CYTOCHEMICAL INVESTIGATION OF LOCALIZATION OF ALKALINE RIBONUCLEASE IN NORMAL AND LEUKEMIC RAT BLOOD CELLS

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UDC 616.155-392-008.931 : 577.155.2]-092.9

The distribution of alkaline ribonuclease in bone marrow cells of healthy rats and in cells from ascites fluid of rats with transplanted Svec leukemia was studied by the azo-coupling method using synthetic α-naphthyluridine-3'-phosphate as substrate. A decrease in activity of the enzyme was found in the leukemic cells by comparison with the normal ancestral hematopoietic cells.

Determination of the intracellular localization of the ribonucleases is often used to study their biological role in the genesis of cancer and leukemia. Modern histochemical methods can be used for this purpose, notably reactions with azo-dyes and methods using esters of halogenated indoxyl, developed in recent years can be used for this purpose [3, 5, 6].

Bone marrow cells from healthy Wistar rats and cells from the ascites fluid of rats with a transplanted Svec leukemia [4] were used as the test objects. The localization of alkaline ribonuclease in these cells was determined by the azo-coupling method.

No reference could be found in the accessible literature to the histochemical determination of ribonuclease activity by this method in the cells of hematopoietic tissues under normal conditions and in luekemia.

EXPERIMENTAL METHOD

In the absence of any available commercial preparation of the reaction substrate (α-naphthyluridine-3'-phosphate), it was prepared by the method of Zan-Kowalczewska et al. [6], based on the investigations of polynucleotide synthesis undertaken in Khorana's laboratory [1]. Of the several methods suggested by these workers, it was decided to use the method of preparing α-naphthyluridine-3'-phosphate in which dihydropyran is used to protect the 2'- and 5'-hydroxyl groups of the uridine residue and the resulting 2',5'-di-O-tetrahydropyranyluridine is then condensed with α-naphthyl phosphate in dry pyridine in the presence of N,N'-dicyclohexylcarbodi-imide, and the pyranyl groups are then removed.

Method of Laboratory Synthesis of α-Naphthyluridine-3'-phosphate. To 1 mmole (224 mg) uridine in 5 ml dimethyl sulfoxide, 1 ml trifluoroacetic acid and 6 mmoles (0.55 ml) 2,3-dihydropyran were added and the reaction mixture was kept for 18 h at 22°C. Half of the solvent was evaporated under reduced pressure at 36-37°C. After cooling of the mixture and addition of 5 ml concentrated ammonia, the evaporation was repeated in vacuo down to a volume of 2 ml. By analytical ascending chromatography on paper, using water-saturated N-butanol as the solvent, the presence of 5'-O-tetrahydropyranyluridine (R_f 0.61), 2',5'-di-O-tetrahydropyranyluridine (R_f 0.80), and 2',3',5'-tri-O-tetrahydropyranyluridine (R_f 0.96) was demonstrated. After the preparative chromatography has been completed, the 2',5'-di-O-tetrahydropyranyluridine was eluted with methanol and the alcoholic eluate was dried.

Laboratory of the Etiology and Pathogenesis of Leukemia, Kiev Research Institute of Experimental and Clinical Oncology. (Presented by Academician V. V. Parin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 72, No. 9, pp. 90-93, September, 1971. Original article submitted October 14, 1970.

