INHIBITORS OF ERYTHROPOIESIS IN PATIENTS WITH APLASTIC ANEMIA

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An inhibitor of erythropoiesis was found in the urine of patients with aplastic anemia. The inhibitor was concentrated with kaolin and separated from erythropoietin by subsequent fractionation with alcohol and gel filtration on Sephadex G-100 and DEAE-Sephadex A-50. The inhibitor blocked the effect of erythropoietin (standard C) when injected into mice with hypoxic polycythemia 3 h before or along with exogenous erythropoietin; partial neurtralization of standard C also was observed on incubation with the inhibitor.

KEY WORDS: erythropoiesis; inhibitors; aplastic anemia; hypoxic polycythemia; erythropoietin.

An inhibitor of erythropoiesis [3, 6, 9, 20] has been found in the blood of animals and humans with posttransfusion and post hypoxic polycythemia [5, 10], in neonatal plasma [18], in erythremia [21], and so on. The discovery of erythropoiesis inhibitor in the urine of healthy persons [12], in the plasma of persons returning from the hills to the plains [17], in the serum of patients with uremia [16], and also in the plasma of patients with iron-deficiency anemia [4] sheds light on the role of this inhibitor in the regulation of erythropoiesis under normal conditions and in various blood diseases. The serum and urine of patients with aplastic anemia contain large quantities of erythropoietin [22]; an inhibitor of erythropoiesis has also been found in the urine of these patients [14].

This paper describes the results of a study of erythropoiesis inhibitor isolated from the urine of patients with aplastic anemia.

EXPERIMENTAL METHOD

Urine was collected with the addition of 0.1% phenol from patients with aplastic and hypoplastic anemia during an exacerbation when the hemoglobin concentration was low (5.2-8 g%); the samples were kept at −10°C until used.

Erythropoietin and erythropoiesis inhibitor were concentrated by absorption of kaolin and subsequent elution with ammonia and precipitation with acetone [1], the resulting powder was dissolved in 3 M NaCl and centrifuged at 10,000 rpm for 10 min, and the residue was washed twice or three times with the same solution and then centrifuged.

Ethyl alcohol was added to the pooled supernatant (protein content 2-3%) up to a final concentration of 67% at 2°C and centrifuged at 5,000 rpm for 10 min at 0-2°C. The residue precipitated by alcohol in a concentration of 0-67% contained the inhibitor; the erythropoietin remained in solution [15]. The residue was dissolved in distilled water and dialysis was carried out against distilled water for 12-20 h at 4°C. The solution was freeze-dried. The preparation described as fraction B was purified on a column with DEAE-Sephadex A-50 with stepwise elution in 0.005 M phosphate buffer, pH 7.4, or on a column with Sephadex G-100, equilibrated with 0.005 M tris-buffer, pH 7.5. The specific activity of the inhibitor was determined in mice with hypoxic polycythemia [2].

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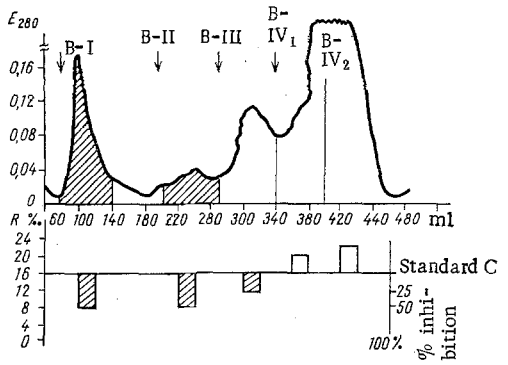

Fig. 1. Chromatography of fraction B with DEAE-Sephadex A-50. Column with Sephadex 1.7 ×45 cm; equilibrated against 0.005 M phosphate buffer + 0.025 M NaCl, pH 7.4. Preparation (70 mg protein in 2 ml of original buffer) dialyzed overnight against buffer and applied to column. Stepwise elution with NaCl of increasing concentration: 0.075, 0.15, and 0.5 M. Volume of fractions 6 ml; rate of elution 50 ml/h. Lower part of figure: inhibitory properties of corresponding fractions in mice with hypoxic polycythemia.

