LOW CONCENTRATIONS OF C_6-SUBSTITUTED PURINES RETARD THE $G_0 \rightarrow G_1$ TRANSITION OF BOVINE LENS EPITHELIUM CELLS

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1. Introduction

During morphogenesis, differentiation and ageing cell movement and the mitotic rate of cells decline. In differentiated tissues of adult organisms most of the cells are in a resting state (G_0). Explantation in a suitable culture medium causes the cells to become reactivated. After the so called 'latent period of cell emigration' cells start to move and subsequently undergo DNA synthesis and cell division (G_1, followed by the other phases of the cycle). The duration of the latent period correlates posetively with the age of the donor organism [1,2,6].

In preceding papers we described an aggregation phenomenon shown by primarily explanted epithelial cells from bovine lenses, which can be strongly enhanced by physiologically occurring C_6-substitued purines, added to the culture medium [3,4,6]. That aggregation phenomenon takes place during the latent period of cell emigration and is dependent on the age of the donor animals [6]. Presenting quantitative data on cell density, flattening and extension of the cell sheet, mitotic rate, protein- and DNA-synthesis we show that low concentrations of physiological purine derivatives retain bovine lens cells in the G_0-state and delay cell spreading and proliferation normally triggered by in vitro conditions.

2. Materials and methods

Lenses of one to two year-old animals were used. Details of the culture procedure are described in [3,6]. To quantify the cell aggregation and the spreading of the aggregated or non-aggregated epithelium sheet the central zone of the anterior lens capsule [7] was cut into four pieces. Each capsule-epithelium preparation was immediately transferred to a Petri dish containing 4 ml of medium, epithelial cells facing upwards. The preparations were fixed with pieces of glass at their edges. A circular area of 2.95 mm^2 was marked by means of a syringe cone. The epithelium outside of the

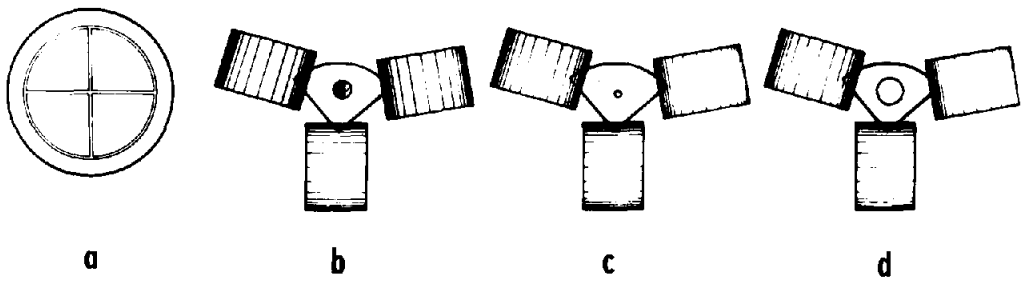

Fig.1. Scheme of the capsule-epithelium preparations and the time-course of the test. (a) The central zone of the anterior lens capsule is cut into four pieces. (b) The marked area of the epithelium at the beginning of the explantation; the epithelium outside of the circle is scraped off. (c) Epithelium in aggregation, about 5 h after explantation. (d) Epithelium in spreading, about 35 h after explantation.

circle was scraped off. The area of the marked epithelium was estimated at various times (fig.1).

In those experiments designed to determine cell density, diameters of the nuclei, and the mitotic rate the preparations were fixed in 4% formol-Ca solution and stained with methylene blue after 72 h of explantation. Then the area of the whole epithelium was determined along with the number of mitosis. Cell density was calculated by counting cell nuclei from ten squares of 0.3 mm^2 each located in various places of the preparation. The area analysed amounted to about 10% of the whole preparation. For the estimation of the mitotic rate the total cell number was calculated from the mean value of the cell density and the corresponding area of the cell layer. The mean values of the diameters of the nuclei were roughly estimated by measuring the largest diameters of 10 nuclei per preparation.

Protein- and DNA-synthesis in primarily cultured lens epithelium cells: lens capsules with adhering epithelium cells of the central zone were cultured with and without adenine in Petri dishes. [^{3}H]thymidine and [^{14}C]leucine were added to the culture medium in a final concentration of 1 μCi/ml and 0.25 μCi/ml, respectively. After incubation, the cells were washed in physiological salt solution, scraped off from the lens capsule and homogenized in an all glass micro-homogenizer. The washing procedure for liquid scintillation counting was done as reported [10]. The radioactivity incorporated in the macromolecular fraction was related to the protein content of the homogenates, which was estimated according to a modified Lowry procedure [11].

