RADIOIMMUNOASSAY OF SERUM ALBUMIN PRODUCED BY HUMAN EMBRYONIC LIVER IN MONOLAYER CULTURE

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INTRODUCTION

Fetal liver contains hematopoietic and mesenchymal cells in addition to hepatic parenchymal cells. Culture conditions would be expected to favor survival of certain of these cell types at the expense of others (3). In order to study the survival of hepatic parenchymal cells in culture, a marker that is specific for differentiated parenchymal cells is highly desirable (5, 12). Morphologic characterization is obviously inadequate. Although histochemical localization of intracellular enzymes or glycogen may be helpful, such markers are often nonspecific and require sacrifice of the cells. Albumin, on the other hand, is a secreted protein and is thought to be synthesized exclusively in hepatic parenchymal cells *in vivo* (8). In the present report, a radioimmunoassay for human albumin is described and utilized to monitor albumin production by human fetal liver in monolayer culture.

MATERIALS AND METHODS

Tissue Culture

The cultivation of primary human embryonic liver is reported elsewhere (2). The culture medium was that of Leibovitz (L-15, Microbiological Associates, Inc., Bethesda, Md.) supplemented with 0.042% arginine (HCl), 0.03% glutamine (Microbiological Associates, Inc.), 0.1% dextrose, potassium penicillin G, 150 μ g/ml, streptomycin sulfate, 250 μ g/ml, and 10% fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.). Inocula of 5×10^6 cells in 0.5 ml of medium were seeded into 16 $\times$ 100 mm constricted

glass tubes (Rochester Scientific Co., Rochester, N. Y.) and incubated in air. The resulting cell growth was washed with medium after 2 days and cultivated a further 3 or more days before the medium was assayed for human albumin. The number of apparent hepatic parenchymal cells between 3 and 10 days of culture was approximately constant by direct cell counts of stained preparations.

A cell line composed of fibroblasts morphologically was derived from a liver culture that appeared to be overrun with fibroblasts on initial passage. The line was maintained in the standard growth medium and continued to resemble fibroblasts, with passage by trypsinization every three or four weeks. HeLa cells, human muscle-skin fibroblasts, and mouse "L" cells were similarly maintained. Because liver parenchymal cells in monolayer generally survived poorly after passage, they were studied in primary culture only.

Cellulose-Conjugated Antibody and Radiolabeled Albumin

Bromoacetyl cellulose (BAC) was prepared as described by Robbins et al. (11), and sheep anti-human albumin (Calbiochem, Los Angeles, Calif.) was conjugated to BAC by the method of Mann et al. (6). Human albumin (grade III, Sigma Chemical Co., St. Louis, Mo.) was labeled in the presence of 1.0 mCi Na-¹²⁵I (New England Nuclear Corp., Boston, Mass.) (7). The labeled albumin was freed of unreacted ¹²⁵I by dialysis against several liters of 0.14 M saline at 4°C and had an initial specific activity of 0.4 mCi/mg. Of the radioactivity in the final preparation, 92% was precipitable by antialbumin.

Incubations of BAC-antibody and albumin (6)

were carried out in Veronal-buffered saline (VBS) containing 0.1% gelatin, pH 7.40. For the study of *de novo* synthesis of albumin, Eagle's minimal medium minus leucine (MEM-L) was obtained from Grand Island Biological Co., Berkeley, Calif., and leucine- ^{14}C (specific activity, 72 mCi/mole) was obtained from New England Nuclear Corp.

FIGURE 1 Titration of BAC-antibody. Albumin- ^{125}I , 0.13 μg (3000–5000 cpm), was incubated in duplicate with serial dilutions of BAC-antihuman albumin in VBS, as described elsewhere (6). After pelleting of the BAC-antibody with bound albumin- ^{125}I , supernatant radioactivity was determined in a well-type scintillation counter.

