Effect of 5-bromodeoxyuridine on the appearance of the liver isoenzyme of pyruvate kinase

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Hepatocyte cultures derived from 15-day foetal rats produce the liver form of pyruvate kinase (EC 2.7.1.40) only after 3 days of culture. The appearance of the liver form of the enzyme can be blocked by the addition of 5-bromodeoxyuridine on day 2 of culture, but not by addition on day 3 of culture. The reversibility of the action of 5-bromodeoxyuridine was shown when the inhibitor was added on day 2 and removed on day 4. By day 6 of culture the liver form of pyruvate kinase was detectable. The specificity of the action of 5-bromodeoxyuridine was monitored by following changes in the closely related embryonic form of the enzyme as a control. This was unaltered by the inhibitor.

It has been shown in several systems, such as myogenesis (Bischoff & Holtzer, 1970), erythrocytogenesis (Holtzer *et al.*, 1972; Weintraub *et al.*, 1972; Miura & Wilt, 1971), chondrogenesis (Mayne *et al.*, 1971) and the induction of tyrosine aminotransferase (EC 2.6.1.5) in transformed liver cells (Stellwagen & Tomkins, 1971) and in foetal hepatocyte cultures (Yeoh & Oliver, 1980), that 5-bromodeoxyuridine exerts an inhibitory effect on the acquisition of various specific markers during the developmental process. It does not markedly affect the proliferative rate of differentiating cells or the extent of DNA or RNA synthesis (Rutter *et al.*, 1973).

It has previously been shown that foetal hepatocytes derived from 15-day foetal rats, after 2 days in culture, produce the liver-specific enzyme tyrosine aminotransferase (Yeoh *et al.*, 1979). In the presence of 5-bromodeoxyuridine, the accumulation of tyrosine aminotransferase can be inhibited by up to 85% of control values (Yeoh & Oliver, 1980). If foetal hepatocytes are taken from animals that are already competent with respect to synthesis of tyrosine aminotransferase, no effect on the activity of the enzyme can be detected in the presence of 5-bromodeoxyuridine.

In the present paper, the effect of 5-bromodeoxyuridine on the appearance of the liver form of pyruvate kinase (EC 2.7.1.40) is examined and compared with the closely related gene of pyruvate kinase, which is only expressed in the liver of the

embryonic rat. This study shows that addition of 5-bromodeoxyuridine on day 2 of culture results in a complete inhibition of the appearance of the liver isoenzyme of pyruvate kinase, with no effect on the embryonic form. The effect of 5-bromodeoxyuridine was also shown to be reversible, as addition on day 2 of culture, followed by removal on day 4, resulted in the detection of the liver isoenzyme on day 6 of culture.

Materials and methods

Animals and materials

Rats (*Rattus norvegicus*) of the Wistar Albino strain were used. Gestational age was determined from the time of detection of spermatozoa in the vaginal tract and is accurate to ± 8 h.

[3 H]Deoxycytidine and [3 H]leucine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ADP, phosphoenolpyruvate, NADH, fructose 1,6-bisphosphate and 5-bromodeoxyuridine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cell-culture media, fungizone and glutamine were products of Grand Island Biological Co., Grand Island, NY, U.S.A. Penicillin and streptomycin were purchased from Calbiochem, La Jolla, CA, U.S.A. Foetal-calf serum was supplied by Commonwealth Serum Laboratories, Parkville, Vic., Australia. Eagle's minimum essential medium was bought from Flow Laboratories, Annandale, N.S.W., Australia. Coomassie Brilliant Blue was purchased from Bio-Rad

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Laboratories, Richmond, CA, U.S.A. All other reagents were of analytical grade.

Hepatocyte culture

Hepatocytes were obtained from foetal animals of 15 days gestation. The foetuses were delivered by caesarian section and the livers excised and placed in balanced salts solution (Hanks & Wallace, 1949), as in the method of Yeoh *et al.* (1979). The livers were disaggregated with a Pasteur pipette, washed three times in balanced salts solution and plated out on 90mm-diam. collagen-coated culture dishes (Sterilin Products, Teddington, Middx., U.K.). The cultured hepatocytes were maintained at 37°C in a water-saturated atmosphere of air/CO₂ (19:1).

Fresh medium was given to the cultures every 24h. 5-Bromodeoxyuridine was dissolved in balanced salts solution, and added to the culture medium to give a final concentration of 150 µg/ml.

