ACID GLYCOSIDASES OF BOVINE LYMPHOCYTES FROM NORMAL COWS AND COWS WITH CHRONIC LYMPHATIC LEUKEMIA

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Changes in leukocytic acid glycosidase activity have been discovered by several workers in different forms of leukemia. In myeloid leukemia a tendency has been found for activity of the leukocytic acid glycosidases to increase, whereas in lymphatic leukemia, activity of these enzymes falls [5, 10, 11]. Changes in activity of certain glycosidases are accompanied by changes in the relative proportions of their molecular forms. In particular, an increase in the relative content of the I-form of N-acetyl-hexosaminidase has been observed in children with lymphoblastic leukemias [6-8, 12]. It has been suggested that changes observed in acid glycosidase activity be used as an additional test for the differential diagnosis of leukemias [11], but this would require a more detailed study of the causes of these changes. Since it is difficult to carry out such an investigation on leukocytes from human patients, because of the restricted quantity of material, we decided to use animals (cows) with chronic lymphatic leukemia (CCL) as the model. This disease is found quite frequently, and it is similar in many features with CLL in man [3].

The aim of this investigation was to compare activity of five acid glycosidases: α-D-mannosidase, N-acetyl-β-D-hexosaminidase, β-D-galactosidase, β-D-glucuronidase, and α-D-glucosidase. A marked decrease in α-mannosidase activity and a smaller decrease in α-glucosidase activity were found in lymphocytes from cows with CLL. Activity of the other enzymes was unchanged.

EXPERIMENTAL METHOD

Blood was taken from the jugular vein and heparin was used as anticoagulant (10 U/ml). The blood leukocyte count of animals with leukemia varied from 20,000 to 182,000/μl. Lymphocytes were isolated by passing whole blood through a column packed with cotton fiber [2], when granulocytes and monocytes were absorbed. Erythrocytes were destroyed by hemolysis. The isolated lymphocytes were suspended in 0.9% NaCl or phospate buffer, pH 7.2, made up in 0.9% NaCl, with the addition of 0.42 g/liter KCl, and were counted in a Goryaev's chamber. The suspension was made up to a concentration of 20,000-30,000 cells to 1 ml of 0.9% NaCl, and 1% Triton X-100 was added up to a concentration of 0.1%. The suspension was homogenized for 1-2 min in a glass homogenizer and allowed to stand overnight for extraction at 4°C. The resulting extract was centrfuged at 800g (20 min) and the supernatant was used as the enzyme preparation. In some cases the cell suspension was frozen and kept at -18°C. Keeping for 1 month did not cause any decrease in glycosidase activity by more than 20%.

To determine glycosidase activity, the following 4-methylumbelliferyl (MUF) glycosides were used as substrate: MUF-α-D-mannoside, MUF-β-D-galactoside, MUF-β-D-glucuronide, MUF-α-D-glucoside (from Koch-Light, England), and MUF-N-acetyl-β-D-glucosaminide (from Serva, West Germany). To determine activity of all the glycosidases except glucosidase, samples of 60 μl contained 20 μl of a solution of substrate, 20 μl phosphate-citrate buffer (0.1M citric acid + 0.2M sodium phosphate), pH 4.0, and 20 μl of the enzyme preparation. To determine glucosidase activity the sample of 200 μl contained 50 μl of substrate solution, 50 μl of water, 50 μl of phosphate-citrate buffer, pH 4.0, and 50 μl of the enzyme preparation. The reaction (30 min, 37°C) was stopped by the addition of 5 ml of 0.1M Na_2CO_3. Fluorescence was measured on a BIAN-130 fluorometer (excitation wavelength 365 nm, emission wavelength 436 nm). The following final substrate concentrations were used (in mM): for mannosidase 1.66, for galactosidase

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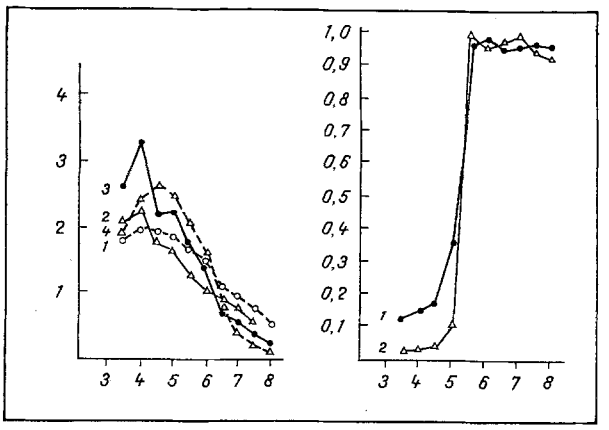

Fig. 1 Fig. 2

Fig. 1. Activity of glycosidases from lymphocytes of leukemic animals as a function of pH. Abscissa, pH values; ordinate, activity (in units/mg protein). 1) β-D-Glucuronidase; 2) β-D-galactosidase; 3) α-D-mannosidase; 4) N-acetyl-β-D-hexosaminidase (reduced 20-fold).

