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Biochemical Estimation of the Basic Dye-binding Capacity of RNA from Rat Hepatoma[1]

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SUMMARY

Areas of hyperplastic livers that acquire hyperbasophilic properties at advanced stages of carcinogenesis apparently represent the sites of neoplastic transformation, and hyperstaining of cytoplasmic RNA with basic dyes also characterizes the cancer cells. Estimations of the RNA content of cell fractions from normal rat liver and solid Novikoff hepatoma provided no evidence that the intense staining of cancer cells could be explained on the basis of an increase in cytoplasmic RNA content.

The possibility that cytoplasmic fractions of Novikoff hepatoma show greater affinity for basic dyes than corresponding normal fractions has been examined by means of a test-tube toluidine blue-binding assay. The results revealed that the dye-binding capacity of total cytoplasmic fractions from tumors is 75% higher than normal after Carnoy fixation which retains mostly ribosomal RNA. Assays on fresh ribosomes indicated that tumor ribosomes bind 71% more toluidine blue per mg of RNA than the ribosomal preparation from normal liver.

This study thus demonstrates a greater affinity of tumor RNA for basic dyes, and a comparison of biochemical and cytophotometric analyses suggests that an increase in basophilia by a factor of about 2 would be due to a qualitative alteration in ribosomal RNA molecules and/or ribosome structure in cancer cells.

INTRODUCTION

Histochemical studies have suggested that areas of hyperplastic nodules that acquire hyperbasophilic properties at advanced stages of hepatocarcinogenesis are the actual sites of tumor development (4-6, 15). This hyperstaining of cytoplasmic RNA with basic dyes, which also characterizes neoplastic cells, has been tentatively explained on the bases of increased RNA content (12, 15-17), incorporation of exogenous RNA (4, 7), changes in RNA distribution, or physicochemical modification of RNA molecules (7). More recently, results obtained in this laboratory have suggested that the phenomenon of hyperbasophilia might be associated with alterations in rRNA molecules and/or ribosome structure (10).

In the present study, the RNA content of cell fractions has been determined in normal rat liver and solid Novikoff hepatoma to verify the possibility of an increased cytoplasmic RNA content in tumors. Since the results of these experiments revealed comparable amounts of RNA in the cytoplasm of normal and neoplastic hepatocytes, measurements of the basic dye-binding capacity of subcytoplasmic fractions were carried out using a test-tube toluidine blue-binding assay to determine whether a greater affinity of tumor RNA for basic dyes could be demonstrated by biochemical methods as well as by histochemical analyses.

MATERIALS AND METHODS

Animals. Male albino Sprague-Dawley rats (150 to 175 g) were used in this study. The animals were fed Purina laboratory chow and water *ad libitum*. Solid Novikoff hepatomas were obtained by s.c. injections of approximately 5×10^7 cells of the ascites form in the inguinal region. The tumors used were 7-day-old transplants. The animals were killed by decapitation, and normal livers or tumors were excised with scissors and placed on ice.

For studies on extraction of labeled RNA, some animals were given i.p. injections of 1 ml of tritiated orotic acid (0.25 mCi/ml of 0.85% NaCl solution) and were killed 8 hr later.

Preparation of Tissue Homogenates and Cell Fractions. The normal livers were minced with a plastic squeezer, whereas the tumors (less than 1 cm in diameter) were cut into small pieces with scissors after removal of attached tissue and macroscopically recognizable necrotic areas. An exact amount of tissue was homogenized in a glass test tube with a Teflon pestle to give a 10% homogenate in 0.25 M sucrose. The cellular fractionation was carried out according to the method of Schneider and Hogeboom (19).

Ribosome Preparation and Fractionation. Ribosomes were prepared according to the method of Tashiro and Siekevitz (20) with minor modifications (1). Membrane-bound and free ribosomes were obtained by centrifugation of the microsomal suspension in 0.001 M Tris-maleate buffer, pH 7.6, containing 0.001 M $MgCl_2$. Two ml of the

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suspension were layered over 28 ml of a 2 M sucrose solution and spun at 25,000 rpm for 24 hr in a swinging bucket Rotor SW 25.1. The top zone and the reddish membrane band were collected together as the membrane-bound ribosome preparation, while the ribosomes that sedimented or migrated in the bottom 10 ml composed the free-ribosome fraction. The ribosomes of both fractions were purified according to the method of Tashiro and Siekevitz (20).

