ISOLATION OF A CYTOTOXIC FACTOR FROM BLOOD SERUM OF NUDE RATS

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Tumor cells are known to survive poorly in nude animals [3]. By passage of tumors through nude rats, E. S. Revazova found that if cells from a number of animal (including human) tumor lines are injected into nude rats at the age of 2-8 weeks, they give rise to tumors which may attain a large size, and cause death of the animals. After injection of tumor cells of the same lines into rats aged 8-16 weeks, large tumors were formed but the animals do not die, because the tumors are absorbed.

It was decided to study whether the blood serum of rats of this age contains a cytotoxic factor which is responsible for tumor absorption.

We found that blood serum of nude rats aged 8-16 weeks, unlike serum from normal rats of the same age, has a cytotoxic action on mouse cells of line L929. This cytotoxicity of the serum may be due to various factors and, in particular, to tumor necrosis factor (TNF) [4].

To determine the nature of this cytotoxic factor, we attempted to obtain it in a homogeneous state. The cytotoxic factor of rat serum was found not to be adsorbed on DEAE-cellulose under conditions when adsorption of TNF on this ion-exchanger takes place [1]. For the first stage of isolation we therefore used chromatography on the cation-exchange resin CM-Toyoperl 650 M. The rats' serum was diluted 3 times with bidistilled water, the pH adjusted to 7.2, and the sample was allowed to stand for 30 min at +4°C. The serum was then clarified by centrifugation (1500g, 15 min, +4°C) in a TJ-6R centrifuge. The supernatant was applied to a column with CM-Toyoperl 650 M (2.5 × 30 cm), equilibrated beforehand with 0.01 M ammonium acetate solution, pH 7.2. After rinsing with start buffer, gradient elution was carried out with 0.01-0.3 M ammonium acetate, pH 7.2. The column was then washed successively with 0.3 M and 1.0 M solutions of ammonium acetate, pH 7.2. As a result of this chromatography, most of the ballast proteins were removed. The cytotoxic activity of the fractions thus obtained was tested on L929 cells. For this purpose, 100 μl of Dulbecco's medium, in which the test material had previously been dissolved, was added to $2 \cdot 10^4$ cells, and the sample was incubated for 48 h. The number of dying cells was determined after staining with 0.5% trypan blue solution. It was accepted that the unit of cytotoxic activity should be the activity possessed by a given preparation if its addition to the cells in the test described above caused death of 50% of the cells in one well.

Cytotoxic activity was found in fractions (I) and (II), which were eluted by 0.3 M (fraction I) and 1.0 M (fraction II) solutions of ammonium acetate respectively. Fraction II was subjected to chromatography on hydroxyapatite. The choice of this fraction for further purification was based on its higher specific activity than fraction I, but determination of the nature of fraction I and its connection with fraction II are matters for further research.

Fraction II was diluted tenfold with bidistilled water and applied to a column with Biogel HTP (2.5 × 20 cm), equilibrated with a 0.05 M solution of sodium phosphate, pH 6.8. Gradient elution then followed, with 0.05-0.5 M sodium phosphate solution, pH 6.8.

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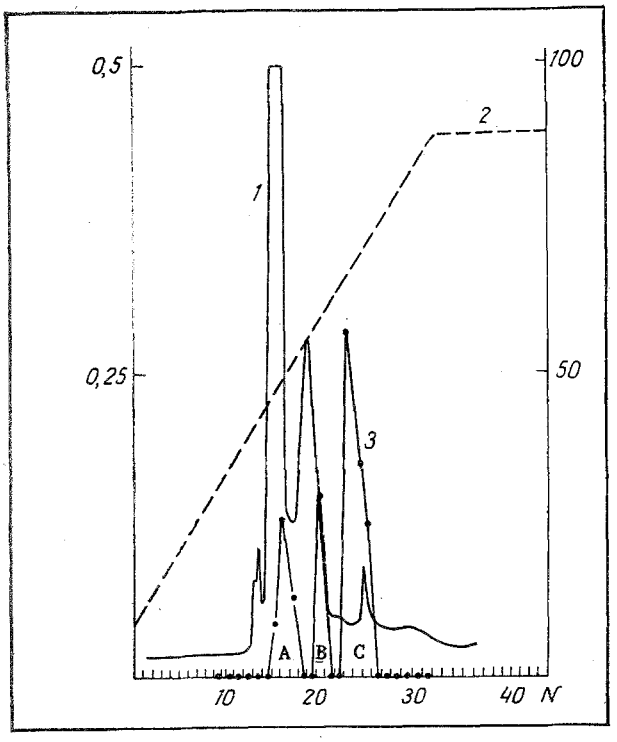

