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Purification of a Chalone-like Inhibitor for Ehrlich Ascites Mammary Carcinoma Cells from Bovine Mammary Gland

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Abstract—*A factor which inhibits the resumption of proliferation of stationary Ehrlich ascites mammary carcinoma (EAC) cells* in vitro *was isolated from the normal, lactating bovine mammary gland. After fractionation of the* 50,000 *g supernatant of the tissue homogenate by ammonium sulfate precipitation and gel filtration, it was further purified* 2600-*fold by ultrafiltration and lectin affinity chromatography. Polyacrylamide electrophoresis revealed one band containing all the activity. SDS gel electrophoresis showed a main band corresponding to a protein with a molecular weight of about* 15,000 dalton. *At this state of purity the factor concentration for half-maximal inhibition of proliferation was* 12 ng/ml *or* 8×10^{-10} M. *The effects of factor concentrations of* 4×10^{-9} *or* 4×10^{-8} M *are abolished by* 5×10^{-9} M *insulin almost completely or to* 70%.

INTRODUCTION

EAC CELLS from the stationary phase of growth *in vivo* (12–14 days after transplantation) resume proliferation in suspension culture and increase in number by 70 to 120% within 24 hr. Recently we described a new factor inhibiting this process with the following properties: its inhibitory effect is reversible [1]; it inhibits only the resumption of growth but does not, within the times investigated and the concentrations used so far, inhibit the ongoing growth of rapidly proliferating cells obtained either from the exponential phase of growth *in vivo* or stimulated to resume growth by serum before addition of the factor [1, 2]; the activity of the factor is counteracted by insulin and proinsulin [2, 3], both known as growth factors, insulin being an especially effective stimulator of normal and malignant mammary epithelium cell proliferation [4–7]. An almost maximal inhibition by the factor can be prevented by physiological concentrations of insulin [2, 3]; and the factor could be isolated from EAC cells (especially from the plateau phase) or the cell-free ascites fluid and from bovine mammary glands, but could not be detected in lung, liver, spleen, kidney, heart and leukemia L1210 of mice and in malignant lymph node, thymus, kidney and liver of the cow [2].

With regard to the non-cytotoxic action, the specificity as to the tissue of origin and its non-species specificity, the factor resembles a chalone according to the definition given by Bullough [8], while other properties, such as target cell specificity, have not been extensively investigated as yet. Therefore we call the factor 'chalone-like' for the time being. This report describes that, after purification from bovine mammary gland, the inhibitory activity can be found in a protein appearing as a single band in gel electrophoresis.

MATERIALS AND METHODS

Materials

Medium 199 with Hanks salts and calf serum were obtained from the Staatliches Institut für Immunpräparate, G.D.R. Proinsulin-free insulin was prepared by M. Krabiel, VEB Berlin Chemie. All other agents were of p.A. grade of purity.

Test system

A hyperdiploid line of the Ehrlich ascites mammary carcinoma was used. Once a week 10^7 cells in 0.5 ml of sterile isotonic NaCl solution

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were injected intraperitoneally into female AB mice. Twelve to fourteen days later the test cells in the plateau phase of growth were collected freshly for each experiment.

The cells were cultivated in suspension according to Negelein *et al.* [9]. The complete test medium consists of 6.4 mg medium 199 with Hanks salts per ml, supplemented with 4% calf serum, 2 mM disodiumphosphate, 2 mM monopotassium phosphate, 5.2 mM glucose, 2.8 mM fructose, 6 mM glutamate, 29 mM sodium bicarbonate, 0.2 mM reduced glutathion and antibiotics (100 I.U. penicillin and 50 μg/ml streptomycin). About 25×10^3 cells/ml were incubated under permanent shaking in Warburg flasks in a gas atmosphere of 1.5% O_2, 5% CO_2 in N_2 at 37°C for 24 hr. All samples were incubated in duplicate. Proliferation was evaluated by counting the cells in a hemocytometer. The differences between duplicate samples were below 5%.

Assay for the inhibitory activity

The inhibitory preparation (no more than 0.1 ml), or an equal volume of the appropriate solvent (control), were mixed with 2 ml test medium before adding the cells. After 24 hr the inhibitory effect was estimated from the difference between the increase in cell number in the controls and in the inhibitor-containing samples, and was expressed as % inhibition. The units of inhibitory activity are defined as follows: 1 unit/ml is the concentration of the inhibitor which gives half maximum inhibition of cell proliferation [2]. Under our conditions the maximal inhibition obtained is usually about 40–60% [2].