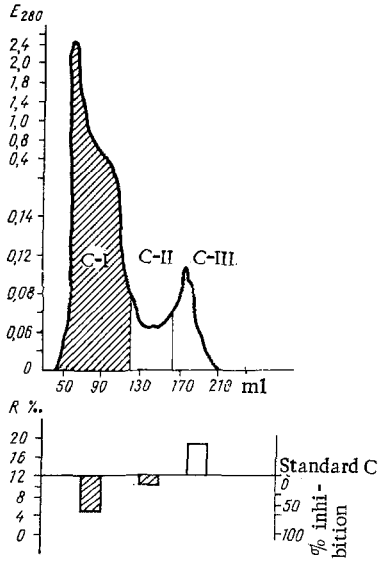

Fig. 2. Gel filtration of fraction B on Sephadex G-100 (size of column 1.6 ×74 cm) in 0.005 M tris-HCl buffer + 0.1 M $CaCl_2$, pH 7.5. Volume of fractions 4 ml, rate of elution 50 ml/h. Lower part of figure the same as in Fig. 1.

The material for testing was injected subcutaneously in a dose of 0.5 mg protein 3 h before injection of 0.5 unit of standard C or along with it. This material for testing also was incubated together with standard C for 1 h at 37°C and the incubation mixture was injected subcutaneously into mice with polycythemia. The inhibitory effect was judged from the difference between the number of reticulocytes in the blood of the experimental animals and of animals receiving standard C in a similar dose. The standard C was obtained from the urine of patients with aplastic anemia [1] and calibrated against International standard B.

EXPERIMENTAL RESULTS AND DISCUSSION

Fraction B had an inhibitory action (40%). It was separated into five fractions (B-I, B-II, B-III, B-IV_1, and B-IV_2) with the aid of DEAE-Sephadex A-50 (Fig. 1). The inhibitor was found in fractions B-I (50%), B-II (50%), and to some extent in fraction B-III (27%). Fractions B-IV_1 and B-IV_2, when injected along with the standard C, had a marked erythropoietic action.

On electrophoresis of the original fraction B on polyacrylamide gel three bands with the mobilities of prealbumin, albumin, and α_1–α_2-globulin were detected.

Fraction B-II formed two bands with the mobilities of albumin and α_1-globulin.

It is stated in the literature that the inhibitor of normal plasma has the mobility of albumin and β-globulin [19], that of the plasma of patients with iron-deficiency anemia has the mobility of γ- and β-globulin [4], and the inhibitor in the urine of healthy persons and patients with anemias the mobility of β- and α_1-globulin [12]. On purification of the inhibitor from the urine of patients with aplastic anemia [14] by gel filtration on Sephadex G-100, evidence of its low molecular weight was obtained.

On fractionation of fraction B on Sephadex G-100 in the present experiments (Fig. 2) three fractions were obtained (C-I, C-II, and C-III). Fraction C-I (56%) had well-marked inhibitory properties; traces of inhibitor (17%) were found in fraction C-II; fraction C-III contained erythropoietin. Under the experimental conditions used the inhibitor was found at $V_e/V_0 \approx 1.2$, whereas in other experiments [14] $V_e/V_0 = 3.0$. These differences were probably due to preliminary treatment of the urine with kaolin and alcohol in the present experiments, which could lead to some changes in the properties of the inhibitor. Possibly in this case the inhibitor formed a complex with protein, as has been described for erythropoietin [8, 13].

After incubation of 0.5 mg of fraction C-I with 0.5 unit of standard C, followed by injection of the incubated mixture into mice with polycythemia, the erythropoietic action of the standard C also was reduced (by 37%). These findings indicate that the inhibitor blocked erythropoietin somehow. The block possibly took place at the stage of undifferentiated erythroid precursors, for the inhibitor exhibited its effect both when injected

simultaneously with the erythropoietin and before its injection; i.e., it acted in the same way as antierythropoietic serum. Since in these experiments the action of the inhibitor was weaker in vitro than in vivo, the existence of two inhibitors can be postulated, as other workers also have done [4, 5]. However, the possibility cannot be ruled out that the inhibitor is a thermolabile substance and undergoes partial denaturation during incubation [11].

The results suggest that in certain hematological diseases changes in the erythropoietin molecule in the presence of inhibitor play an important role. An erythropoiesis inhibitor has in fact been found in the serum and urine of patients with tumors of the thymus accompanied by aplasia of the erythroid cells. Thymectomy restored the normal blood picture and caused the inhibitor to disappear from the plasma and urine [7].

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