Preparation of extracts

Cells were washed in 10ml of balanced salts solution and harvested with a Teflon scraper, centrifuged at 400g for 5 min, and resuspended and washed with homogenizing buffer [50mM-Tris (pH 7.5)/250mM-sucrose/1mM-EDTA]. After the final wash, 200 µl of homogenizing buffer was added and the sample sonicated for 15s with a Branson sonifier (at 1.5A). A 175 µl portion of sonicated extract was centrifuged in a Beckman Airfuge (A 100 rotor) at 160000g for 15 min. The supernatant was collected and used for pyruvate kinase assay.

Pyruvate kinase assay

Pyruvate kinase activity was measured at 25°C by a coupled assay method similar to that described by Bucher & Pfeleiderer (1955). The assay buffer contained 50mM-Tris/HCl (pH 7.5), 100mM-KCl, 5mM-MgSO₄, 2mM-ADP, 2mM-fructose 1,6-bisphosphate, 2mM-phosphoenolpyruvate, 0.04mM-NADH and 1 unit of lactate dehydrogenase/ml. The rate of decrease of NADH was measured at 340nm with a Beckman DU-8 spectrophotometer. The limit of detection was 0.15nmol of NADH reduced/min per µg of protein.

Electrophoresis

Electrophoresis was performed as described previously (Scott & Yeoh, 1983), on cellulose acetate, for 5h at a constant 250V at 4°C; this allowed complete separation of the isoenzymes of pyruvate kinase. The cellulose acetate was then cut into 30.2mm strips, and the isoenzymes were eluted and assayed as described previously.

Protein synthesis

Total protein synthesis was measured by the method of Mans & Novelli (1961). It was measured by [³H]leucine incorporation into trichloroacetic acid-precipitable protein. Cultures were exposed to [³H]leucine for 45 min, then washed thoroughly with balanced salts solution. The cells were then homogenized with a Branson sonifier set at 1.5A for 15s. A 5 µl sample of homogenate was taken and incubated in 500 µl of 1.5% H₂O₂ in 1M-NaOH for 15 min. The protein was then precipitated by addition of 1ml of 25% (v/v) trichloroacetic acid plus 2mM-leucine, filtered through Whatman GF/C glass microfibre filters and washed with 20ml of ice-cold 5% trichloroacetic acid plus 2mM-leucine. The glass filters were placed in an aqueous scintillant (Beckman Ready-Solv TM HP) and counted for radioactivity in a Beckman LS 3800 instrument.

Protein assays were performed on all samples by the method of Bearden (1978), with Coomassie Brilliant Blue G-250 dye in a dilute acid medium. Bovine serum albumin (Sigma) standards were included in all determinations.

DNA synthesis

DNA synthesis was measured by [³H]deoxycytidine incorporation into DNA. Cells were incubated for 24h before harvest in [³H]deoxycytidine, washed with 5 × 5ml of balanced salts solution and homogenized in 500 µl of 5% trichloroacetic acid. The homogenate was then incubated for 1h at 90°C and centrifuged at 1000g for 10 min. A 5 µl sample of supernatant was dissolved in an aqueous scintillant and its radioactivity determined in a Beckman LS 3800 instrument.

DNA assays were performed on all samples by the fluorimetric method described by Hinegardner (1971), with highly polymerized calf thymus DNA (Sigma) as standard.

Results

The addition of 5-bromodeoxyuridine on day 2 of culture results in no significant difference in the rate of DNA synthesis (Fig. 1a). At a concentration of 150 µg/ml there was no evidence of any cytotoxic effect of the mutagen, which was corroborated by the finding that incorporation of [³H]leucine into protein was not altered significantly with respect to control cultures (Fig. 1b).

The effect of 5-bromodeoxyuridine on the embryonic and liver forms of pyruvate kinase is shown in Fig. 2. The M2 isoenzyme was present throughout the culture period, whereas the L isoenzyme was detected after day 3 of culture. In the presence of 5-bromodeoxyuridine the activity of the M2 isoenzyme was not significantly altered.

Fig. 1. Effect of 5-bromodeoxyuridine on (a) DNA synthesis and (b) protein synthesis

Bromodeoxyuridine was added on day 2 of culture (final concn. 150 μ g/ml). Incorporation of (a) [3 H]deoxycytidine into DNA or (b) [3 H]leucine into total protein was then determined. Each point represents the mean $\pm$ S.E.M. for (a) four or (b) eight culture dishes. The effects were tested with Student's *t* test and found to be insignificant ($P > 0.05$). ■, Control; □, + bromodeoxyuridine.