Preincubation experiments

After removal from the mouse, the cells were incubated in normal medium for 4 hr, centrifuged, resuspended and cultured as described before. After preincubation, cells from the plateau phase were no longer inhibited by our factor. This is in contrast to the effect of other cytostatic or cytotoxic agents, e.g. oxidized polyamines or synthetic inhibitors such as nucleoside analogues which are strongly inhibitory towards preincubated cells. Therefore this procedure is a simple way of avoiding the purification of cytotoxic activities and is performed at each purification step.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* [10], with bovine serum albumin as a standard.

EXPERIMENTS AND RESULTS

Preparation of a crude extract from bovine mammary gland

After removing most of the fat and connective tissue, the mammary gland (lactating stage) of a cow was deep-frozen and stored at -20°C until needed or used immediately. All further steps were performed at 4°C. About 1 kg of the tissue was homogenized in a Waring blender for 3 min at 15,000 rpm in 3 vol. of 10 mM phosphate buffer (with 10 mM NaCl), pH 7.4. After two successive centrifugations, first at 11,000 *g* for 15 min (Beckman J21 with rotor JA-10), then at 50,000 *g* for 60 min (Beckman L-3 with rotor 19), ammonium sulfate was slowly added to the supernatant up to 30% saturation. After 2.5 hr the resulting precipitate was removed by centrifugation at 6000 *g* (Beckman J21 with JA-10) for 30 min. The supernatant was brought to 60% ammonium sulfate saturation. After another 2.5 hr the precipitate was collected by centrifugation at 6000 *g* for 30 min and dissolved in 100 ml 10 mM phosphate buffer (with 10 mM NaCl). After a final centrifugation at 75,000 *g* for 45 min the almost clear supernatant (200 ml; 18 g protein, 30% of the protein in the first supernatant) was used for G-75 gel filtration.

It could be shown with a preparation purified by ultrafiltration as described earlier [1] that all inhibitory activity is recovered in the precipitate obtained between 30 and 60% ammonium sulfate saturation.

Further purification of the dissolved $(NH_4)_2SO_4$ *precipitate by G75 Sephadex chromatography and XM50 ultrafiltration*

The dissolved precipitate was chromatographed over a 7.5×1200 cm column ($V_0 = 1600$ ml) of Sephadex G-75 resin equilibrated in 10 mM phosphate buffer (with 10 mM NaCl), pH 7.4. Fractions of 200 ml were collected at a flow rate of about 20 ml/hr using equilibration buffer as the eluent. The crude fractions (sp. act. about 30 U/mg protein) were then further purified by XM50 ultrafiltration without loss of activity (ultrafiltration was used by Bichel [11] in his pioneer work on the chalone of an ascites tumour, the JB-1 plasmocytoma, and then by us for the factor for EAC cells [1, 2]). After ultrafiltration fraction 3 showed the highest specific activity (8000 U/mg) and was therefore chosen for further purification (Table 1). Fractions 4–7 were pooled and also further purified, solely to see whether their activity is due to the same protein as that in fraction 3.

Fractions 8 and 9 also showed an inhibitory activity which, however, was excluded from further purification because it could not be

Table 1. *Data on the inhibitor-containing G-75-fractions after XM50 ultrafiltration*

Fraction	Vol. (ml)	V_E/V_0 (G75)	mg protein/ml	Units/ml	Units/mg protein
1	200	1.0–1.2	n.d.	0	—
2	200	1.2–1.3	n.d.	0	—
3	200	1.3–1.4	0.025	200	8000
4	200	1.4–1.5	0.05	200	4000
5	200	1.5–1.6	0.08	100	1200
6	200	1.6–1.7	0.11	100	900
7	200	1.7–1.8	0.14	80	700

prevented by preincubating the cells (see Materials and Methods).