RESULTS

The titration of BAC-antibody against a standard quantity of albumin- ^{125}I is represented in Fig. 1. An amount of BAC-antibody that bound approximately 50% of the added labeled albumin was chosen for the standard assay. When this amount of BAC-antibody and the standard quantity of albumin- ^{125}I were incubated with unlabeled albumin, label was displaced to the supernatant. The amount of label displaced was proportional to the amount of unlabeled albumin present, when this was in the range 0.05–0.50 μg (Fig. 2), and the resulting curve served as a reference for assay of unknown samples. Because it appeared that the assay was most accurate in a narrower range (0.08–0.20 μg), unknowns were assayed until a reading on this portion of the reference curve was obtained. Volumes of culture medium up to 0.4 ml were assayed without affecting accuracy (6).

Dilutions of whole human serum with comparable amounts of albumin gave a displacement curve that was essentially identical to the reference curve (Fig. 2). Because the culture medium usually contained 10% fetal calf serum, bovine albumin was also assayed. It showed no significant cross-reactivity, however, over the range present (0.1–0.5 mg) in the sample volumes usually assayed.

FIGURE 2 The reference curve for radioimmunoassay of human serum albumin (HSA). A standard amount of BAC-antibody (see text) and albumin- ^{125}I , as in Fig. 1, were incubated with known amounts of unlabeled crystalline human serum albumin ($\bullet$), whole human serum ($\circ$), or pure bovine serum albumin (BSA) in culture medium ($\triangle$). After pelleting of the BAC-complex (6), the ratio of counts per minute released by unlabeled albumin to the total bindable counts per minute was determined (R/TB). The vertical lines represent the standard deviations for a series of determinations carried out with the same reagents over a period of several weeks.

TABLE I
Albumin Production by Various Cell Cultures

Culture	Albumin ($\mu\text{g}/\text{tube}$ per day)
Human embryonic liver (5.0, M)*	1.0
" (11.5, M)*	0.6
" (4.0, F)*	0.8
" (12.7, M)*	1.0
" (6.0, F)*	0.7
Liber fibroblast	<0.02 (<0.02)‡
HeLa cells	<0.02 (<0.02)‡
"L" cells	<0.02
Human muscle-skin fibroblast	<0.02

* Fetal crown-rump dimension (cm), sex.

‡ Cell lysate

Monolayers of human embryonic liver, prepared as described in Materials and Methods, were grown for 2 days, then washed. The albumin produced during the subsequent 3 days was measured (see legend to Fig. 2). The line cells were grown to full monolayers (5–10 days). Lysates were prepared by repeated freezing and thawing, suspended in the original volume of medium, and assayed for albumin. The standard inoculum of trypsinized liver contained 1.07 μg albumin.

TABLE II
Leucine- ^{14}C Incorporation into Albumin by Various Cell Cultures

Culture	Total secreted albumin ($\mu\text{g}/\text{tube}$)			Leucine- ^{14}C in albumin ($\mu\text{moles}/\text{tube}$)		
	Day 5	Day 7	Day 5/Day 7	Day 5	Day 7	Day 5/Day 7
Liver cells						
HS	1.5	2.5	0.60	26	38	0.68
HS	—	—	—	21	37	0.56
FCS	0.5	0.8	—	8	—	—
FCS	0.3	0.4	—	7	—	—
Liver fibroblast	—	<0.02	—	—	0	—
Disintegrating liver cells	—	<0.02	—	—	0	—
HeLa cells	<0.02	—	—	0	—	—

The cultures were grown as described in Materials and Methods. Liver cell cultures contained either 1% human serum (HS) or 10% fetal calf serum (FCS). At day 2, the cultures were washed and then incubated in MEM — L, supplemented with glutamine, antibiotics, as above, and 1.0 $\mu\text{Ci}/\text{ml}$ leucine- ^{14}C . After 3 or 5 days of further incubation, 0.2 ml of culture medium was taken for assay of albumin- ^{14}C . The sample was treated initially with a nonspecific BAC conjugate (to guinea pig serum protein) to remove extraneous labeled material, which was pelleted by centrifugation at 1500 g for 20 min (6) and washed once. The combined supernatants were divided between two tubes, each containing BAC-antialbumin. Unlabeled albumin was added in excess (100 μg) to one tube of each pair, the final volumes were adjusted to 0.5 ml with VBS, and the tubes were shaken for 3 hr at room temperature. After centrifugation, the BAC-antibody with bound label was washed with cold leucine and collected on a Millipore filter (0.45 μ). The filters were dried and their radioactivity was determined by liquid scintillation counting. Albumin- ^{14}C was estimated from the amount of radioactivity displaced (100–700 cpm/0.1 ml of original sample) from the BAC-antibody by cold albumin. Total secreted albumin was measured by the standard radioimmunoassay on the same samples of culture medium.

