Factors in mouse uterine fluid that inhibit the incorporation of [³H]uridine by blastocysts *in vitro*

H. M. Weitlauf

*Department of Anatomy, School of Medicine, University of Oregon Health Sciences Center,
Portland, Oregon 97201, U.S.A.*

Summary. Fluid flushed from the uteri of 'delayed implanting' and 'implanting' mice was fractionated by gel-filtration. The fractions were freeze-dried, individually re-suspended in culture medium containing [³H]uridine, and used for incubating 'implanting' blastocysts. Several factors were found that reduced incorporation of [³H]uridine by the blastocysts. The amount of inhibitory activity in corresponding factors was generally similar in flushings from both types of uteri, but there was significantly more inhibitory activity in the void volume fractions of flushings from 'delayed implanting' animals and it is suggested that this factor may be responsible for the metabolic dormancy of embryos during the diapause associated with delayed implantation.

Introduction

It has not yet been determined how 'delayed implanting' mouse and rat embryos are rendered dormant and then reactivated to implant at a later time. Brambell (1937) originally suggested that the 'delayed implanting' uterus (1) secretes an inhibitory factor or (2) fails to secrete an essential stimulatory factor (see McLaren, 1973, for discussion).

Several attempts have been made to identify factors in fluid flushed from the uteri of 'implanting' and 'delayed implanting' rats and mice that have stimulatory or inhibitory effects on blastocyst metabolism (Psychoyos & Bitton-Casimiri, 1969; Psychoyos, 1973; Psychoyos, Bitton-Casimiri & Brun, 1975; Weitlauf, 1976). In these experiments, significant decreases in the rate of [³H]uridine incorporation by blastocysts were interpreted as indicating inhibitory effects of the uterine fluid. However, fluid flushed from the uteri of 'implanting' and 'delayed implanting' females caused significant inhibition of [³H]uridine incorporation by blastocysts and it was concluded that a non-specific inhibitory effect was introduced by the methods used to collect or assay the fluid (Weitlauf, 1976). It seems clear that valid interpretations of results in such experiments will be possible only if non-specific factor(s) can be eliminated.

Therefore, in the present experiments fluid was flushed from the uteri of 'delayed implanting' and 'implanting' mice and was fractionated into various components by means of gel filtration. Comparisons between 'delayed implanting' and 'implanting' animals were then made on the basis of the effect that the individual components had on incorporation of [³H]uridine by blastocysts *in vitro*.

Materials and Methods

Virgin female white Swiss mice (6–8 weeks of age) were selected at random stages of the oestrous cycle, ovariectomized bilaterally, and allowed to recover for 7 days. The animals to be used as donors of 'delayed implanting' uterine fluid were injected subcutaneously with progesterone (Sigma, St Louis, 2.0 mg/day: Group 1) or the sesame seed oil vehicle (0.1 ml/day: Group 3) for 3 days (Weitlauf, 1971); those to be used as donors of 'implanting' uterine fluid were injected with progesterone (2.0 mg/day) for 2 days and 2 mg progesterone + 25 ng oestradiol-17 β (Sigma, St Louis) on the 3rd day (Group 2) (Weitlauf & Greenwald, 1968). The animals were killed by cervical dislocation 24 h after the last injection and their uteri were removed and flushed with glass-distilled water as previously

described (Weitlauf, 1976). Flushings were collected from 30 females in each treatment group; the samples were pooled, centrifuged (106 000 *g* for 1 h) to remove debris, and the protein content of the supernate was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Aliquots of the uterine fluid containing 30 µg protein were freeze-dried, reconstituted in 50 µl buffer (0.1 M-NH₄HCO₃, pH 8.5), placed on a Sephadex column (Sephadex G-25, 50 × 0.7 cm), and eluted with the same buffer supplied continuously by a peristaltic pump. Fractions (10 drops each) were collected with a fraction collector. The void volume of this column, as determined with blue dextran and bovine serum albumin, was at Fractions 22–23; the salt volume, as determined with (NH₄)₂SO₄, was at Fraction 40. Each fraction was freeze-dried, reconstituted with 100 µl glass-distilled water and freeze-dried again. The process was repeated 8 times to remove NH₄HCO₃; the residue was stored at –20°C.

