental NAA consisted in irradiating the samples, which were sealed in quartz vials (Suprasil AN.Heraeus, Hanau), for 72 h in a neutron flux of $8 \cdot 10^{13}$ n · sec^{-1} · cm^{-2} in the F.R.II reactor at Karslruhe. After a cooling period of about 25 days, samples and standard solutions were measured in a high resolution Ge/Li well type detector coupled with a computerized multichannelanalyzer (TN 11, Tracor Northern Middleton, Wisconsin). By this procedure, concentrations of Co, Rb, Se and Zn were determined in the lymphocytes. Additionally in plasma samples Cs content could be measured.

Determination of Au, Co, Cu, K, Mn, Rb, Sb and Zn in lymphocytes was done by irradiating the samples for 60 h in a neutron flux of $2 \cdot 10^{12}$ n · sec^{-1} · cm^{-2} in the Triga Mark I reactor at Heidelberg. Immediately after irradiation, samples were wet ashed with a mixture of concentrated sulfuric acid and hydrogen peroxide, and the above elements were separated by passing the resulting strong acid solution through small columns containing inorganic precipitates, ion exchangers and chelating agents (table 1). A more detailed description of this chemical separation is given by Schuhmacher et al.[8]. Because of the very low activity of the separated radionuclides, the gamma spectra had to be measured with a $5 \times 6''$ NaI/Tl well type detector. The resulting concentrations were computed by applying a least square fit of known standard spectra to the sample spectrum.

Table 1. Chemical separation scheme

1	Wet ashing (H_2O_2/H_2SO_4)	
2	Rubidium-12-molybdophosphate (RbMP)	^{86}Rb
3	Hydrated antimony pentoxide (HAP) + 6n NaOH	^{24}Na
4	Coppersulfide (CuS)	^{198}Au, ^{64}Cu
5	Ionexchanger Bio Rad AG 1×8 Cl^-	^{69m}Zn, ^{65}Zn
6	Ionexchanger Bio Rad AG 1×8 J^- + 6n NaOH	^{122}Sb, ^{124}Sb
7	Pyrrolidinedithiocarbamate (APDC)	^{60}Co
8	Chelex 100	^{56}Mn
9	Effluent	^{42}K

Table 2. Increase of number of leukocytes and of tumor weight of male Sprague-Dawley rats bearing a solid Walker 256 carcinosarcoma

Days after transplantation	Leukocytes/ml blood (million cells ± SD)*		Tumor weight (g ± SD)*
	Control	Tumor	
3	11.1±2.1	14.3±1.7	0.18±0.15
5	12.7±2.1	16.0±1.7	1.04±0.78
7	11.8±2.2	22.7±3.6	8.54±2.27

* n = 5.

Table 3. Trace element content of peripheral lymphocytes from healthy Sprague-Dawley rats [ng ± SD/10^6 cells]. n = 15

Co	0.048±0.037*	K	797±81**	Zn	10.8±1.5
Cu	0.22 ±0.05	Rb	1.83±0.17		
Mn	0.036±0.01	Se	0.19±0.025		

* Co content in tumor-bearing animals and in controls showed a very high significant time response by the 2-way variance analysis, resulting in a large SD. A reason for decrease of Co content during the whole experiment might be the changed standard diet 2 days before transplantation. ** n = 8.

Results and discussion. The increase of number of leukocytes and of tumor weight during the 7 days of the experiment is given in table 2. The data of trace element analysis of peripheral rat lymphocytes for Co, Cu, Mn, Rb, Se and Zn are shown in figure 1. Additionally table 3 gives the average of trace element content per 10^6 cells from the 15 healthy control animals. Zinc values are in the same range as determined by Dennes[5] for human leukocytes (14±1.9 ng Zn/10^6 cells). As indicated by the 2-way variance analysis in figure 1, there is no significant change in trace element content at any time after transplantation of a Walker carcinosarcoma. This result is in contrast to the failing condition of tumor-bearing animals, which 1 or 2 days later (8–9 days after transplantation) die from cachexia, hypercalceaemia and soft tissue calcification[9]. In addition, trace element content of plasma is strongly affected by tumor growth. As seen in figure 2, a 50% decrease of the Zn level was measured, also a significant loss of Co and an increase of Rb. As reported by Wesch et al.[10], plasma copper also increases during growth of a Walker tumor.

Apart from the elements mentioned above Au, K and Sb were also analyzed. Concentrations of these elements in lymphocytes of tumor-bearing animals, as well as in control animals, varied considerably: Au 0.001–0.170 ppm, Sb 0.064–2.55 ppm and K 2010–13,100 ppm. Au and Sb are nonessential trace elements and their variation may result from different uptake levels prior to this experiment. The difference in potassium content seemed to be a result of varying shrinkage of cell cytoplasma during separation on the Ficoll-Isopaque gradient. From 30 potassium determinations, 8 gave results of 11,000–13,100 ppm. All others were uniformly distributed from 2010–10,000 ppm, so the upper extreme of 11,000–13,100 ppm is assumed to be correct.

In summary, no influence on trace element metabolism of lymphocytes could be detected by the fast frowing Walker carcinosarcoma. Even a servere loss of zinc in plasma of tumor-bearing animals did not change the zinc content of lymphocytes. These findings are in contrast to those of other authors[6,11], who found a decreased zinc content in granulocytes of patients with neoplastic diseases. An expla-

nation may be that centrifugation of blood on a Ficoll-Isopaque gradient mainly separates lymphocytes and monocytes (mononuclear cells).

