EFFECT OF BENZO(a)PYRENE ON MONOLAYER CULTURES OF NORMAL AND MALIGNANT MOUSE LIVER CELLS

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Cells of a monolayer culture of embryonic mouse liver, like cells of a culture of highly malignant hepatoma 22A, maintained by transplantation for 20 years, actively metabolized the carcinogenic hydrocarbon benzo(a)pyrene and are highly sensitive to its toxic action. Considering that liver tissue in vivo is resistant to carcinogenic hydrocarbons, the authors suggest that this resistance is due to factors acting at the organ or organism level but not at the cell level. The problem of the mechanism of preservation of the sensitivity of hepatoma 22A to the toxic action of benzo(a)pyrene also is discussed.

KEY WORDS: *benzo(a)pyrene; liver; hepatoma; tissue culture.*

Nonspecific oxidases of microsomes, converting polycyclic hydrocarbons from "procarcinogenic" and "protoxic" compounds into carcinogenic and toxic metabolites, are most active in cells of the liver [9]. Meanwhile the liver in vivo is resistant to the action of these compounds [10, 12]. This fact requires explanation, more especially because most investigations into the metabolism of carcinogenic hydrocarbons have been carried out with liver tissue homogenates. Since the resistance of the liver to such compounds may be due to special features acting both at the organ and at the cell level, it was regarded as important to compare the ability of a culture of liver cells to metabolize benzo(a)pyrene (BP) with its sensitivity to this carcinogen. In addition, the metabolism of BP was investigated in a culture of the highly malignant hepatoma 22A and its action on the cells of that tumor in order to discover whether these indices change during the malignant transformation of liver cells.

EXPERIMENTAL METHOD

The liver of mouse embryos (12-14 days of development) of strain C3HA was dispersed in a mixture of equal volumes of trypsin and versene solutions. A cell suspension ($5 \cdot 10^6$-$8 \cdot 10^6$ in 1 ml) was introduced into Carrel's flasks or petri dishes and cultured in Eagle's medium with a double concentration of amino acids and vitamins and 20% bovine serum [5]. The culture of tumor cells was adapted to growth in vitro by Perova by cloning the ascites form of hepatoma 22A obtained in 1953 by Gel'shtein [3]. Tumor cells were seeded at the rate of $0.1 \cdot 10^6$-$0.2 \cdot 10^6$ cells/ml and cultured under the same conditions as the embryonic liver cells.

BP metabolism was determined by a fluorescence-spectral method [4] and with the aid of radioactive labeling. In both cases BP was added to the cell monolayer (3-5 days of culture) up to a final concentration of 0.5-0.6 or 10 μg/ml. In the second method, together with "cold" BP, [^{3}H]BP was added up to a final activity of 2 μCi/ml. The residue of unmetabolized carcinogen was extracted with n-octane after 1, 2, and 3 days and determined quantitatively by the fluorescence-spectral method or from the radioactivity of the octane extract.

The toxic effect of BP was determined from the change in the mitotic index and density of the cell monolayer. Cells were cultured on coverslips in petri dishes. After formation

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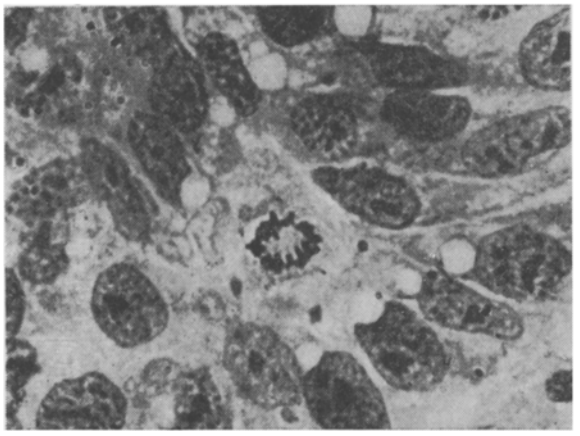

Fig. 1. Monolayer of mouse hepatocytes (fourth day of culture). Mayer's hematoxylin, 700 ×.