Fig. 1

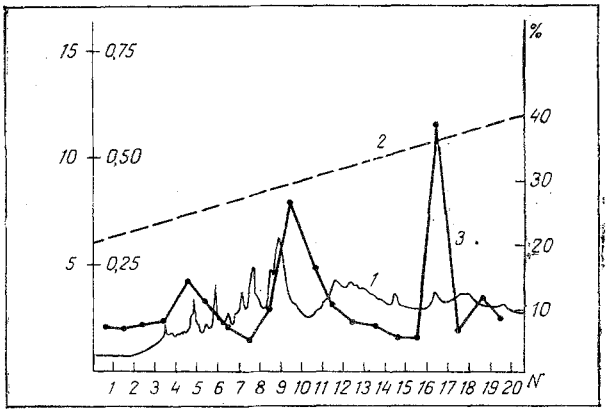

Fig. 2

Fig. 1. Chromatography of partially purified preparation of cytotoxic factor on Biogel HTP. N) No. of fraction, 1) absorption at 280 nm; 2) sodium phosphate concentration in eluting buffer; 3) cytotoxic activity of fractions.

Fig. 2. HPLC of preparation of cytotoxic factor, partially purified on Biogel HTP. Dialyzed fraction after chromatography on Biogel HTP was lyophilized, dissolved in 2 ml of 0.1% TFA solution and applied to an Ultrapore RPSC column, 4.6 mm × 7.5 cm ("Beckman"), equilibrated with 0.5% TFA solution. Gradient elution carried out with N-propanol, containing 0.05% TFA. N) No. of fraction, 1) absorption at 206 nm, 2) N-propanol concentration in eluting buffer, 3) cytotoxic activity of fractions.

As the chromatogram indicating separation of the proteins of fraction II on Biogel HTP shows, cytotoxic activity was found in fractions, A, B, and C. The ratio of the peaks B and C of activity and also the elution volume of peak B changed depending on the batch of serum. The cytotoxicity of fractions corresponding to peak A was perhaps due to the presence of interleukin I, which exhibits similar behavior during chromatography on hydroxyapatite and has a cytotoxic action on L929 cells [5].

Part of fraction C obtained by chromatography on Biogel HTP was used to estimate the molecular weight of the cytotoxic factor. Proteins of this fraction were separated in a polyacrylamide gel slab in the presence of sodium dodecylsulfate, after which the gel was cut into strips 3 mm wide, proteins were eluted from them, and their cytotoxicity determined. The cytotoxicity corresponded to a wide zone of proteins with mol. wt. of 28,000-45,000. These data agree with estimation of the molecular weight of the cytotoxic factor obtained by exclusion chromatography on Toyoperl HW50F in 0.2 M ammonium acetate solution (pH 7.2), as a result of which a value of 30,000-40,000 was obtained.

The rest of fraction C was subjected to reversed-phase chromatography on an Ultrapore RPSC column (Fig. 2). Only fraction 17 possessed cytotoxic activity.

The protein which we found in the serum of nude rats is evidently not identical with the TNF described previously [1, 6], for unlike the latter, it is not adsorbed on DEAE-cellulose under the conditions given in [1]; it has a relative molecular weight of 30,000-40,000, whereas the molecular weight of TNF is 17,000 daltons [6].

It is currently considered that the relative resistance of nude mice and rats to growth of tumor cells is due to an increased number of natural killer (NK) cells [3]. It therefore seems likely that the protein factor is related to the so-called NKCF proteins [2], which are

secreted by NK cells on contact with target cells. Recently, in a study of proteins secreted by NK cells, the writers found polypeptides with mol. wt. of 25,000-40,000 daltons, possessing cytotoxic activity. To establish the connection between these proteins and serum cytotoxic factor of nude rats, the study of their primary structures is essential.

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