Table 1. Effect of adding 5-bromodeoxyuridine on day 3 of culture

5-Bromodeoxyuridine was added on day 3 of culture (final concn. 150 μ g/ml). Enzyme activity is expressed as nmol of NADH reduced/min per μ g of protein. Each value represents the mean $\pm$ S.E.M. for eight culture dishes (N.D., not detectable).

	Control	+ 5-Bromodeoxyuridine
Day 3		
L	N.D.	N.D.
M2	4.5 $\pm$ 0.6	2.9 $\pm$ 0.4
Day 4		
L	1.5 $\pm$ 0.5	0.9 $\pm$ 0.6
M2	2.6 $\pm$ 0.3	1.8 $\pm$ 0.4

In contrast, the appearance of the L isoenzyme was prevented.

If addition of 5-bromodeoxyuridine was postponed until day 3 of culture, the L isoenzyme

Fig. 2. Effect of 5-bromodeoxyuridine on the two forms of pyruvate kinase

Bromodeoxyuridine was added on day 2 of culture (final concn. 150 μ g/ml). Isoenzymes were separated electrophoretically, eluted from the cellulose acetate membrane and then assayed for activity. Enzyme activity is expressed as nmol of NADH reduced/min per μ g of protein. Isoenzymes: ●, control L; ○, test L; □, control M2; ■, test M2. Each point represents the mean $\pm$ S.E.M. for eight culture dishes.

Table 2. Effect of adding 5-bromodeoxyuridine to, and then removing it from, cultures

5-Bromodeoxyuridine was added on day 2 of culture (final concn. 150 μ g/ml), then removed on day 4 of culture. Enzyme activity is expressed as nmol of NADH reduced/min per μ g of protein. Each value represents the mean $\pm$ S.E.M. for six culture dishes (N.D., not detectable).

	Control	+ 5-Bromodeoxyuridine
Day 3		
L	N.D.	N.D.
M2	4.4 $\pm$ 0.2	4.5 $\pm$ 0.4
Day 6		
L	2.0 $\pm$ 0.2	1.0 $\pm$ 0.1
M2	2.3 $\pm$ 0.4	2.0 $\pm$ 0.2

appeared on day 4. The activity of the isoenzyme was, however, decreased to approx. 60% of the control values (Table 1). To test the reversibility of the 5-bromodeoxyuridine effect, the mutagen was added on day 2 of culture and removed on day 4 (Table 2). When cultures were assayed for enzyme activity on day 6, both isoenzymes were present.

Discussion

An interruption in the normal pattern of development by 5-bromodeoxyuridine has been shown to block the emergence of luxury gene products (Holtzer *et al.*, 1972). Previous experiments on systems differentiating *in vitro* (see the introduction) have shown that 5-bromodeoxy-

uridine addition interferes with the process in a unique manner. The effects of 5-bromodeoxyuridine on these systems are reversible (Rutter *et al.*, 1973).

Previously it has been shown that hepatocytes derived from 15-day foetuses synthesize the liver form of pyruvate kinase after 3 days in culture (Scott & Yeoh, 1983). In the present paper, the effect of 5-bromodeoxyuridine, a molecule that inhibits cytodifferentiation, on this event is reported.

At the dose chosen for this study, 5-bromodeoxyuridine has little effect on the normal cellular processes of DNA and protein synthesis. However, under these conditions foetal hepatocytes fail to initiate synthesis of the L isoenzyme of pyruvate kinase. By monitoring the activity of the M2 isoenzyme, the possibility of non-specific actions of 5-bromodeoxyuridine can be eliminated. It was shown that this isoenzyme remains expressed throughout the period of exposure to 5-bromodeoxyuridine. These results suggest that the expression of the M2-isoenzyme gene and the gene which is about to be switched on are differentially affected by 5-bromodeoxyuridine, since only the L gene is prevented from being expressed.

The time at which 5-bromodeoxyuridine is added to the cultures was found to be critical. If 5-bromodeoxyuridine was added on day 2 of culture, the appearance of the L isoenzyme was completely inhibited. If addition occurred 24h later, the L isoenzyme appeared, implying perhaps that some cells have passed through a critical cell cycle which renders the L-isoenzyme gene transcribable. The situation is analogous to that reported by Holtzer *et al.* (1972) and Miura & Wilt (1971), who showed that expression of the haemoglobin gene in developing erythroid cells is only sensitive to 5-

bromodeoxyuridine at a particular stage of development. Once the programme for haemoglobin synthesis is initiated, 5-bromodeoxyuridine is no longer effective.

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