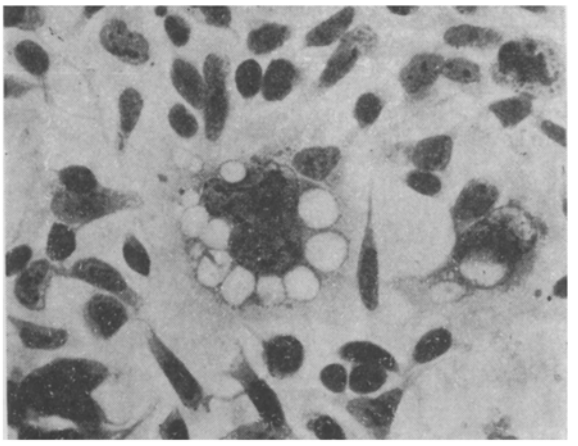

Fig. 2. Hepatoma 22A (fourth day of culture). Mayer's hematoxylin, 300 ×.

TABLE 1. Metabolism of BP by Cultures of Mouse Embryonic Liver Cells and Hepatoma 22A (M ± σ)

Culture	Quantity of BP metabolized, percent of original quantity of BP					
	incubation with BP in concentration of 0.5-0.6 μg/ml				incubation with BP in concentration of 10 μg/ml	
	expt. No.	24 h	48 h	72 h	expt. No.	72 h
Mouse liver cells	1	37±3	98±0	100±0	8	55±3
	2	57±8	86±3	—	9	82±3
	3	89±3	100±0	100±0	10	92±4
	4	86±4	93±7	95±1	11	100±1
Hepatoma 22A cells	5	17±1	64±9	88±2	12	40±1
	6	57±2	81±2	89±3	13	74±8
	7	49±7	93±2	98±0	14	83±2

Legend. Experiments No. 1-3 and 8-14 performed by fluorescence-spectral method, remainder with the aid of [^{3}H]BP.

TABLE 2. Toxic Action of BP on Cultures of Mouse Liver and Hepatoma 22A Cells (72 h of incubation with the carcinogen; M ± σ)

Culture	Concentration of BP, μg/ml	Experiment No.	Density of monlayer		Mitotic index, percent	
			control	experiment	control	experiment
Mouse liver cells	1	1	148±40	78±35	1,75±0,9	0,95±0,2
		2	95±21	40±4	1,8±0,5	0,7±0,3
		3	120±27	138±18	0,9±0,3	0,3±0,2
		4	152±4	47±9	1,5±0	0,2±0,2
	10	5	148±40	30±10	1,75±0,9	0
		6	161±21	28±4	1,1±0	0
		7	190±21	190±10	0,7±0,35	0
		8	169±36	61±5	1,3±0,85	0
Hepatoma 22A cells	10	9	86±2	15±8	0,6±0,3	0
		10	62±17	35±1	0,65±0,28	0
		11	59±8	42±8	0,54±0,27	0

Legend. Density of monolayer implies number of cells in field of vision of microscope (magnification 300 ×).

of the monolayer, BP was added to the culture medium in concentrations of 0.1, 1.0, or 10 μg/ml. The cells were fixed 3 days later and stained with Mayer's hematoxylin. To determine the mitotic index, the number of mitoses was counted in 800-1000 cells per coverslip. At each point of the experiment 3 to 5 coverslips were used. The change in density of the monolayers was determined from the change in the mean number of cells per field of vision of the microscope. From 10 to 20 fields of vision were counted in each preparation, and 3 or 4 preparations were examined at each time. The significance of differences between the control and experimental values was determined by Student's criterion. Morphological changes in the cells were studied in the optical microscope in living cultures and stained sections.

The ability of the cells to synthesize α-fetoprotein was determined by the immunoprecipitation test in gel.

EXPERIMENTAL RESULTS

With a seeding density of $5 \cdot 10^6$-$8 \cdot 10^6$ cells/ml a monolayer of embryonic liver cells was formed on the third to fifth day. At that time the cells still continued to divide (mitotic index 1.3 ± 0.6%) and to actively synthesize α-fetoprotein. Up to 80% of the cells of the primary culture were epithelial cells, polygonal in shape, with large nuclei and a granular cytoplasm (Fig. 1). The cells were well spread out on the coverslips and in close contact with one another.

Cells of hepatoma 22A formed a monolayer on the second to fourth day after seeding. The mitotic index at that time was 0.6 ± 0.3%. The monolayer of tumor cells was less dense than that of the liver cells and it was composed of polymorphic cells of epithelial and fibroblast-like types. The former often attained a giant size. From 2 to 8% of the cells were ring-shaped (Fig. 2).