Fig. 2. α-D-Glucosidase activity as a function of pH: 1) in absence, 2) in presence of turanose. Remainder of legend as to Fig. 1.

0.83, for glucuronidase 3.3, for glucosidase 0.25, and for hexosaminidase 1.0. The quantity of enzyme catalyzing removal of 1 nanomole 4-methylumbelliferone from the corresponding substrate in 1 min was taken as the unit of activity.

EXPERIMENTAL RESULTS

Most animal and human cells contain two types of glucosidases: acid (with optimum of action at acid pH values) and neutral (optimum at neutral pH). The ratio between these forms of glycosidases differs in different types of cell. To determine the relative contribution of these two types of glycosidases to total glycosidase activity of lymphocytes from healthy and sick animals and the possibility of determining acid glycosidase activity, dependence of glycosidase activity on pH was studied for each of the glycosidases. The results showed that the shape of the curves of glycosidase activity as a function of pH for healthy and sick animals was virtually identical. For all enzymes studied except glucosidase, the activity curves had a maximum at pH 4.0-4.5, evidence of the relatively high activity of acid glycosidases in lymphocytes (Fig. 1). Unlike the other glycosidases, glucosidase activity had a marked optimum at a pH above 6.0 (Fig. 2). To detect acid α-glucosidase activity the specific inhibitor of this enzyme, the disaccharide turanose, was used. Glucosidase activity was found to be completely inhibited by turanose at pH 4.0. This showed that activity of acid α-glucosidase is exhibited at pH 4.0. and confirmed that it can be determined at this pH value. Similar relations between acid and neutral α-glucosidases were found by the writers previously in human lymphocytes [4].

A marked reduction in acid α-mannosidase activity was found in lymphocytes from leukemic animals: on average down to 17.5% of normal ($p<0.001$). Some decrease in acid-glucosidase activity also was observed. On average the acid α-glucosidase activity in lymphocytes of the leukemic animals was only half of that in healthy cows ($P<0.01$). Activity of other glycosidases remained within normal limits. Activity of acid α-L-fucosidase also was detected in lymphocytes from two healthy and two leukemic cows. The mean activity of this enzyme was found to be twice as high in lymphocytes from healthy cows as in those from leukemic cows (2.3 and 5.3 per 10^8 cells and 1.1 and 1.3 units per 10^8 cells respectively). It cannot be definitely concluded from these results that fucosidase activity is reduced by 50% in leukemia, but they do suggest that there is a tendency for the activity of this enzyme to fall.

Activity of glycosidases was measured on three different occasions during development

of the disease in one of the cows. An increase in the leukocyte count in the blood was observed (123,000, 166,000, and 182,000 cells in 1 μl in May, June, and September respectively). The increase in the leukocyte count was accompanied by a decrease in acid α-mannosidase activity (7.4, 5.8, and 3.9 units/10^8 cells). Activity of the other glycosidases did not change significantly under these circumstances.

The results show that as the disease progresses there is a further decline in acid α-mannosidase activity.

Since the greatest decrease in activity was observed for α-mannosidase, some properties of this enzyme for normal cows and cows with CLL were compared. One possible cause of the reduced α-mannosidase activity in lymphocyte extracts from sick cows could be a firmer bond between it and the cell, with consequent incomplete extraction under conditions which, as our data showed, ensure complete extraction from normal lymphosytes. To test this hypothesis α-mannosidase was extracted from lymphocytes of leukemic cows by different methods, including triple freezing and thawing, treatment with ultrasound (35 kHz, twice 20 sec each time, with an interval of 1 min at 0°C), and addition of KCl to the extracting fluid up to a concentration of 1 mM) activity also was determined in the whole homegenate. None of these methods led to the obtaining of higher activity.

