The effect of exogenous nucleosides on postimplantation development of mouse embryos

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Summary. Exogenous nucleosides, either singly or in combination, do not enhance postimplantation development of mouse embryos *in vitro*, and adenosine, guanosine and thymidine are toxic to the embryos at high concentrations.

Techniques for culturing postimplantation mouse embryos have advanced in recent years, and it is now possible to grow mouse embryos from the 2-cell or the blastocyst stage to the egg-cylinder stage (Spindle & Pedersen, 1973; Pienkowski, Solter & Koprowski, 1974) and even to the early somite stage (Hsu, 1972; Hsu, Baskar, Stevens & Rash, 1974). However, the nutritional requirements for postimplantation development of mouse embryos are poorly understood, except that amino acids, serum (Gwatkin, 1966; Spindle & Pedersen, 1973) and glucose (Wordinger & Brinster, 1976) are required for hatching, attachment and outgrowth of blastocysts. This study was undertaken to determine whether exogenous nucleosides would stimulate development of mouse blastocysts *in vitro* to postimplantation stages.

Randomly bred Dub: (ICR) mice (Flow Laboratories) were used as the source of embryos. Female mice (6–10 weeks old) were induced to superovulate by injecting 5 i.u. PMSG (Sigma) and 5 i.u. HCG (Ayerst) 48 h later and were caged with Dub: (ICR) male mice overnight. Two-cell embryos were flushed from the oviducts 45–48 h after HCG injection and were cultured in modified standard egg-culture medium (Spindle & Goldstein, 1975). After incubation for 3 days at 37°C in an atmosphere of 5% CO₂ in air, blastocysts were transferred to eight-chamber culture slides (Lab-Tek) (15 embryos/chamber) containing modified Eagle's basal medium (BME) (0.5 ml/chamber), which contains optimal concentrations of essential amino acids (Spindle & Pedersen, 1973). This medium was supplemented with 1 or 10% dialysed serum (equal parts of fetal calf serum (GIBCO) and newborn calf serum (Flow Laboratories)) and with various concentrations of adenosine, cytidine, guanosine, thymidine or uridine (Sigma). Before the addition, the serum was dialysed for 3 days in phosphate-buffered saline, which was changed twice each day, to remove any nucleosides that might have been present. Stock solutions of nucleosides were made by dissolving them singly in modified BME at a concentration of 10⁻² M (adenosine, cytidine and uridine) or 10⁻³ M (guanosine and thymidine), and series of 10-fold dilutions were made from the original stock solutions by adding modified BME. The calculated osmolarity of modified BME was 280 mosmol, and the osmolarity of the medium that contained the highest concentration of nucleosides (10⁻² M) was still within the limit that is well tolerated by mouse embryos (240–310 mosmol: A. I. Spindle, unpublished observation).

The growth response of embryos was examined under a dissecting microscope (×140) after 5 days of culture, and the number of trophoblast outgrowths, the total number of inner cell masses (ICMs), and the number of ICMs that differentiated into endoderm and ectoderm were counted. Significant differences between control and experimental groups in the number of embryos surviving to these three morphological endpoints were determined by Student's *t* test.

When each nucleoside was added alone (Table 1), none stimulated development of embryos at any of the concentrations tested and thymidine, guanosine and adenosine were toxic at high concentrations. At concentrations of 10⁻⁵ M and higher, thymidine reduced the total number of ICMs and the number of ICMs with two germ layers (*P* < 0.01) but it did not affect trophoblast outgrowth, even at 10⁻³ M. By contrast, guanosine reduced the number of trophoblast outgrowths (*P* < 0.05 for

Table 1. Effects of individual nucleosides on the development of postimplantation mouse embryos (15 blastocysts for each concentration of nucleoside; 4–9 replicate experiments)

Nucleoside and developmental endpoint	Mean no. ($\pm$ S.E.M.) of embryos developing to endpoints for various nucleoside concentrations (M)					
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Adenosine						
Trophoblast outgrowth	14.3 ± 0.8	14.2 ± 0.8	14.5 ± 0.6	14.5 ± 0.6	14.8 ± 0.5	11.8 ± 2.6
Total ICMs	11.8 ± 1.7	11.6 ± 3.1	13.0 ± 0.8	12.5 ± 0.6	9.8 ± 2.4	$3.0 \pm 1.6^{**}$
ICMs with 2 germ layers	5.5 ± 2.6	4.8 ± 2.8	6.0 ± 2.8	4.3 ± 1.9	3.5 ± 1.7	0**
Guanosine						
Trophoblast outgrowth	14.0 ± 1.1	14.0 ± 0.7	13.5 ± 1.1	$10.2 \pm 2.7^*$	$4.2 \pm 0.8^{**}$	—
Total ICMs	12.3 ± 1.5	11.4 ± 1.7	9.2 ± 2.3	$2.6 \pm 1.7^{**}$	$0.8 \pm 1.3^{**}$	—
ICMs with 2 germ layers	5.2 ± 1.3	4.8 ± 0.5	2.8 ± 2.6	0**	0**	—
Cytidine						
Trophoblast outgrowth	14.2 ± 0.9	14.1 ± 1.0	14.4 ± 1.1	14.6 ± 0.6	13.8 ± 1.8	14.4 ± 1.3
Total ICMs	11.1 ± 1.9	11.5 ± 1.2	12.1 ± 1.9	14.2 ± 1.3	12.4 ± 2.0	11.4 ± 1.7
ICMs with 2 germ layers	5.1 ± 1.5	5.3 ± 1.4	6.6 ± 2.5	7.0 ± 2.6	4.8 ± 2.6	2.8 ± 1.9
Uridine						
Trophoblast outgrowth	14.2 ± 1.0	14.0 ± 0.9	14.3 ± 0.5	14.0 ± 0.7	14.5 ± 0.6	13.3 ± 1.9
Total ICMs	12.2 ± 1.0	12.7 ± 1.2	13.8 ± 1.0	13.2 ± 1.8	13.3 ± 2.2	11.3 ± 2.7
ICMs with 2 germ layers	5.2 ± 2.1	5.5 ± 1.8	7.8 ± 1.5	6.2 ± 1.9	6.8 ± 1.5	4.7 ± 2.4
Thymidine						
Trophoblast outgrowth	13.9 ± 1.1	14.4 ± 0.6	14.2 ± 0.8	14.6 ± 0.6	13.0 ± 1.6	—
Total ICMs	11.3 ± 1.8	10.6 ± 1.5	$3.5 \pm 2.2^{**}$	$1.8 \pm 0.5^{**}$	$1.0 \pm 1.4^{**}$	—
ICMs with 2 germ layers	5.6 ± 1.7	5.4 ± 2.3	$0.5 \pm 0.8^{**}$	0**	0**	—