Protein- and DNA-synthesis in subcultured lens epithelium cells: Lens epithelium cells were cultured in Eagle MEM medium substituted by 10% of calf serum and subcultured twice a week. [^{3}H]thymidine and [^{14}C]leucine were added in a final concentration of 0.1 μCi/ml and 0.05 μCi/ml, respectively. The method for measuring incorporated radioactivity is given in [12].

Materials: L-[^{14}C]leucine(U), 185 mCi/mmol, (UVVVR, Prague, CSSR); thymidine-(methyl)-T, 3 Ci/mmol (Institut für Angewandte Isotopenforschung, Berlin, DDR); Zellzüchtungsmedium nach Eagle (MEM) (Staatliches Institut für Immunpräparate und Nährmedien, Berlin, DDR); calf serum, abattoir collected, membrane filtered; Adenine, p.A., (Serva, Heidelberg, BRD).

3. Results

Some data on the progress of the activation of bovine lens cells in vitro without and with purine derivatives are listed in table 1 and 2. After 25 h of

Table 1
Effects of purine derivatives on the activation process of bovine lens epithelium in the primary culture

Effector	Area of epithelium 25 h $\bar{x}$	± s	50 h $\bar{x}$	± s	Diameters of cell nuclei 72 h $\bar{x}$	± s	Cell density 72 h $\bar{x}$	± s	Mitotic rate 72 h $\bar{x}$	± s	N
Purine	92.9	27.7	96.7	29.5	95.2	7.9	119	17.8	104	148	9
Adenine	13.0	10.7	17.1	12.7	83.8	8.6	212	45.8	1.1	3.4	17
Guanine	38.3	20.6	30.1	12.1	85.8	4.3	198	51.9	0.0	0.0	8
Hypoxanthine	26.6	23.1	32.4	26.2	88.1	9.1	175	61.2	34.3	20.2	23
Xanthine	93.2	27.8	102	24.4	101	8.1	88.1	69.7	480	738	7
Uric acid	103	30.6	106	36.5	101	8.7	101	16.2	121	139	16
6-Mercaptopurine	101	35.0	96.8	25.3	96.5	5.7	110	10.6	98.9	95.6	10
6-Carboxypurine	106	20.0	106	16.2	95.4	8.7	114	24.0	199	241	10

The data are given as per cent of the controls as estimated with capsule-epithelium preparations described in fig.1. The corresponding data of the controls are: area of the epithelium: 2.95 mm^2 at the beginning, 1.43–3.13 mm^2 after 25 h, and 4.93–8.4 mm^2 after 50 h, respectively; diameters of cell nuclei: 17.90–19.34 μm; cell density: 400–500 cel nuclei per mm^2; mitotic rate: 1.0–7.7 per 1000 cells. Effector concentration 10^{-5} M. N gives the numbers of experiments carried out with lenses from different animals. h = time after explantation.

Table 2
Effect of 10^{-5} M adenine on protein and DNA synthesis of primarily cultured bovine lens epithelium

Radioactive precursors present during	[^{14}C]Leucine[dpm·μg protein^{-1}] Control	Adenine	[^{3}H]Thymidine[dpm·μg protein^{-1}] Control	Adenine	N
1–21 h	186	74[b]	(113)	(84)	5
24–45 h	88	49[a]	(133)	(145)	6
48–69 h	107	27[a]	717	319[a]	5
70–91 h	157	56[b]	1730	178[a]	4

Adenine was present from the beginning of the cultivation. The protein content per sample was 10–20 μg. N gives the number of experiments carried out with paired lenses. Statistical evaluation of the paired dates:
[a] p smaller than 0.01.
[b] p smaller than 0.05.
( ) = cpm in the tritium channel too low for a correct analysis.

explantation the controls occupy 50 to 110% and after 50 h 170 to 290% of the originally marked area (legend of table 1). After 72 h of explantation the cell density amounts to 400 to 500 nuclei/mm^2 (legend of table 1) in comparison with more than 3000 nuclei/mm^2 in vivo [7]. Protein synthesis could be demonstrated during all periods investigated, DNA synthesis became measurable during the period from 48 to 69 h. Mitosis could be observed (in unstained preparations) not earlier than 60 h after explantation. Culturing with adenine or hypoxanthine or guanine, 10^{-5} M, results in a stimulation of cell aggregation (not shown here) and in a retardation of cell spreading. Simultaneously these substances delay the enlargement of the cell nuclei as well as protein- and DNA-synthesis with a consequent delay in the initiation of mitosis. Adenine is the most effective substance, followed by hypoxanthine and guanine. Nearly related compounds like xanthine, uric acid, 6-mercaptopurine and 6-carboxypurine are ineffective as well as purine itself. As can be seen from table 3, lens cells lose much of their sensitivity to adenine during subculturing.