The albumin produced by cultures of five different human fetal livers is shown in Table I. The time course of such production is presented elsewhere (2). Neither the culture medium nor cell lysates of various other cells contained detectable albumin.

The data of Table I suggest that *de novo* synthesis of albumin is occurring, in that the albumin secreted, over the period tested, exceeds by 50–200% the amount of albumin in the initial cell inoculum (see legend to Table I). Moreover, albumin secretion during the first two days in culture has been disregarded. Further evidence for the *de novo* production of albumin was the incorporation by liver cell cultures of a labeled amino acid precursor into secreted albumin. Electrophoresis of media from liver cell cultures that had been incubated with leucine- ^{14}C demonstrated a peak of radioactivity migrating with albumin. The medium from simi-

larly treated HeLa cell cultures gave no such peak (Fig. 3). The albumin- ^{14}C of liver cell cultures could also be measured immunologically through its ability to reversibly complex with specific BAC-antibody. No albumin- ^{14}C was found in media from fibroblast and HeLa cultures treated in a similar fashion (Table II). Moreover, in the liver cultures, immunologically measurable albumin- ^{14}C increased with time to the same extent that total secreted albumin increased, as measured by the standard radioimmunoassay. Because the intracellular pools of albumin and leucine were unknown, a quantitative comparison of total albumin secretion and *de novo* synthesis was not possible. The data suggest, however, that secretion and synthesis closely parallel one another in liver cell cultures.

DISCUSSION

Albumin secretion by cultures of rat hepatoma cells has been demonstrated previously by immunoprecipitation of newly labeled albumin (9) and by complement fixation (10). Both techniques, however, are more laborious and generally less sensitive than the present method. The coupling of antibody to BAC renders the antibody insoluble and thereby avoids the time-consuming precipitation steps of other radioimmunoassays, a run of several dozen tubes can be completed in less than 4 hr. Its sensitivity, like that of other radioimmunoassays, depends primarily on the specific activity of the labeled albumin. The sensitivity of the present assay proved to be particularly convenient since the albumin of most cultures could be measured without extensive dilution of the unknown sample.

The incorporation of labeled precursor into albumin by fetal liver was first demonstrated several years ago with liver slices and indicates that human fetal liver is capable of albumin synthesis (4). The specificity of this function for hepatocytes in culture, however, has been questioned with the report of albumin synthesis by HeLa cells (1). We have been unable to detect albumin in cultures of similar cells (Tables I and II); if albumin is produced by HeLa cells, the amount appears to be two or more orders of magnitude less than that produced by hepatic parenchymal cells in primary culture. The parenchymal cells appear, moreover, to be solely responsible for the albumin measured, since the amount secreted correlates well with the apparent extent of parenchymal cell growth in a mixed culture of human embryonic liver and with

FIGURE 3 Electrophoresis of culture fluid from liver cells and HeLa cells incubated with leucine- ^{14}C . The media analyzed were those of the HS liver cell culture and HeLa culture of Table II. The liver cell medium, with added cold leucine and carrier albumin (200 μg), was dialyzed against isotonic saline and then concentrated 40-fold with respect to the volume of the initial sample. HeLa cell medium was treated similarly for a 200-fold concentrate. Each concentrate was electrophoresed on cellulose acetate strips in 0.05 M Tris-barbital buffer, pH 8.8, at 1.5 v/cm for 2 hr. The strips were assayed for radioactivity in a gas-flow scanner (Tracerlab, Waltham, Mass.) and compared to a reference electrophoresis of normal human serum stained for protein which demonstrated the portion of serum albumin (ALB) and globulins (α_1 , α_2 , β , and γ). Bold line, liver cell medium; light line, HeLa medium.

other indications of parenchymal cell differentiation (2). Albumin secretion, therefore, may be particularly useful for evaluating parenchymal cell survival and differentiation in tissue culture of embryonic liver.

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