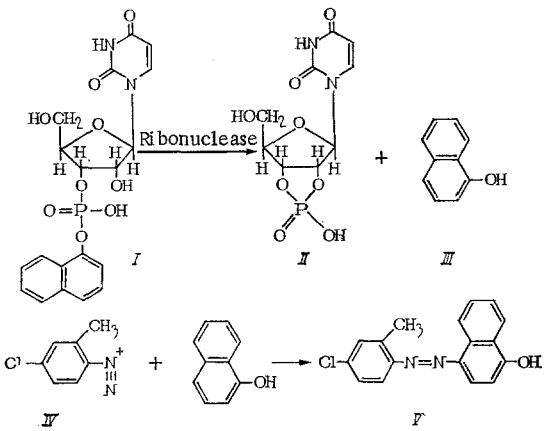
To 0.5 mmole (206 mg) 2',5'-di-O-tetrahydropyranyluridine dissolved in 5 ml dry pyridine 2.5 mmoles (600 mg) α-naphthyl phosphate and 5 mmoles (1030 mg) N,N'-dicyclohexocarbodi-imide were added, and the mixture was kept for 24 h at 29°C. After the addition of 5 mmoles (0.4 ml) pyridine in 2.5 ml water the reaction mixture was dried in vacuo at 35°C. After addition of 1.5 ml water and drying for a second time the residue was separated by filtration. The filtrate was neutralized with a hot saturated solution of barium hydroxide, and the mixture was filtered and dried under reduced pressure. By analytical chromatography on paper the presence of two bands corresponding to α-naphthyl-2'-di-O-tetrahydropyranyluridine-3'-phosphate (R_f 0.56) and α-naphthyl phosphate (R_f 0.10) was demonstrated. The residue was transferred to cold acetone, filtered, and allowed to stand at room temperature for 24 h. A white precipitate was thrown down. The L-naphthyl phosphate present as an impurity was then removed by chromatography on paper. The α-naphthyl-2',5'-di-O-tetrahydropyranyluridine-3'-phosphate was eluted with purified methanol. The eluate was dried, the residue was dissolved in water, the solution was agitated in the cold with Dowex-50 (H^+), and the filtrate was evaporated in vacuo and treated with 80% acetic acid solution at room temperature in order to remove the protective tetrahydropyranyl groups. The solvent was removed under reduced pressure and the product was dried from aqueous solution, redissolved in 5 ml water, neutralized with 1 M NaOH, and again dried.

The synthesized α-naphthyl-uridine-3'-phosphate was identified by chromatography on paper (R_f 0.23) and by qualitative reactions which demonstrated the stability of the resulting substrate in the presence of deoxyribonuclease, phosphomonoesterase, and phosphodiesterase from snake venom. Hydrolysis of the α-naphthyl ester of uridine-3'-phosphate by pancreatic ribonuclease in the presence of the dye fast red TP was accompanied by the development of a red color with an absorption maximum at 550 nm.

For the cytochemical determination of alkaline ribonuclease, freshly prepared films were dried in air and fixed for 3 min in the vapor of 10% neutral formalin, and then treated with a mixture containing 18 mg α-naphthyluridine-3'-phosphate (the sodium salt) and 24 mg fast red TP in 5 ml 0.1 M tris- HCl buffer (pH 9.0). After incubation for 2 h at room temperature, during which the medium was twice replaced with fresh to prevent spontaneous breakdown of the substrate, the films were washed, dried, and examined under the microscope.

EXPERIMENTAL RESULTS

During the action of intracellular ribonuclease on α-naphthyluridine-3'-phosphate (I) the phosphodiester bond is ruptured with the formation of uridine-2',3'-cyclic phosphate (II) and with liberation of α-naphthol (III). The α-naphthol reacts with the fast red TP (5-chloro-o-toluidine) (IV) present in the incubation mixture in accordance with the stand azo-coupling technique [2] to give an insoluble red compound (V) detectable in structures possessing ribonuclease activity.



The accuracy of ribonuclease localization is ensured by the fact that the reaction takes place in one stage, that incubation is brief, that the azo-coupling reaction takes place rapidly (at pH 9.0), and that the resulting product is only sparingly soluble.