Affinity chromatography

Up to 20 ml of the solution from the preceding ultrafiltration step was applied to a wheatgerm agglutinin-Sepharose 4B column (1.5 × 30 cm; flow rate: 7 ml/hr; 2–4°C), which was then washed with dilute phosphate buffer (10 mM phosphate, 10 mM NaCl, pH 7.2) until the absorbance at 280 nm was down to background. Almost all of the activity was retained by the column and then eluted with 0.1 M 2-acetamido-2-deoxy-D-glucose in the dilute phosphate buffer. After exhaustive dialysis against this buffer practically all the activity could be recovered, and the specific activity of fraction 3 was 80,000 units/mg protein (Fig. 3). Compared to the specific activity of 30 units/mg after G-75 gel filtration (before ultrafiltration) this corresponds to a purification factor of 2600. However, in this fraction 90% of the protein present in the crude 50,000 *g* supernatant (for which an activity assay could not be done) had already been removed. Assuming no loss of activity, this would correspond to a purification of up to 26,000-fold. The pooled fractions 4–7 processed in the same way had a specific activity of 10,000 units/mg. protein. The factor was also retained by concanavalin A-, helix pomatia-, lens culinaris- and ricinus communis-Sepharose 4B, but not by peanut- and activated Sepharose 4B (not shown).

Disc gel electrophoresis

The eluate from the affinity column was exhaustively dialyzed in order to remove 2-acetamido-2-deoxy-D-glucose, concentrated again by ultrafiltration (Diaflo UM 10 membranes) and subjected to disc gel electrophoresis (8 μg protein/gel, gel dimension 0.5 × 5 cm, 2 mA/gel, 3 hr at room temperature). The electrophoresis was performed according to conventional methods using 7.5% polyacrylamide gel with 0.2% methylenebisacrylamide in Tris buffer, pH 8.9; electrode buffer: Tris/glycine, pH 8.3. One of the gels was stained for protein with Coomassie G 250, revealing the presence of one main band (see Fig. 1). The other gels were sliced according to the staining pattern. The protein bands were eluted with buffer in a Potter-type homogenizer, dialyzed again and tested for biological activity. In all preparations the activity was found in the main band section. All other sections of the gel did not show any activity (see Fig. 1). The pooled G-75 fractions 4–7 showed a few protein bands, the active band being in the same position as the one from fraction 3. Therefore all the activity found in the original fractions 3–7 was due to one and the same protein.

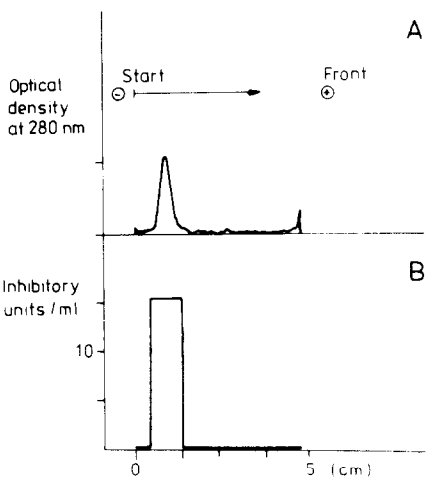

Fig. 1. *Disc gel electrophoresis. (A) Gel densitogram at* 280 nm *after fixation with trichloroacetic acid and (B) inhibitory activity in the polyacrylamide gel.*

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed as described by Fairbanks *et al.* [12] and Weber and Osborn [13], and revealed one protein band after staining with Coomassie blue as well as by the less sensitive but more specific u.v. ($E_{280\,nm}$) scan of the unstained gel (Fig. 2). The position of the bands as judged from that of the reference proteins corresponds to a protein with a molecular weight of 15,000 dalton. Only traces of proteins were detectable at positions of higher molecular weight.

