THE PURIFICATION AND PARTIAL CHARACTERIZATION OF A SERUM FACTOR ASSOCIATED WITH A NEOPLASM IN GOLDEN HAMSTERS

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SUMMARY

A previous report by Stevens and Schwenk (1) showed that serum from hamsters carrying ascites tumors in contrast to normal serum contained a factor that appeared to be proteinaceous in nature that induced cleavage in multinucleated hamster cells in tissue culture when estrogen was present. In this paper a method for purification of the factor(s) from ascites fluid that produces a 5000-fold purification is described. The factor has an electrophoretic mobility in acrylamide gel at pH 8.3 similar to prealbumin and concentrates in the pH gradient on electrofocusing in the region of pH 5.5-5.8. Although the chemical nature of the factor(s) is still uncertain, the electrophoretic properties are consistent with it being a simple or complex polypeptide or protein.

INTRODUCTION

Stevens and Schwenk (1) made the interesting observation that the relative number of polynucleated cells decreased from 20 to 10% in a culture of hamster ascites tumor cells when they were washed and incubated in medium containing estrogen and serum from cancer-carrying hamster, rats, or humans. Further they showed that this reduction in multinucleated cells resulted from enhanced cytokinetic activity of these cells rather than death of multinucleated or increased growth of uninucleated cells. In contrast to serum from cancer-carrying mammals, normal serum did not, under similar conditions, produce this effect. These results indicated that a factor(s) in the serum of cancer-carrying animals in the presence of estrogen promotes or induces cleavage in multinucleated cells.

Previous results suggested that the factor(s) in the serum of cancer-

carrying mammals was proteinaceous in nature (1). Therefore, using techniques common to protein chemistry, we have purified the factor(s) 5000-fold. Electrophoresis at pH 8.3 in polyacrylamide gels of both impure and pure samples of the active factor(s) showed that the cytokinetic activity migrated slightly ahead of albumin in a relatively sharp amido black-positive band and concentrated in the pH gradient in the region 5.5-5.8 on electrofocusing.

MATERIALS AND METHODS

Female golden hamsters, Mesocricetus auratus,[1] which had been ovariectomized at least fourteen days before, were injected intraperitoneally with 0.25 ml of hamster ascites tumor. Ten days later the hamsters were sacrificed and the ascites tumor was removed from the peritoneal cavity with a sterile needle and syringe. It was centrifuged for five minutes at 4,340 X g and the supernatant, henceforth called ascites fluid, was decanted. The active fraction was isolated in relative pure form from ascites fluid using the following procedure. An equivalent volume of saturated ammonium sulfate solution was added to the ascites fluid with constant shaking and the resulting precipitate was removed by centrifugation at 22,000 X g at 4°C for forty-five minutes. The supernatant which contained the active factor was decanted and crystalline $(NH_4)_2\ SO_4$ added to it to bring the salt concentration to 65% saturation. The precipitate that formed was collected by centrifugation and redissolved in 1.5 volumes of 1 M Tris, pH 9.2, (i.e., 1.5 ml for each 1 ml of original ascites fluid). Finally the solution was placed in a water bath maintained at 100°C and stirred constantly for 20 minutes. Under these conditions the factor(s) remained soluble and biologically active while other contaminating proteins were insoluble and precipitated from solution. After centrifugation at 22,000 X g for 30 minutes, the supernatant was removed and dialyzed against

[1]Obtained from Dennen Animal Industries, Inc., 405 Essex Avenue, Gloucester, Mass.

three changes of distilled water for 36 hours at 4°C and lyophilized and stored at 4°C until used. This material will be referred to as boiled ascites fluid. Finally the boiled ascites fluid was fractionated by acrylamide gel electrophoresis and the cytokinetic activity extracted from gel slices.

At each step in the purification procedure, fractions were assayed for heterogeneity by discontinuous polyacrylamide gel electrophoresis using the pH 8.3 anionic system as described by Davis (7).

Electrofocusing experiments were done as described in detail by Weller and co-workers (6). After measuring the pH the fractions were stored at 4°C until tested for activity, usually within 3-4 days. Initially, all fractions (i.e., entire contents of separational arm) were assayed for pH as well as for ability to induce cytokinesis in multinucleated hamster cells (1). However, activity was found only in the pH range of 5-6. Therefore, in later experiments only fractions in this range were tested.

All experiments were repeated at least twice and the results were statistically analyzed for significance.

RESULTS AND DISCUSSION

Electrophoretic analysis of fractions of the ascites fluid from animals carrying the tumor following various manipulations or treatments during purification showed clearly that the pre-albumin band (i.e., position A, Figure 1) increased in relative concentration until only it was seen in the electrophoretogram. This band, when sliced from the gel, extracted, and assayed, showed cytokinetic activity. In contrast, slices from other sections of the same gel column treated in a similar manner were inactive.

Serum from hamsters not carrying the active tumor (i.e., normal animals) was carried through the purification procedure along with that from tumor-bearing animals. Again the electrophoretogram showed solely pre-albumin. However, cytokinetic activity was not extracted from the pre-albumin region of the gel or from any other. Thus it is unlikely that the active factor(s)