Significantly different from respective control value, * $P \leq 0.05$; ** $P \leq 0.01$.

10^{-4} M and $P < 0.01$ for 10^{-3} M) as well as the total number of ICMs and the number of ICMs with two germ layers ($P < 0.01$). Inhibition of trophoblast outgrowth by guanosine was largely due to its interference with the attachment of denuded blastocysts: about 50% of denuded blastocysts failed to attach in the presence of 10^{-3} M-guanosine, whereas 100% of untreated denuded blastocysts attached. Adenosine was less toxic to embryos than thymidine or guanosine, but it inhibited development of ICMs at 10^{-2} M ($P < 0.01$). Cytidine and uridine were not toxic to the embryos even at 10^{-2} M.

There was no additive effect when the five nucleosides were tested together at 10^{-6} M, the concentration at which none of the nucleosides was toxic to embryos (Table 2).

The lack of a stimulatory effect by exogenous nucleosides indicates that mouse blastocysts are capable of synthesizing the nucleic acid precursors which are necessary for their postimplantation growth *in vitro*. This lack of stimulation is probably not due to the inability of embryos to transport nucleosides across the cell membrane, because radioactive nucleosides are readily taken up into the

Table 2. Postimplantation development of mouse embryos (15 blastocysts for each experiment and 6 replicates of each) in the presence of 10^{-6} M adenosine, cytidine, guanosine, thymidine uridine

Medium	Serum conc. (%)	Mean ($\pm$ S.E.M.) no. of embryos developing to endpoints		
		Trophoblast outgrowth	Total ICMs	ICM with 2 germ layers
Control	1	14.83 ± 0.41	12.67 ± 2.88	5.33 ± 1.75
Supplemented	1	14.83 ± 0.41	12.03 ± 1.64	6.83 ± 1.47
Control	10	14.07 ± 0.52	14.00 ± 1.10	7.50 ± 2.07
Supplemented	10	14.67 ± 0.52	13.67 ± 1.03	8.00 ± 2.61

cells and incorporated into the RNA of mouse blastocysts (Daentl & Epstein, 1971; Epstein & Daentl, 1971) and the DNA of postimplantation-stage mouse embryos (Pedersen & Cleaver, 1975). However, the synthesis of nucleic acids in cultured embryos may be limited by some other factors, such as an inadequate supply of folic acid or nicotinamide, which are constituents of coenzymes involved in nucleic acid synthesis (Friedkin, 1963; Laurent, Moor & Reichard, 1964).

Exogenous nucleosides have also been found to be nonstimulatory or toxic to development of preimplantation mouse embryos *in vitro* (TenBroeck, 1968; Snow, 1973a). However, the toxic effect of nucleosides on postimplantation mouse embryos is different from that on preimplantation mouse embryos in three respects: thymidine is more toxic to postimplantation embryos (specifically to development of the ICM) than to preimplantation embryos, adenosine is less toxic than guanosine to postimplantation embryos but is more toxic than guanosine to preimplantation embryos, and cytidine and uridine are toxic only to preimplantation embryos. These differences may indicate differences in nucleic acid metabolism between pre- and postimplantation mouse embryos. Indeed, amino acids and serum are first required by blastocysts (Gwatkin, 1966; Spindle & Pedersen, 1973), indicating that a marked change in embryo metabolism occurs at this stage of development.

The toxic effects of adenosine, guanosine and thymidine observed in this study, and the absence of such effects by cytidine and uridine, could reflect the regulatory roles that these nucleosides have in the inhibition of nucleic acid metabolic pathways. It has been shown that deoxyribonucleoside triphosphates of adenine, guanine and thymine inhibit DNA synthesis by interfering with the activity of ribonucleotide reductase but that deoxycytidine triphosphate does not (Reichard, Canellakis & Canellakis, 1961).

The reason for the higher toxicity of guanosine than adenosine is not clear, but guanosine has also been shown to be more toxic than adenosine to 4-day-old chick embryos (Karnofsky & Lacon, 1961). The inhibitory effect of guanosine on trophoblast outgrowth, which is probably due to attachment failure, is intriguing because treatments of blastocysts with thymidine and with various other agents (Snow, 1973b; Sherman & Atienza, 1975; Glass, Spindle & Pedersen, 1976; Pedersen & Spindle, 1976) do not affect trophoblast outgrowth but severely interfere with the development of the ICM. Because omission of leucine or arginine from the medium also causes attachment failure of mouse blastocysts (Gwatkin, 1966; Spindle & Pedersen, 1976), guanosine may be interfering with the synthesis of proteins that are needed for attachment of blastocysts.

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