It will be clear from Table 1 that cultures of liver cells metabolized BP completely if present in low concentrations during incubation for 48 h. This corresponds to the intensity of metabolism of these same BP concentrations by cultures of embryonic fibroblasts of the same strain of mice [1]. The higher metabolic activity of the liver cultures than of fibroblasts is shown by the fact that even when BP was present in a concentration of 10 μg/ml the liver hepatocytes oxidized more than three-quarters of the added carcinogen in the course of 72 h. Cultures of embryonic fibroblasts during the same period oxidized only 65% of the BP added in a concentration of 1.6 μg/ml [1]. This is in good agreement with data on the higher activity of nonspecific oxidases in microsomes isolated from hepatocytes than from fibroblast cultures [6, 11].

It is interesting to note that cultures of hepatoma 22A cells metabolized BP almost as intensively as cultures of normal hepatocytes (Table 1). This distinguishes it considerably from the cultures of tumor fibroblasts studied, for most of them had lost this property [1].

Since the toxic effect of chemical carcinogens is connected with their metabolism [1, 7], it might be expected that both normal and tumor hepatocytes would be highly sensitive to the toxic action of BP. This was confirmed by the experimental results given in Table 2. Starting with a concentration of 1 μg/ml BP regularly reduced both the density of the monolayer and the mitotic activity of the hepatocytes; with a BP concentration of 10 μg/ml mitotic activity was completely suppressed. Clearly the sensitivity of the hepatocytes to BP in the culture was comparable with the sensitivity of cultures of mouse embryonic fibroblasts (mice of the same strain), on which BP has both a toxic and a carcinogenic action in vivo and in vitro [1].

The high sensitivity of the liver cells to BP in culture (see also [2, 6]) thus suggests that the resistance of the liver to this carcinogen is a phenomenon which depends not on the resistance of individual hepatocytes to BP, but on factors acting at the organ level.

In high concentrations BP (10 μg/ml) acted on the culture of hepatoma 22A cells in the same way as on the culture of liver cells, causing a marked reduction in the density of the monolayer and complete suppression of mitotic activity. Under the influence of BP the morphological picture of the hepatoma culture also changed: There was a sharp increase (more than tenfold) in the number of ring-shaped cells. It is interesting to note than 10 years ago explants of the solid form of hepatoma 22A were more sensitive to the toxic action of BP than explants of embryonic liver [2]. The high sensitivity of hepatoma 22A to the toxic action of BP, which distinguishes it from most cultures of sarcomas [1], can be explained, first, by

the fact that it was induced by o-aminoazotoluene, a carcinogen which differs chemically from BP and, second, by the independent progression of the features of the malignant tumors [8].

At the cell level, correlation was thus found between the intensity of BP metabolism by hepatocytes and its toxic action on them. It was also shown that even sharply dedifferentiated cells of malignant tumors of the liver can retain their ability to metabolize BP and to exhibit sensitivity to its toxic action.

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SENSITIVITY OF MOUSE, RAT, AND HUMAN EMBRYONIC LUNGS TO THE ACTION OF NITROSOMETHYLUREA IN ORGAN CULTURE

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In response to the direct action of nitrosomethylurea (NMU) in a concentration of 0.05 mg/ml on organ cultures of embryonic lungs of strain A mice, Wistar rats, and man, a varied degree of degenerative changes and hyperplastic proliferation of the epithelium developed in the cultures. In the early stages of the experiment the toxic effect of the cultures predominated. Tissue of rat embryonic lungs was most sensitive to the toxic action of NMU, mouse lung tissue least sensitive. The frequency of hyperplastic proliferation, on the other hand, was highest in cultures of mouse lungs and lowest in cultures of rat lungs. During culture the sensitivity of the human and rodent embryonic lungs to the toxic action of NMU decreased when the substance was repeatedly added to the nutrient medium. Meanwhile an increase in the survival of the experimental cultures was observed compared with the intact control.

KEY WORDS: *nitrosomethylurea; organ culture; embryonic lungs.*

The comparative study of the sensitivity of the tissues of animals and man to the action of carcinogenic agents is of theoretical interest and may be of the utmost importance to the

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