The entire procedure for collecting fluid and its fractionation was repeated 3 times so that 3 entirely different batches of uterine flushings were prepared for each hormone treatment.

Assay of uterine flushings

At the time of assay, fractions were selected randomly and the freeze-dried residue was reconstituted with 25 µl Brinster's medium containing [³H]uridine (sp. act. 39 Ci/mmol: New England Nuclear, Boston) at a concentration of 6×10^{-7} M. Blastocysts were recovered from intact females killed on Day 5 of pregnancy (Day 1 = vaginal plug) as previously described (Weitlauf, 1976); 4–9 blastocysts were incubated in the radioactive medium for 2 h at 37°C in a humid atmosphere of 5% CO₂ + 95% air. After incubation, the blastocysts were removed and rinsed in medium with unlabelled uridine (6×10^{-4} M), and twice in 0.9% (w/v) NaCl (1.0 ml). Blastocysts were placed individually on Millipore filters (25 mm diameter, 0.45 µm pore size), and washed on a Millipore suction device with TCA (10%, 4°C) followed by glass-distilled water. The filters were dried and radioactivity in the acid-insoluble material was estimated with a scintillation counter as previously described (Weitlauf, 1974).

Fractions before the void volume fraction were used to establish the control level of [³H]uridine incorporation by blastocysts for each batch of uterine flushings (intra-assay coefficient of variation < 25%; inter-assay coefficient of variation < 20%). Incorporation of [³H]uridine was expressed as a percentage of control and transformed to logs for statistical analysis. Nested analysis of variance for groups of unequal sizes showed that the results with batches of fluid within hormone treatment groups were not different and the data were combined (Snedecor & Cochran, 1967). The same analysis indicated significant differences between fractions within treatment groups. Therefore, incorporation of [³H]uridine by blastocysts in the individual fractions was compared with that by blastocysts in the control fractions for each treatment group by one-way analysis of variance (Snedecor & Cochran, 1967).

Results

As shown in Text-fig. 1, several fractions of uterine flushings from each treatment group contained material that influenced incorporation of [³H]uridine by blastocysts *in vitro*. For convenience the active materials in the fractions are hereafter referred to as 'factors'. Factors with inhibitory activity were numbered consecutively from those in the void volume towards the salt volume. As shown in Table 1, some factors were present in flushings from animals in all three groups in amounts sufficient to inhibit blastocyst activity significantly; other factors were either not present in flushings from one or another of the treatment groups, or were present in amounts insufficient to affect the blastocysts significantly.

The responses of blastocysts to those factors common to all groups were compared by one-way analysis of variance followed by Duncan's multiple range test (Text-fig. 2). Factor 1 was more inhibitory in flushings from the 'delayed implanting' animals than in those from the 'implanting' animals. Factors 4, 6, 7, 10 and 11 were not significantly different in flushings from any group. Factors 8 and 9 were significantly more inhibitory in flushings from Group 1 animals than in those from Groups 2 or 3, and Factor 12 was significantly more inhibitory in flushings from Group 1 than from Group 2 animals.

Text-fig. 1. Semi-log plot of the incorporation of [^3H]uridine by mouse blastocysts incubated in the various fractions of flushings from uteri of 'delayed implanting' (Group 1, progesterone only, and Group 3, oil vehicle only) and 'implanting' (Group 2, progesterone + oestrogen) mice (expressed as % of the incorporation with pre-void volume fractions).

Table 1. Factors in fluid flushed from 'implanting' (Group 2) and 'delayed implanting' (Groups 1 and 3) mouse uteri that influence incorporation of [^3H]uridine by blastocysts *in vitro*

Group	Treatment	Designated factor no.												
		1	2	3	4	S	5	6	7	8	9	10	11	12
1	Progesterone	+	*	+	+	*	—	+	+	+	+	+	+	+
2	Progesterone + oestrogen	+	—	*	+	+	+	+	+	*	+	+	+	*
3	Oil vehicle	+	*	—	+	*	+	+	+	*	+	+	+	+

Comparison of uridine incorporation with that of control blastocysts showed no effect (—), a non-significant effect (*) or a significant effect (+, $P < 0.05$).