4. Discussion

Both, the mechanism holding most cells in adult organisms in the G_0-state as well as the mechanism triggering the $G_0 \rightarrow G_1$ transition after explantation of tissue fragments in suitable culture media are unknown. It has been suggested that environmental factors play an important role in the regulation of cell division. Harding and co-workers have reported on serum fractions and insulin stimulating mitosis in organ cultured frog and rabbit lenses [8,9]. Most cells, when trypsinized or handled as tissue fragments, in vitro first adhere to and then begin to move on the substratum. So in most cases cell-substratum adherence, cell flattening and cell movement as well as RNA- and protein synthesis precede the initiation of both DNA synthesis and mitosis. One may suggest that these events are various signs of one and the same activation cascade triggered by the culture conditions. Obviously, the purine derivatives interfere with that activation process at its very beginning.

It should be noticed that the physiologically occurring purine derivatives inhibit both protein and DNA synthesis of primarily cultured lens epithelium cells in concentrations as low as 10^{-5} M (table 2). If adenine is added at the beginning of the primary culture a delay in cell spreading can be demonstrated down to $3 \cdot 10^{-6}$ M adenine [6].

The remarkable specificity of the purine derivatives in inhibiting the activation process (see table 1) points at a possible mechanism by which these substances may exert their effects. Adenine, hypoxanthine, and guanine, effective in retarding the activation of primarily cultured lens cells, have been shown to be effective in depressing the concentration of PRPP (more than 99%) in Ehrlich ascites cells when added to the culture medium

Table 3
Influence of adenine on protein synthesis ([^{14}C]leucine incorporation) and DNA synthesis ([^{3}H]thymidine incorporation) of subcultured lens epithelium cells

Experiment (N)	Culture conditions S	t_c	t_p	t_i	Adenine (M)	[^{14}C]Leucine [dpm] $\bar{x}$ ± s	% of control	[^{3}H]Thymidine [dpm·10^{-3}] $\bar{x}$ + s	% of control
A (10)	2	0	20	4	0	171 24	–	78 13	–
					10^{-5}	196 36	115	82 13	105
					10^{-4}	190 43	111	81 9	104
					$4{\cdot}10^{-4}$	185 48	108	74 12	95
B (15)	7	0	20	4	0	310 45	–	82 12	–
					10^{-5}	284 46	91	75 9	91
					10^{-4}	300 39	97	82 9	100
					$7{\cdot}10^{-4}$	281 55	90	63 11	77
C (5)	23	41	1	4	0	496 54	–	74 17	–
					10^{-5}	502 25	101	63 10	85
					10^{-4}	535 55	108	66 8	89
					10^{-3}	471 31	95	57 12	78
D (5)	23	24	18	4	0	471 60	–	160 15	–
					10^{-5}	510 33	108	123 19	77
					10^{-4}	357 49	76	129 16	80
					10^{-3}	256 37	54	93 12	58

s: Number of subcultures.
t_c: Time of culturing before adding adenine (h).
t_p: Time of preincubation (h) with adenine.
t_i: Time of incubation (h) with radioactive precursors in the presence of adenine.

[15]. Nearly related compounds like xanthine and uric acid which show no effect on lens cells are ineffective in depressing the concentration of PRPP too [15].

The high sensitivity of lens cells to adenine already decreases during the primary cultivation even when adenine is present [6]. In subcultured lens cells adenine effects both the synthesis of proteins and the synthesis of DNA only in concentrations higher than 10^{-4} M (table 3). This action on subcultured lens cells is comparable with the growth-inhibitory effect of high concentrations (10^{-3} M) of exogenous adenine on L cells [13] and *E. coli* [14]. Since the action of 10^{-3} M adenine on L cells and *E. coli* can be overcome by cytidine or uridine but not by the corresponding bases it has been proposed that this growth-inhibitory effect of adenine is also caused by depressing the intracellular level of PRPP followed by an inhibition of the synthesis of pyrimidine nucleosides [14].

The drastic reduction of the sensitivity to adenine during culturing points out an interesting difference between 'in vivo-cells' and 'in vitro-cells' of bovine lens epithelium. Such a reduction may be expected if the PRPP producing machinery becomes stimulated during the $G_0 \rightarrow G_1$ transition of the lens epithelium cells. Recently it has been shown that after mitogenic stimulation of lymphocytes by phytohaemagglutinin the intracellular level of PRPP increases [16] within 5 min after exposure to the mitogen [17]. With respect to the importance of PRPP in regulating purine and pyrimidine metabolism we feel that an early increase of PRPP may play an essential role in initiating the in vitro-activation of bovine lens cells. In this case the purine derivatives presumably would exert their effects by utilizing PRPP for their transmembranal transport by means of adenine phosphoribosyl transferase and hepoxanthine-guanine phosphoribosyl transferase. Another possibility is that the purine derivatives directly effect the outer membrane of the lens cells.

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