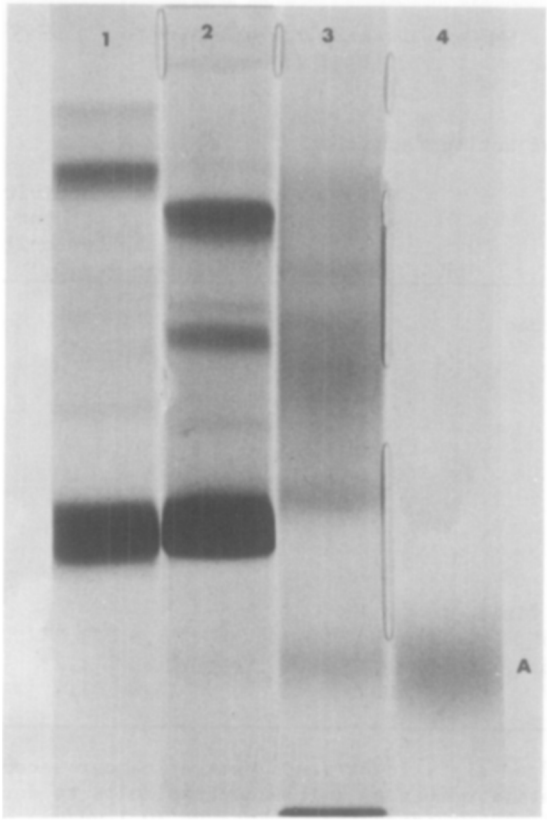

Fig. 1. Disc electrophoresis of (1) total ascites fluid, (2) 65% $(NH_4)_2SO_4$ insoluble, (3) boiled ascites fluid, and (4) pre-albumin band sliced from (3) and rerun. Cytokinetic activity was found only in the bands at point A. Migration is from top to bottom.

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is pre-albumin. It does migrate with the serum protein under the conditions of electrophoresis (i.e., Tris buffer pH 8.3).

The purification procedure outlined gives a substantial purification, since the minimum concentration of protein in the incubation medium required for activity (i.e., specific activity) decreases from 5mg/ml for the unfractionated ascites fluid to 0.1 μg/ml in the most highly purified fraction (Table 2). Furthermore the electrophoretograms show that the concentration of other serum proteins decreases relative to pre-albumin until they are no longer seen in the electrophoretogram of the most highly purified fraction. Similar results have been reported recently by Herranen (5).

TABLE 1

Isoelectric pH of active factor(s).

Material	pH of fraction	Cytokinetic activity of fractions: percent multinucleated cells (Mean ± S.D.)	percent decrease from control
Total ascites fluid			
Tube number 15	5.4	17.6 ± 1.5	7
16	5.8	11.3 ± 1.0	41*
17	6.5	16.7 ± 2.2	12
65% $(NH_4)_2SO_4$ insoluble			
Tube number 15	5.4	16.1 ± 1.0	15
16	5.6	10.8 ± 1.3*	43*
17	5.9	17.1 ± 1.5	10
Boiled ascites fluid			
Tube number 15	5.5	17.9 ± 1.3	6
16	5.8	11.8 ± 1.2*	38*
17	6.1	17.1 ± 1.4	10

*Decrease is judged significant since hamster cancer serum (0.1 ml/ml of culture) caused the relative number of multinucleated cells to decrease from 18.9±2.4% (control) to 11.4±1.6% (standard). Estrone (0.1 μg/ml) was added to all samples including control.

TABLE 2

Specific activity of fractions.[2]

Fraction[1]	mg/ml protein in incubation medium[2]
Total ascites fluid	5
65% $(NH_4)_2SO_4$ insoluble	4
Boiled ascites fluid	0.014
Pre-albumin band	0.0001

[1] See Figure 1 for corresponding acrylamide gel patterns of fractions.

[2] Specific activity defined as minimum concentration of protein required to give a significant decrease in the number of multinucleated cells.

Since the factor(s) exhibited properties expected of a protein, an attempt to determine its isoelectric pH was made using the technique of

electrofocusing. Three different samples, each more highly purified than the previous one, were electrofocused. It can be seen in Table 1 that the cytokinetic activity concentrated in the pH gradient in the region 5.5-5.8. It was not possible to obtain minimum effective dose data which would give an indication of concentration and relative purity of the active factor because of limited amounts of material. However, activity was detected in only a single fraction. The corresponding absorbance profile for the ascites fluid when corrected for absorbance of carrier ampholytes showed a peak near pH 5 that was probably due to albumin. The absorbance in the fractions showing cytokinetic activity was low, indicating that electrofocusing had separated the factor from albumin. The heterogeneity of the active fraction from the electrofocusing experiments has not been determined by acrylamide gel electrophoresis because of the limited amount of the material. However, electrofocusing obviously could be an important final step in the purification procedure. Accordingly, such experiments are planned.

In a control series of experiments the ampholytes alone (i.e., without sample added) were electrofocused and the contents of the separational arm showed no cytokinetic activity. In addition, a 2% ampholine solution did not inhibit cytokinesis in the presence of the active factor. Thus the carrier ampholites do not interfere with the biological assay.

While the exact nature of the factor(s) present in serum and peritoneal fluid of the hamsters carrying ascites tumors is not known, a procedure has been developed that gives a 5000-fold purification of it. Thus a more detailed characterization of the factor can now be done.

Its electrophoretic mobility as well as its apparent pI are consistent with the factor(s) being a simple or complex polypeptide or protein. However, the possibility that the active factor is adsorbed to pre-albumin has not been excluded, for separation of activity from pre-albumin has not been possible using technique employed in this study.

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REFERENCES

1. Stevens, D. F., and Schwenk, E., Experimentia 15:479 (1959).
2. Stevens, D. F., Exptl. Cell Res. 25:59 (1961).
3. Stevens, D. F., Exptl. Cell Res. 25:654 (1961).
4. Herranen, A., and Stevens, D. F. (Unpublished data)
5. Herranen, A., Proc. Amer. Assoc. Ca. Res. 10:38 (1969).
6. Weller, D. L., Heaney, A., and Sjogren, R. E., Biochim. Biophys. Acta 168:876 (1968).
7. Davis, B. J., Annals N.Y. Acad. Sci. 121; Art. 2:404 (1964).