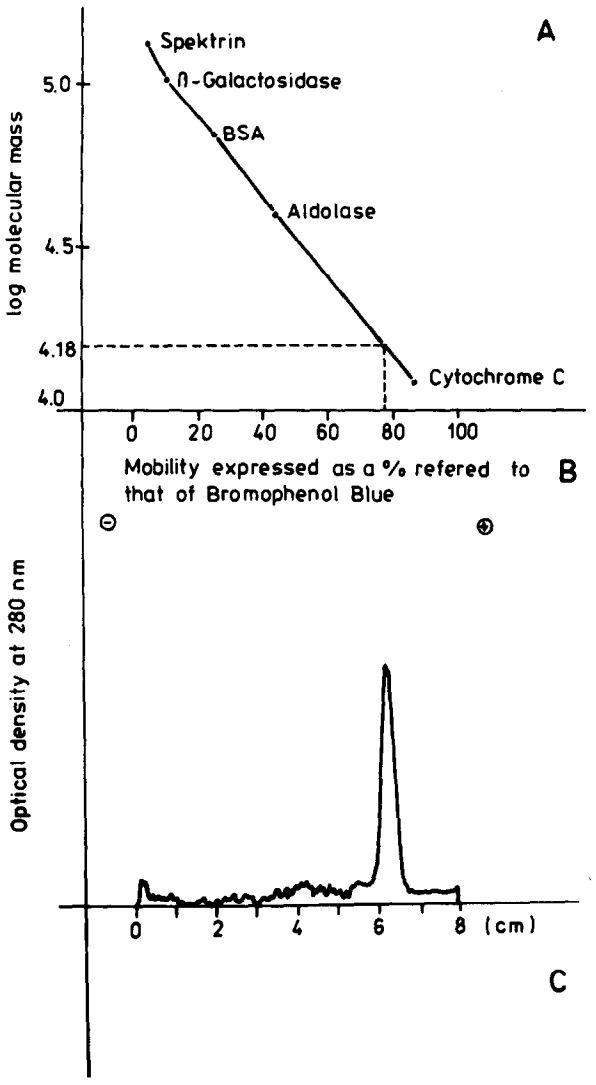

Fig. 2. SDS-polyacrylamide gel electrophoresis. (A) Molecular mass equilibration curve, (B) gel densitogram at 280 nm *after fixation with trichloroacetic acid and (C) gel after staining with Coomassie blue.*

Biological experiments

Dose-response relationship. Figure 3 shows the dose-response curve of the purified inhibitor. In accordance with results described earlier [2], this curve levels off at higher concentrations, resulting in a maximal inhibition of 50% (probably not all cells of the test cell population respond to the factor, G_2-phase cells being less responsive [2]). The factor concentration for half maximal inhibition (1 U/ml) is 12 ng/ml. This corresponds to a specific activity of 80,000 units/mg protein and, based on a molecular weight of 15,000 dalton, to a concentration of 8×10^{-10} M.

Insulin effect. Using a partly purified factor from ascites fluid of the EAC, we found that insulin, a well-known growth stimulator, abolishes the effect of the factor [3]. To obtain further proof for the identical biological properties of the factors from ascites fluid and from cow udder, we investigated the effect of insulin at

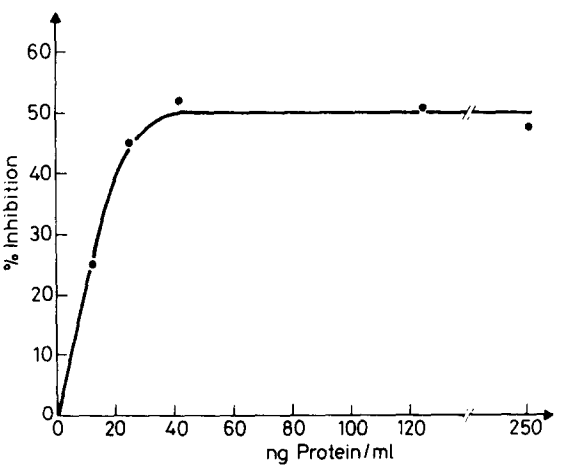

Fig. 3. Dose-response curve of the purified factor.

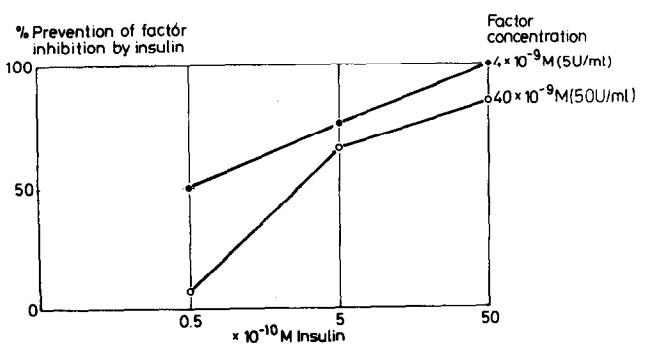

Fig. 4. Effect of insulin on two different factor concentrations. On the ordinate is given the percentage by which the inhibition by the factor (45%) is prevented by insulin. Cells were incubated without serum and with the insulin concentrations given in the presence and absence of the factor. After 4 hr *serum was added to a final concentration of* 4% *and* 20 hr *later the increase in cell number was estimated.*

two different factor concentrations. As shown in Fig. 4, the inhibitory effect of 5 or 50 factor units/ml (60 and 600 ng/ml or 4×10^{-9} M and 4×10^{-8} M respectively) is abolished almost completely or to 70% by about 300 ng/ml of insulin (5×10^{-9} M). This is in good agreement with the results published earlier for the factor preparation from ascites fluid [3].