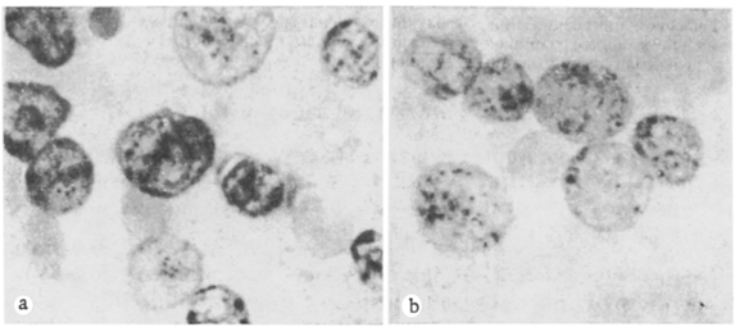


Fig. 1. Alkaline ribonuclease activity in bone marrow cells of a healthy rat (a) and in cells of ascites fluid of a rat with transplanted Svec leukemia (b). Here and in Figs. 2 and 3: immersion, 630 ×.

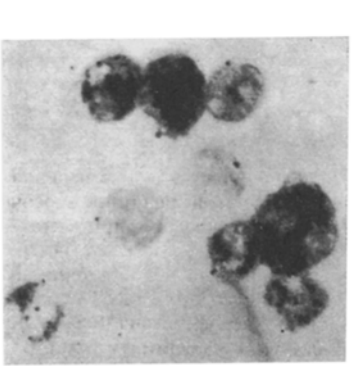


Fig. 2

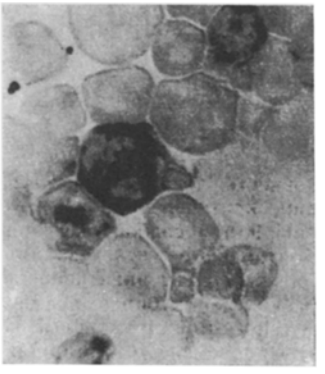


Fig. 3

Fig. 2. Phosphodiesterase I activity in bone marrow cells of a healthy rat.

Fig. 3. Nonspecific alkaline phosphatase activity in bone marrow cells of a healthy rat.

Control tests were carried out with bone marrow films in which the enzyme was inactivated by heating to 90°C for 10 min, and also with films incubated in medium not containing the reaction substrate. Under these circumstances the structural components of the cells remained unstained. If the α-naphthyluridine-3'-phosphate was replaced by α-naphthylthymidine-5'-phosphate and by the α-naphthylphosphate used to synthesize the substrate, the localization of the end product corresponded to the distribution of phosphodiesterase I activity and nonspecific alkaline phosphatase activity in the cells.

The azo-coupling method with α-naphthyluridine-3'-phosphate as the substrate can be used to determine the intracellular localization of ribonucleases rupturing phosphodiester bonds between pyrimidine and other nucleotides reacting by a similar mechanism with pancreatic ribonuclease [polyribonucleotide-2'-oligonucleotide transferase (cyclizing), 2.7.716]. Enzymes of the T_2-ribonuclease type were not discovered, for the cytoplasm of the hematopoietic cells gave only a weak, diffuse coloration in the reaction with synthetic α-naphthylinosine-3'-phosphate, a substrate for both T_1- and T_2-ribonucleases simultaneously.

Investigation of films of bone marrow from healthy rats revealed alkaline ribonuclease activity as red granules in the cytoplasm of the myeloid cells and also in the lymphocytes, erythroblasts, and normoblasts (Fig. 1). By contrast, phosphodiesterase I activity was detected only in the cells of the myeloid and erythroid series, and nonspecific alkaline phosphatase activity was detected only in the leukopoietic cells starting from the myelocyte stage (Figs. 2 and 3).

In the reticulum cells and the ancestral hematopoietic cells (hemocytoblasts, myeloblasts) the reaction for alkaline ribonuclease was weak. Its intensity was sharply increased in the myelocytes. The greatest increase in the number of granules and in the intensity of their staining was observed in the leukocytes: stab cells, polymorphs, and eosinophils.

The reaction for alkaline ribonuclease was considerably reduced in intensity in the cells from the ascites fluid of rats with a transplanted Svec leukemia (having undergone 268 passages in the writers' laboratory over a period of 7.5 years), most of which were reticulum cells and undifferentiated cells of the hemocytoblast and proerythroblast types.

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