The separation of the ribosomes into monosomes and polysomes of various sizes was carried out by centrifugation on linear 5 to 30% sucrose gradients containing 0.001 M $MgCl_2$. The gradients were spun at 25,000 rpm for 2 hr in a swinging bucket Rotor SW 25.1. Fractions of 1 ml were collected after centrifugation (8).

Protein and RNA Determinations. Proteins were measured by the biuret (9) or Folin (11) methods with bovine serum albumin as standard. The RNA was determined by the orcinol method (2) using highly polymerized yeast RNA as standard.

Dye-binding Assay. A test-tube assay has been developed to quantify the binding of basic dyes to cellular RNA in conditions comparable to the ones currently used for the staining of tissue sections. The histochemical method consists of staining of rehydrated tissue sections for 5 min in a 0.1% solution of toluidine blue (G. T. Gurr) in 0.3 M Veronal buffer, pH 5.0, and removing the excess dye with *tert*-butyl alcohol (6). The biochemical method used the same conditions except that a 1:10 dilution of the dye solution was used to permit spectrophotometric measurements.

One ml of an RNA solution or of an isolated cellular fraction was mixed with 4 ml of a 0.01% solution of toluidine blue (G. T. Gurr, London, England) in 0.03 M Veronal buffer, pH 5.0. After standing at room temperature for 5 min, trichloroacetic acid was added at a final concentration of 3%. The precipitated dye-RNA complex was recovered by centrifugation and washed with 3 ml of *tert*-butyl alcohol. The washed sediment was completely dissolved in concentrated formic acid and the absorbance of the solution was measured at 630 nm. An aliquot was used for the determination of RNA by the orcinol reaction. The values were corrected for the absorbance due to formic acid and dye.

Fixation of Cytoplasmic Fractions. In experiments on the effect of Carnoy (6:3:1) fixation, the cytoplasmic fractions obtained by centrifugation were put in a dialyzing bag, and the bag was immersed in Carnoy's fluid for 24 hr at room temperature. The amount of Carnoy's fluid relative to fresh tissue was 50 ml/g. After fixation, the fixed material was suspended in water.

RESULTS AND DISCUSSION

RNA Distribution. The RNA contents of tissue homogenates of normal liver and Novikoff hepatoma did not show any significant differences on a wet-weight basis (10.0 and 11.0 ± 0.5 mg (S.D.), respectively). The amount of RNA in the nuclear fraction was slightly lower in normal liver (2.0 ± 0.2 mg) than in hepatomas (2.7 ± 0.3 mg). The reverse was observed for the mitochondrial fraction with respective values of 1.7 ± 0.2 and 0.6 ± 0.01 mg. The microsomal and supernatant fractions of normal liver contained, respectively, 4.6 and 1.7 ± 0.4 mg of RNA per mg of tissues, whereas the values for Novikoff hepatoma were 1.7 ± 0.4 and 5.3 ± 0.6 mg.

These agree with those previously published (18) and reveal no significant difference between the cytoplasmic RNA content of normal and tumor tissues (8.0 and 7.8 mg, respectively). The different distribution of RNA noted in tumor postnuclear fractions is probably due to the presence in this tissue of a large amount of free ribosomes which do not sediment with the membrane-bound ribosomes of the microsomal fraction but remain in the supernatant solution. This altered distribution would thus simply reflect a modification of the ratio of membrane-bound to free ribosomes, rather than a change in proportions of ribosomal *versus* other types of RNA.

However, if total cytoplasmic RNA remains constant in tumors, the situation might be different for the amount of RNA retained in fixed tissues, and this point was verified by studying the effect of Carnoy fixation on RNA extraction. The results indicated that Carnoy's solution extracts about 30% of labeled cytoplasmic RNA (35 and 27%, respectively, for normal liver and Novikoff hepatoma). The extracted RNA appears to be mainly cytosol RNA, since supernatant fractions dialyzed against Carnoy's fluid show RNA losses of the order of 50% (47 and 45%, respectively, for normal and tumorous tissues). Thus the cytoplasmic RNA retained after Carnoy fixation represents approximately 70% of the original amount and must consist mostly of rRNA. The analyses further revealed that the amount of cytoplasmic RNA retained in fixed tumor tissue is comparable to normal.

rRNA Content. Since rRNA may be responsible for the hyperbasophilic properties of tumor cells in tissue sections (10), it was felt desirable to pursue the biochemical analyses on purified ribosomes to determine more precisely the rRNA content of normal and neoplastic hepatocytes.