DISCUSSION

With the methods described here we obtained a preparation showing only one single band in normal polyacrylamide electrophoresis which contains all the detectable activity. This band appears in the same position as the active band from earlier, less pure preparations, containing 4 other bands in addition. Altogether this has been highly reproducible, the active band now being found in the same position on the gel in about 15 different preparations (obtained, in part, with different methods and from 8 different udders). In SDS electrophoresis one main band, corresponding to a protein with a molecular weight of 15,000 dalton, is visible. Since they strongly depend on the amount and the dye-binding properties of proteins, as well as on the extent of staining and destaining, gel electrophoretic methods alone certainly yield insufficient evidence for the homogeneity of a protein with a very high specific activity.

However, the data for the specific activity of 80,000 units/mg protein and the molecular weight of 15,000 dalton allow some quantitative considerations and comparisons with other peptide hormones. For 1 kg of bovine mammary gland we find about 140,000 units (6 preparations from 2 different cow udders; the udder could be kept at -20°C for up to 6 months at least without loss of activity). This corresponds to about 1.5 mg of factor/kg tissue, a value some orders of magnitude higher than suggested earlier for chalones [14]. Furthermore, half maximal inhibition is obtained at a factor concentration (8×10^{-10} M) within the range of physiological concentrations of other peptide hormones (e.g. insulin $2\text{–}6 \times 10^{-10}$ M [15]). In our studies on the antagonism between insulin and the factor, both were effective at similar concentrations (see Fig. 4). The specific activity is certainly subject to some variations due to the biological test system (as all bioassays are), and at present we can neither prove nor disprove the possibility that the activity is due to a small molecule 'riding' on a larger one [16]. The protein nature of our inhibitor is demonstrated by its sensitivity towards trypsin and heat [1]. In addition, the molecule contains a carbohydrate moiety, indicated by its specific binding to immobilized lectin. The activity is unstable to repeated freezing and thawing but stable in 10^{-4} M mercaptoethanol solution for up to 2 months at -18°C. Preliminary results obtained with storage in the lyophilized state have been promising.

Its properties distinguish our factor from other inhibitors described for the EAC. All these factors are detected and followed by their drastic inhibitory effect on thymidine incorporation into DNA (an effect not exerted by our factor), while data on the inhibition of proliferation have not been reported so far. Gonzales and Verly purified a factor from bovine mammary gland acting somewhat more strongly on cells from mammary glands (or mammary carcinomas, such as EAC) than on other cells (e.g. HeLa). This factor was highly purified and has a molecular weight of 2000–3000 dalton [17]. Nakai [18, 19] investigated the effect of a partially purified 'chalone' preparation from EAC cells on nascent DNA synthesis and DNA polymerase in a 50–200 μg/ml concentration range. The author assumes a molecular weight of 10,000–15,000, similar to our factor, but his inhibitor is not retained on Concanavalin A-Sepharose. Rothbarth *et al.* found an inhibitor of 500–1000 dalton released from EAC cells and also points out its chalone-like character [20].

We have only a few preliminary results regarding the target cell specificity of our factor. A human mammary carcinoma cell line was inhibited to 60% by the factor (2 units/ml), while a human ovarial carcinoma was not inhibited by up to 20 units/ml. Also, the PHA stimulation of lymphocytes was not inhibited (10 units/ml). These results, together with others from investigations still under way, will be described in detail elsewhere.

This factor may be analogous for mammary epithelium to the one described by Holley *et al.* [21, 22], which has a high degree of specificity for kidney epithelium.

Our failure to inhibit rapidly proliferating EAC cells by the factor might be due to the test system used not properly reflecting *in vivo* conditions (e.g. cell density), so that influences on proliferating cells or the regain of sensitivity at special points within the cell cycle might be missed. Again, tumour cells, especially those with such a long history of malignant growth as the EAC cells, might be less responsive, so that an effect is detected only under the conditions described here. After all, it is surprising that these cells still respond to a factor from mammary tissue.

A lower response of rapidly proliferating cells

was also described for interferon [23], epidermal- [24–28], granulocytic- [29] and JB-1 ascites chalone [30]. The investigations on the factor will be continued with further biochemical characterizations and a study of its effects on the cell membrane and on its *in vivo* activity. It is tempting to speculate that the factor is a constituent of the recognition system on the cell surface [31, 32].

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