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Comparison of X-prolyl dipeptidyl-aminopeptidase activity in human cerebrospinal fluid with that in serum

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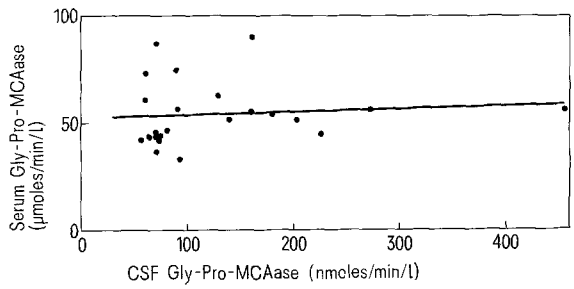
Summary. X-Prolyl dipeptidyl-aminopeptidase activities in cerebrospinal fluid and serum from the same patients without neurological diseases, undergoing surgery under lumbar anesthesia, were assayed fluorometrically with a newly synthesized fluorogenic substrate, 7-glycylproline-4-methylcoumarinamide; the values were 129.1±19.5 nmoles/min/l and 54.17±3.11 μmoles/min/l (mean±SEM, n=23), respectively, and there was no correlation between both activities (r=0.0894).

X-Prolyl dipeptidyl-aminopeptidase[2] cleaves N-terminal X-proline from peptides. The enzyme was purified from porcine and lamb kidney[3-6] and human submaxillary gland[7]. The N-terminal amino acid (X) can be an amino acid with a free amino terminal such as Gly, Ala, Arg, Lys, Glu and Asp, and glycine gives the highest activity[8]. The presence of Pro in the penultimate position is substantial, but Ala and Hyp can give a weak activity[9]. The physiological role of this enzyme is not clear, but since the enzyme preferentially hydrolyzes Gly-Pro sequence which is rich in the collagen molecule, it may work on the degradation of peptides with the N-terminal Gly-Pro sequence derived from collagen in the connective tissue.

We found the enzyme in human serum[10] and saliva[11]. We have recently synthesized a new fluorogenic substrate, 7-(Gly-Pro)-4-methylcoumarinamide for the enzyme[12], and developed a highly sensitive fluorescence assay for X-prolyl dipeptidyl-aminopeptidase. By using this method, the enzyme was found also in human cerebrospinal fluid[12]. Preliminary data showed that the enzyme activity in cerebrospinal fluid was only about 0.2% of the serum enzyme activity. Since the cerebrospinal fluid protein concentration is about 0.3% of serum protein, the enzyme activity of cerebrospinal fluid could be directly related to the serum protein content of the spinal fluid. To examine this question, we have measured the enzyme activity in cerebrospinal fluid and serum simultaneously from the same patients.

Cerebrospinal fluid was obtained from patients at Fujita-Gakuen University School of Medicine Hospital by lumbar puncture. The patients were undergoing surgery under lumbar anesthesia. No patient suffering from central or peripheral neurological diseases was included, and the general physical and nutritional states of the patients were within normal range. Care was taken to avoid any contamination of blood into cerebrospinal fluid. The first 5 ml was removed for chemical and cytological examinations, and the next 5 ml was used for the assay of X-prolyl dipeptidyl-aminopeptidase activity. The samples of cerebrospinal fluid were all clear, and no red cells were detected. Blood samples were obtained by venipuncture and serum was removed. X-Prolyl dipeptidyl-aminopeptidase incubation mixture (total volume 100 μl) contained 40 μl of 0.15 M glycine-NaOH buffer (pH 8.7), 25 μl of 2 mM 7-(Gly-Pro)-4-methylcoumarinamide tosylate, and 35 μl of cerebrospinal fluid or 35 μl of diluted serum containing 1 μl of serum. The control tube contained no enzyme. All the tubes were incubated at 37 °C for 30 min, and the reaction was stopped by adding 1.0 ml of 1 M sodium acetate buffer, pH 4.2. The same amount of enzyme was added to the control, after stopping the reaction. The fluorescence intensity was read at 460 nm with excitation at 380 nm, using a Shimadzu RF-500 spectrofluorometer, and 7-amino-4-methylcoumarin liberated by the enzyme reaction was measured.

X-Prolyl dipeptidyl-aminopeptidase activity in cerebrospinal fluid and serum (mean±SEM, n=23) were 129.1±19.5 nmoles/min/l and 54.17±3.11 μmoles/min/l, respectively. Thus, the enzyme activity in cerebrospinal fluid was 0.24% of the serum enzyme activity. As shown in the figure, a correlation coefficient between the activity in cerebrospinal fluid and that in serum was 0.089, and there was no significant correlation between the activity in cerebrospinal fluid and that in serum. The result suggests that the enzyme in cerebrospinal fluid could be derived from the brain and not from the blood. It should be noted in this connection



Correlation between X-prolyl dipeptidyl-aminopeptidase activity in human cerebrospinal fluid (CSF) with that in serum. 7-(Gly-Pro)-4-methylcoumarinamide was used as substrate, and 7-(Gly-Pro)-4-methylcoumarinamidase (Gly-Pro-MCAase) activity was expressed as 7-amino-4-methylcoumarin liberated (nmoles or μmoles)/min per l of cerebrospinal fluid or serum.