The results on purified ribosomes and aggregates prepared by the method of Tashiro and Siekevitz (20) give values of 1.8 ± 0.2 and 1.9 ± 0.7 mg of RNA per g of tissue for normal liver and Novikoff hepatoma. Values of 0.3 ± 0.1 and 0.4 ± 0.2 mg were obtained for the respective aggregate fractions. The ribosomes obtained by this method include the ribosomes detached from the microsomal membranes by deoxycholate and the free ribosomes, while the aggregate fraction consist of low-speed-sedimentable material remaining after solubilization of the microsomal membranes with sodium deoxycholate. The RNA contents of both the ribosomal and aggregate preparations were similar for normal liver and Novikoff hepatoma, and the amount of RNA found in the aggregate fraction of both tissues represented about 20% of the value obtained for the ribosomal preparation.

These results on the rRNA content of normal liver are consistent with values previously reported (8), and the experiments on Novikoff hepatoma reveal no significant increase in rRNA of cancer cells. It thus seems that the increased RNA staining shown by tumor sections could be

attributed to neither an increase in total cytoplasmic RNA content of fresh or fixed tissue nor to a change in the amount of rRNA in tumors cells.

Dye-binding Assays. To determine whether cytoplasmic and, more particularly, rRNA show increased dye-binding capacity in neoplastic hepatocytes, a dye-binding assay has been devised and applied to cell fractions of normal liver and Novikoff hepatoma. Measurements of the amounts of toluidine blue that bind to known amounts of highly polymerized yeast RNA in test-tube assays gave a linear curve of basic dye binding with increasing amounts of RNA. Similar results were obtained for toluidine blue binding with increasing amounts of various cellular fractions.

Dye-binding Capacity of Cytoplasmic Fractions. The assays with crude cytoplasmic fractions are reported in Table 1. Differences were noted in the dye-binding capacity of various cytoplasmic fractions of normal liver, and all of the values obtained for tumor fractions were lower than the corresponding normal values. Taking into account the RNA content and the dye-binding capacity of each fraction, the total dye-binding capacity of Novikoff hepatoma crude cytoplasmic fractions averages 80% of the value obtained for normal liver.

These results could not be easily interpreted in terms of cellular constituents because of the differences in the dye-binding capacity of the various fractions and the different distribution of RNA in tumor cell fractions. These experiments were thus pursued on fixed tissues and purified ribosomes to facilitate comparisons with histochemical analyses.

Dye-binding Capacity of Fixed Cytoplasmic Fractions. The results of toluidine blue binding to fresh and fixed cytoplasmic fractions are shown in Table 2. While the dye-binding capacity of total cytoplasmic RNA of normal liver is not significantly altered by Carnoy fixation, the amount of basic dye that binds to cytoplasmic RNA retained in fixed tumor tissue is about twice that obtained for dye-binding per mg of RNA in the fresh preparation. The basic dye-binding capacity of fixed tumor cytoplasm is then 75% higher than that of the corresponding fixed cytoplasmic fraction from normal liver.

Thus the biochemical assays reveal that cytoplasmic RNA of fixed tumor tissue does show hyperbasophilic properties, as noted in histochemical analyses. The differences between fresh and fixed material presumably result from the selective retention of rRNA in fixed tissue, and assays on purified ribosomes were carried out to clarify this point.

Dye-binding Capacity of Isolated Ribosomes. The results of dye-binding assays on purified ribosomes and ribosomal subfractions are summarized in Table 3. The total ribosomal fraction of Novikoff hepatoma was observed to bind 71% more toluidine blue per mg of RNA than the corresponding fraction from normal liver.

Analyses of membrane-bound and free ribosomes indicated that the membrane-bound ribosomes show similar dye-binding capacities in normal and neoplastic tissues, whereas the free ribosomes from hepatomas bind 61% more toluidine blue per mg of RNA than the corresponding fraction from normal liver. Assays on monosomes and polysomes of various sizes further revealed that the increased basophilia of tumor ribosomes results mainly from increases in the dye-binding capacity of the polysomes.

