REVERSIBLE INHIBITION OF CELL MULTIPLICATION BY A SMALL CLASS OF LIVER PROTEINS*

Jerome J. Freed+ and Sam Sorof

The Institute for Cancer Research, Fox Chase, Philadelphia, Pa. 19111

Received November 12, 1965

Metabolites of three types of carcinogens, the aminoazo dyes (1,2), N-2-fluorenylacetamide[1] (3), and polycyclic hydrocarbons (4), are bound preferentially *in vivo* to a small electrophoretic class (h_2) of relatively basic soluble proteins of the target organs (liver, liver, and skin, respectively). In contrast, the three subsequently induced tumors not only do not form h_2 protein-carcinogen conjugates[1] (2,4-6), but also have markedly reduced amounts of h_2 proteins themselves (2,4,7).

These observations have led to the proposal that certain proteins of this small class may function in normal cells as metabolic regulators, inhibiting autonomous growth and leading to cellular differentiation (8). Such an interpretation of the normal function of certain h_2 proteins would be considerably strengthened by the demonstration that proteins in this group are capable of controlling cell growth rate. In experiments reported here, a reversible inhibition of the multiplication of cultured cells of several lines of diverse origins has been produced by the soluble proteins of normal

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*Supported by Grants CA-05959, CA-05945, CA-06927 from the Public Health Service; Grant E-73 from the American Cancer Society; and by contract AT (30-1) 2356 with the U. S. Atomic Energy Commission. The competent and devoted assistance rendered by Susan Schatz, Emily M. Young, Carol B. Coffey, and Mary Porter is gratefully acknowledged.

+Public Health Service Career Development Awardee (CA-3401).

[1]Sorof, S., Young, E. M., McBride, R. Z., and Coffey, C. B. Manuscript in preparation.

rat liver. The activity is reversible and is confined to the h_2 proteins. The activity is non-dialyzable, is heat labile, and does not involve interaction with the non-protein components in the culture medium.

METHODS

Isolation of proteins. - Each preparation was isolated at 1-4° C from 4-5 adult male rats (Carworth CFN) as previously reported (2). Livers were perfused with 0.08 M sodium phosphate buffer, pH 7.8, containing 0.075 M NaCl; homogenized (1:1) in this buffer using a Potter-Elvehjem apparatus; and centrifuged at 105,000xg for 1 hr. Supernatant macromolecules were concentrated by dialysis in 0.20 M NaCl + 0.01 M sodium phosphate, pH 7.4, containing 20-22% purified clinical dextran. The concentrates were dialyzed against sodium veronal buffer, pH 8.6, ionic strength 0.02, containing 0.03 M NaCl. The extracts were diluted to 8.3-10.4% protein ("column extract"), and were electrophoresed (80 ma, 114 hrs, 2.2° C) in a column of purified ethanolized cellulose (225 cm x 3.1 cm). Protein concentrations of eluted fractions were assayed at 284 mμ yielding profiles of all the soluble liver proteins, as reported earlier (2).

To facilitate assay in cell culture, adjacent fractions throughout profiles were pooled and concentrated at 1-4° C by alternate dialyses (0.07 M NaCl + 0.01 M sodium phosphate, pH 7.4) and pervaporations. After final dialysis in 0.20 M NaCl + 0.01 M sodium phosphate, pH 7.4, the pools (1.5-2.0 ml at 0.01-1.6% protein) were stored at -15° C until assayed.

Cell culture assays. - Plastic petri dishes (6 cm) were inoculated with 3×10^4 L strain mouse fibroblasts (from Cell Culture Repository, line CCL-1) in 5 ml of growth medium containing Eagle's (9) minimum essential amino acids and vitamins, non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (all Microbiological Associates). Penicillin (100 U/ml) and streptomycin (50 μg/ml) were employed routinely. The multiplication rate of the cells in a single dish was determined by counting cells using a modification of the 'window' method (10). Cell number was

determined microscopically in a series of optically-defined windows representing a known fraction of the growth area of the dish. In estimates based on a series of 'windows' containing a total of 300-500 cells, the 95% confidence interval for the cell number lay within 15% of the mean value. Generation times for individual dishes had an average deviation of 7% from the mean value of 6 replicate dishes.

The protein fractions were incorporated into the growth medium, passed through 0.45 μ Millipore filters, and diluted with medium as required. Control cultures were prepared with equivalent additions of the phosphate -chloride buffer. Cell cultures were first incubated in normal medium for 24 hr at 37° C under 5% CO_2, the cell populations were determined, and the test medium containing protein was added. Cell populations were thereafter examined at 24 hr intervals.