A statistically significant increase ($P < 0.05$) in the incorporation of [^3H]uridine occurred in blastocysts incubated in one fraction (S) of flushings from Group 2 (i.e. 'implanting') females. Although a similar (but statistically non-significant) effect appeared to be present in Fraction s of flushings from 'delayed implanting' uteri (Groups 1 and 3), this factor cannot be reliably evaluated because the blastocysts used in the assay may already have been maximally stimulated *in utero*.

Discussion

The results of the present experiments demonstrate that material flushed from the uteri of mice can be separated into several components by means of gel filtration and that some of these components tend to decrease the incorporation of [^3H]uridine by blastocysts *in vitro*.

Text-fig. 2. The incorporation (mean $\pm$ s.e.m. as % of control level with pre-void volume fractions) of [3 H]uridine by mouse blastocysts (no. at base of each column) incubated with various factors identified in flushings from 'delayed implanting' (Groups 1 and 3) and 'implanting' (Group 2) uteri. Stippled columns = Group 1, progesterone only; open columns = Group 2, progesterone + oestrogen; hatched columns = Group 3, oil vehicle only. Differences were assessed by one-way analysis of variance and Duncan's multiple range test, and columns indicated by paired arrows were significantly different at $*P < 0.01$ or $**P < 0.05$.

As shown in Text-fig. 2, the amount of inhibitory activity in most of the factors common to all groups is similar. However, the factor designated as No. 1 appears to be more inhibitory in fluid from 'delayed implanting' animals, both progesterone-treated and oil-treated, than it is in fluid from 'normal implanting' animals. Since a standard amount of protein from the uterine lumen was used for the gel filtration, differences in the inhibitory activity associated with Factor 1 from the three treatment groups presumably reflect differences in its concentration in the starting materials. It seems unlikely that the observed differences in inhibitory activity are related to dilution of the starting material by non-uterine proteins (e.g. serum) in one group and not another, as one would expect inhibitory activity of all factors in that group to be reduced. Therefore, it is suggested that Factor 1 is more concentrated in fluid flushed from the uteri of 'delayed implanting' animals than it is in fluid from 'normal implanting' animals.

The mechanism by which 'delayed implanting' mouse embryos are rendered dormant and then reactivated to implant at a later time is unknown. However, it has been shown that activation of blastocysts involves at least two distinct steps that appear to be controlled in different ways. The rate of production of CO_2 and the synthesis of protein and RNA by 'delayed implanting' mouse blastocysts increases to the levels characteristic of 'normal implanting' embryos in a simple culture medium

containing bovine serum albumin as the only macromolecular component (Torbit & Weitlauf, 1975; Weitlauf, 1974, 1976). Although this suggests that metabolic activation is achieved by removing the embryos from an inhibitory influence in 'delayed implanting' uteri, and that stimulatory factors are not essential, it does not rule out the possibility that a stimulatory factor is involved in the activation process *in utero*. In contrast, trophoblastic outgrowth occurs *in vitro* only when serum is included in the medium and this step therefore appears to require a specific stimulatory factor (Menke & McLaren, 1970). In the present experiments, metabolically active blastocysts were incubated *in vitro* with fractions of uterine flushings to determine whether factors are present in the uteri of 'delayed implanting' mice that are capable of inhibiting embryonic metabolism. Since such embryos may already be maximally active metabolically, the question of a possible stimulatory factor in metabolic activation, and the broader question of a stimulatory factor necessary for trophoblastic outgrowth, cannot be addressed.

The observation that similar amounts of several inhibitory factors occur in fluid from all treatment groups suggests that these factors are not influenced by the hormone treatments and therefore may be responsible for the non-specific inhibitory effect reported with non-fractionated uterine flushings (Weitlauf, 1976). Factor 1 is more concentrated in fluid from 'delayed implanting' animals than in that from 'normal implanting' animals, suggesting that this factor is influenced by the hormone treatments and could therefore be a specific inhibitor of embryonic metabolism during delayed implantation. The effect of Factor 1 *in vivo* needs to be determined.

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