Comparison of the properties of α-mannosidase from lymphocytes of healthy and sick cows revealed no significant differences between them. In both cases α-mannosidase was activated in the presence of 0.15 mM $ZnSO_4$. Heating to 55°C for 15 min reduced activity by not more than 15% in both cases. The Michealis constant for lymphocytic α-mannosidase from healthy cows was 0.83 mM (mean of 11 determinations) and for α-mannosidase from leukemic cows 0.94 mM (mean of seven determinations), i.e, there was virtually no difference.

The results suggested that the reduced α-mannosidase activity in lymphocytes of leukemic cows is due to a reduction in the rate of biosynthesis of this enzyme and not to a change in its properties.

Comparison of our results with those obtained by other workers on lymphocytes from patients with CLL did not disclose an absolute analogy. It has been shown [10] that activity of all acid glycosidases is reduced in patients with CLL of the B type. In the present case, mean activity of α-galactoside, α-glucuronidase, and N-acetyl-hexosaminidase in leukemic cows remained cows within normal limits. This suggests that the mechanism of the changes in activity that were observed is different. Whereas in man disturbances of certain common stages in the biosynthesis of glycosidases are possible, as is found, for example, in I-cell diseases [1], a disturbance of mechanisms of biosynthesis specific for individual glycosidases and, in particular, for α-mannosidase is a more acceptable explanation.

Since acid (lysosomal) α-mannosidase is an enzyme which partcipates in glycoprotein catabolism (it catalyzes removal of mannose residues from glycoprotein molecules), it can be tentatively suggested that the deficiency of it in the lymphocytes in CLL causes accumulation of mannose-rich compounds that are products of incomplete glycoprotein breakdown. The structure of the glycoproteins of the cell surface is known to be changed in CLL [9]. The possibility cannot be ruled out that α-mannosidase deficiency in the cells plays a definite role in the genesis of these changes.

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CONCENTRATION OF ERYTHROCYTE-BASED MAGNETIC CARRIERS IN THE BLOODSTREAM

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Oriented transport of drugs to a target organ is a promising modern therapeutic and prophylactic technique which enables the action of a drug to be localized, the administered dose to be reduced, and, consequently, its toxic effect to be minimized. Drugs are best transported in biocompatible containers. Erythrocytes [3, 6, 8, 9] are among the most suitable of containers. Erythrocytes "loaded" with a drug can circulated in the bloodstream for up to 10-20 days [1, 4-6, 8]. If, besides a drug, a magnetic material is also introduced into the erythrocyte, such a magentic erythrocyte (ME) can be concentrated locally in a magnetic field [2, 9].

The writers postulated that ME, injected into the bloodstream, may be concentrated in an assigned region of the vascular bed with the aid of the field of a permanent magnet. To test this hypothesis, erythrocytes "loaded" with colloidal magnetite were used, and concentrated in experiments *in vitro* and *in vivo*.

EXPERIMENTAL METHOD

Human erythrocytes were used. ME were obtained by hypotonic lysis in the presence of magnetic material, after which the integrity of the membranes was restored [2]. The magnetic material introduced into the erythrocytes consisted of colloidal magnetite (particle size 10-40 nm), stabilized with dextran T-40 (from Pharmacia, Sweden) [7], which was obtained by precipitation from ferrous and ferric chloride in the presence of dextran [7]. ME were fixed with 1% glutaraldehyde (from Merck, West Germany) for 1 h at 20°C, then washed with phosphate saline buffer (PSB), containing 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.4), by centrifugation at 1000 g for 5 min. The fixed ME were suspended up to a 10% suspension in PSB containing 5 mg/ml of bovine serum albumin (from Sigma, USA) and incubated for 3 h at 20°C, after which they were washed with PSB. A segment of prosthesis to be used for angio-plastic of the femoral artery (Ftorlon† – Lavsan‡, diameter 8 mm, length 40 mm, from the "Sever" Leningrad Production Combine, USSR) was carried out be means of an SP-3 pump (East Germany). The prosthesis was perfused with 30 ml of a 1% suspension of ME (rate of perfusion 500 ml/min). A magnetic field was created by a permanent magnet made from $SmCO_5$, measuring 15 × 38 × 8 mm, with an energy product of over 160 kj/m^3 (the magnet was provided by the All-Union Research Institute of Electromechanics). During perfusion the magnet was fixed on part of the prosthesis. The degree of concentration of ME in the prosthesis was determined from the reduction in the number of cells circulating in the perfusion system. The number of cells was determined by means of a cell counter (Coultronics, France). For the experiments *in vivo*

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