RESULTS AND DISCUSSION

The effects of several concentrations of soluble liver proteins ("column extract") on the growth rate of L cells are shown in Fig. 1. Cells inhibited by 2.7 mg/ml and 0.27 mg/ml of added protein differ characteristically in appearance from cells in control medium. Cells developed long cytoplasmic processes, giving rise to a stellate rather than fibroblastic appearance.

When pooled fractions throughout the electrophoretic profile of the soluble proteins of normal liver (2) were assayed, significant and reproducible inhibition was produced only by the $\underline{h}_2$ proteins. This inhibitory activity has been observed in $\underline{h}_2$ fractions from 7 separate normal liver preparations. The minimum concentration of $\underline{h}_2$ proteins required to produce significant inhibition varied, with the most active preparations inhibiting at 4 μg/ml in the culture medium. The inhibitory fractions were in a narrow electrophoretic region centered at the slow $\underline{h}_2$ proteins. [The principal species of soluble liver azoproteins from rats fed azo carcinogens has previously been found to be in this same very small (1-2%) class of proteins (1,2,11).] Low inhibitions with

out morphological changes were occasionally observed with other fractions.

The h_2 proteins have inhibited L cells grown in suspension cultures, showing that the action was independent of cell-dish attachment.

The inhibition of cell growth is reversed by removal of h_2 proteins from the medium. L cells held at essentially constant cell number for 3 days were rinsed with control medium, and incubated in a second portion. Logarithmic multiplication resumed at a normal rate without measurable lag. Time lapse cinemicrography confirmed that all cells examined underwent division following removal of h_2 proteins.

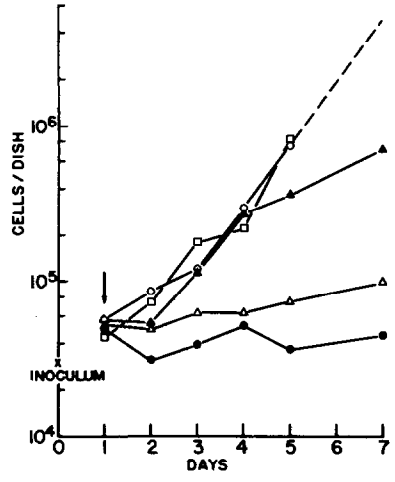

Fig. 1. Growth of L cells in several concentrations of added soluble proteins ("column extract") of normal rat liver. 3 x 10^4 cells were inoculated on day zero (✕). On day one (arrow), protein was added to the following final concentrations: (○) = none, (●) = 2.7 mg/ml, (△) = 0.27 mg/ml, (▲) = 27 μg/ml, (□) = 2.7 μg/ml. Dotted line extrapolates growth of the control culture in which the cell number per window became too high for accurate counting.

The factor responsible for the inhibitory activity of the h_2 proteins in medium is non-dialyzable and remains so after exposure to cell cultures. Medium containing h_2 proteins at a concentration 10 times that required to inhibit L cells strongly was dialyzed for 24 hr against an equal volume of control medium. In other experiments, after L cells, media, and h_2 proteins were incubated for 3 days, the media were separated from the cells and similarly dialyzed. In both cases, the activity failed to pass through the membrane.

The medium of a 3-days inhibited culture was filtered free of cells. Part of the medium was heated at 90° C for 3 min to destroy $\underline{h}_2$ proteins, and assayed on fresh cell cultures. The growth sustained was comparable to that of controls (no $\underline{h}_2$ and heated). The unheated medium continued to inhibit. The heat lability and non-dialyzability of the factor in $\underline{h}_2$-inhibited cultures both indicate that the regulatory effect is probably not a consequence of an interaction with non-protein components of the medium.

The $\underline{h}_2$ proteins were also tested on monolayers of other cell lines. HeLa (human), Cl4FAF28 (Chinese hamster), and RPD65 (grass frog embryo) were inhibited by $\underline{h}_2$ proteins at the same concentration range which arrested multiplication of mouse L cells.

A considerable number of parallel correlations support the hypothesis that the formation of $\underline{h}_2$ protein-carcinogen conjugates may be involved in the carcinogeneses by the aminoazo dyes, N-2-fluorenylacetamide[1], and polycyclic aromatic hydrocarbons [Introduction; (2,4,6,8)]. The present findings are consistent with the view that some proteins of the $\underline{h}_2$ family are able to regulate the multiplication of autonomously growing cells. The action on the cells is reversibly cytostatic, rather than cytotoxic. If certain $\underline{h}_2$ proteins normally function as a group of regulators, this behavior would be expected.

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