The results on Novikoff hepatoma total ribosomal fraction are thus very close to those obtained with the Carnoy-fixed cytoplasmic fraction. They support the view that ribosomes are responsible for the hyperbasophilic properties of fixed tumor tissue, and suggest that the phenomenon rests mainly on some modification of the free polysomes.

It seems that the degree of hyperbasophilia may vary to an appreciable extent from one tumor to another since the variations in ribosomal fractions were somewhat larger in tumors than in normal livers. These variations may be due however to partial hydrolysis by extracellular RNases. The RNA responsible for hyperbasophilia is known to be highly sensitive to RNase (3, 5, 10), and relatively low values were occasionally obtained in subsequent experiments on the dye-binding capacity of ribosomal fractions isolated from

Table 1

Toluidine blue binding of fresh cytoplasmic fractions from normal liver and Novikoff hepatoma

Fraction	Normal liver (A_{630} nm/mg RNA)		Novikoff hepatoma (A_{630} nm/mg RNA)
Mitochondrial	9.6 ± 0.3[a]	(4)[b]	6.5 ± 0.5 (4)
Microsomal	5.2 ± 0.2	(4)	4.1 ± 0.3 (4)
Supernatant	15.5 ± 2.8	(4)	7.2 ± 0.3 (4)

[a] Mean ± S.D.
[b] Numbers in parentheses, number of assays.

Table 2

Toluidine blue binding of fresh and fixed postnuclear fractions from normal liver and Novikoff hepatoma

Postnuclear fraction	Normal liver (A_{630} nm/mg RNA)		Novikoff hepatoma (A_{630} nm/mg RNA)
Fresh	6.7 ± 0.2[a]	(5)[b]	5.3 ± 0.4 (5)
Fixed	6.4 ± 0.4	(5)	11.2 ± 1.2 (5)

[a] Mean ± S.D.
[b] Numbers in parentheses, number of assays.

Table 3

Toluidine blue binding of fresh ribosome preparations from normal liver and Novikoff hepatoma

Fraction	Normal liver (A_{630} nm/mg RNA)		Novikoff hepatoma (A_{630} nm/mg RNA)
Total ribosomes	3.5 ± 0.2[a]	(4)[b]	6.0 ± 2.0 (5)
Membrane-bound ribosomes	3.8 ± 0.5	(3)	3.7 ± 0.7 (4)
Free ribosomes	3.1 ± 0.5	(3)	4.9 ± 1.6 (4)
Monosomes	3.1 ± 0.1	(3)	3.5 ± 0.1 (2)
Di-tetrasomes	3.1 ± 0.1	(3)	4.1 ± 0.3 (2)
Tetra-heptasomes	2.2 ± 0.6	(3)	3.9 ± 0.2 (2)
Large polysomes	2.2 ± 0.3	(3)	3.9 ± 0.0 (2)

[a] Mean ± S.D.
[b] Numbers in parentheses, number of assays.

tumors with large necrotic areas.

The values of 75 and 71% obtained by biochemical analyses for the increases in the dye-binding capacities of the fixed cytoplasmic fraction and the ribosomal fraction of Novikoff hepatoma are in good agreement with the figure of 80% obtained by cytophotometric estimations for the increase in the staining intensity of dividing cells in hyperbasophilic foci and hepatomas, as compared to dividing normal hepatocytes (M. C. Moulin-Camus and R. Daoust, unpublished results). The figures for the resting cells in hyperbasophilic foci and hepatomas were, however, 2 to 4 times higher than the intensities shown by homologous normal tissue in cytochemical analyses (14). It appears from these studies that an increase in basophilia by a factor of about 2 would be due to a qualitative alteration in cytoplasmic RNA or RNA-containing structure, most likely the ribosomes, while the higher staining intensities detected by cytophotometric measurements on tissue sections would result from changes in concentration associated with modifications in nucleocytoplasmic ratio (5, 13).

The present study provides a quantitative evaluation of the phenomenon of hyperbasophilia apparently linked to the onset of the neoplastic transformation (5) and strengthens the view that it rests on some modification of the ribosomes in tumor cells.

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