Changes in the protein content of mouse uterine flushings during normal pregnancy and delayed implantation, and after ovariectomy and oestradiol administration

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Summary. The protein content of the mouse uterine lumen increased significantly ($P < 0.001$) on Day 4 of pregnancy, the day of implantation. This increase was associated with the presence of 14 serum and 22 non-serum proteins in the lumen; the major serum proteins were classed as high molecular weight slow α -globulins, while the dominant non-serum components consisted of slow and fast α -globulins, 6 prealbumins and a large quantity of proteinaceous material migrating near the origin of the gels.

During experimental and lactational delayed implantation the protein levels were constantly low, transferrin, haemoglobin and albumin dominating the protein pattern. After administration of oestradiol-17 β , however, a biphasic uterine response was detected, significant increases in luminal protein concentration being observed within 12 h and again at 40–48 h after injection. The first phase of this response involved an influx of serum and non-serum proteins into the uterine lumen, most proteins migrating as high molecular weight slow α -globulins. The second phase involved an increase in the intensity of many non-serum components, the major proteins having *R_a* values of 0.06, 0.10 and 0.32. The qualitative, but not the quantitative, aspects of this response to oestradiol were identical in the absence of blastocysts.

Introduction

During normal pregnancy in the mouse the morula–blastocyst transition is associated with a sudden increase in embryonic activity (Mills & Brinster, 1967; Woodland & Graham, 1969; Menke & McLaren, 1970; McLaren & Menke, 1971; Weitlauf, 1971; McLaren, 1973), culminating in the initiation of implantation at about 93 h *post coitum* (Finn & McLaren, 1967). If pregnancy is accompanied by lactation, however, these changes do not occur (Weitlauf, 1971; McLaren, 1973) and the blastocysts enter a period of embryonic diapause or delayed implantation. A similar state of diapause can be induced experimentally by ovariectomizing mice on Day 2 of pregnancy and subsequently administering progesterone (Yoshinaga & Adams, 1966). Lactational and experimental diapause can be terminated by the injection of a small dose of oestrogen (Yoshinaga & Adams, 1966; McLaren, 1968; Nalbandov, 1971). The way in which the uterus alternately suppresses and stimulates embryonic activity is not known, but two hypotheses have been put forward: (1) that the uterus elaborates an inhibitor during diapause which is withdrawn in response to oestrogen stimulation, and (2) that oestrogen induces the release of an embryotrophic secretion which is absent during delayed implantation.

The purpose of the present study was to examine the proteins present in the mouse uterine lumen during normal pregnancy, delayed implantation and following the administration of oestradiol in the hope of detecting changes that might be associated with the activation or suppression of embryonic activity.

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Materials and Methods

Animals. Female mice of the randomly bred Q strain were placed with intact or vasectomized males and examined daily; the day on which a vaginal plug was found was designated as Day 0.

Induction and termination of delayed implantation. Delayed implantation was induced experimentally by ovariectomizing mice on Day 2 of pregnancy or pseudopregnancy and administering 1 mg medroxyprogesterone acetate (Depo-provera: Upjohn), a long-acting progestin. Lactational delayed implantation was induced by mating females at the post-partum oestrus and adjusting the litter number to a minimum of 6 young. Experimental delayed implantation was terminated within 7 days by the administration of 1 µg or 50 ng oestradiol-17β (Sigma), both doses proving highly effective in the initiation of implantation (Humphrey, 1967).

Collection of uterine flushings. Uterine flushings were collected between 10.00 and 16.00 h on Days 2, 3 and 4 of normal pregnancy, Days 4, 5, 6 and 7 of pregnancy concurrent with lactation, Days 5, 7 and 9 of experimental delayed implantation and at selected time intervals after the administration of oestradiol. Uterine flushings were also collected from non-pregnant mice which had been ovariectomized at least 8 days previously. The uteri were carefully removed from mice killed by cervical dislocation and placed on cold damp filter paper. The uterine horns were cleared of adhering mesentery, separated and blotted gently to remove all trace of blood. Each horn was flushed rapidly with 0.5 ml 0.001 M-tris-glycine buffer, pH 8.3, into a clean, sterilized watch glass. After 5 animals had been treated in this manner in quick succession, the flushings were pooled, centrifuged at 2000 g for 20 min, frozen to -90°C, freeze dried and finally redissolved in 100 µl double-distilled water.

Chemical analysis. The protein content of the flushings was estimated for 30-µl samples by the technique of Lowry, Rosebrough, Farr & Randall (1951). To compensate for the inhibitory effect exerted by glycine on the colour development of the Folin-Ciocalteu reagent, tris-glycine buffer was also incorporated into the BSA standards.

Qualitative analysis of the uterine proteins was carried out on 70 µl samples using 7% polyacrylamide gels, pH 8.9 (Davis, 1964).

The significance of the means was assessed by Student's *t* and Mann-Whitney tests.

Results

Quantitative analysis

Quantitative analysis of the uterine flushings revealed a highly significant ($P < 0.001$) increase in protein concentration on Day 4 of pregnancy, the day of implantation. In contrast, the protein content of the uterine flushings was uniformly low following ovariectomy and the induction of experimental or lactational delayed implantation (Table 1).

The administration of oestradiol-17β to pregnant animals during experimental delayed implantation elicited a biphasic pattern of protein release into the uterine lumen (Table 2). With the 1 µg dose, significant increases in uterine protein concentration were observed at 4–6 h ($P < 0.005$) and 40–48 h ($P < 0.001$) after injection; similar changes were observed 12 h ($P < 0.005$) and 40–48 h

Table 1. Changes (mean $\pm$ S.D.) in the protein content of mouse uterine flushings during pregnancy, delayed implantation and after ovariectomy

	Day of pregnancy			Experimental delayed implantation		Lactational delayed implantation	Ovariectomy
	2	3	4	Pregnant	Pseudopregnant		
Conc. of protein (µg/mouse)	4.5 ± 1.4	4.8 ± 2.2	19.9 ± 3.8	6.9 ± 1.2	6.3 ± 1.2	3.8 ± 0.7	3.3 ± 1.9
No. of mice (5 mice/sample)	10	40	35	40	15	25	20

($P < 0.025$) after the administration of 50 ng oestradiol-17 β . However, the injection of oestradiol-17 β into ovariectomized pseudopregnant mice did not induce any significant increases in luminal protein content (Table 2).

Table 2. Changes (mean $\pm$ S.D.) in the protein content of uterine flushings after injection of oestradiol-17 β to ovariectomized pregnant or pseudopregnant mice treated with a progestin (i.e. delayed 'implantation')

Oestradiol dose	Time after oestradiol (h)	Pregnant mice		Pseudopregnant mice	
		Conc. of protein (μ g/mouse)	No. of mice (5/sample)	Conc. of protein (μ g/mouse)	No. of mice (5/sample)
1 μ g	0	6.9 $\pm$ 1.2	40	6.3 $\pm$ 1.2	15
	1	7.2 $\pm$ 1.4	15	—	—
	2	—	—	6.5 $\pm$ 1.0	15
	4	10.5 $\pm$ 1.7	15	7.4 $\pm$ 1.3	15
	6	8.5 $\pm$ 0.8	15	7.5 $\pm$ 0.5	15
	12	6.1 $\pm$ 0.8	15	4.9 $\pm$ 2.8	15
	24	7.5 $\pm$ 1.6	15	6.0 $\pm$ 0.4	15
	40	12.8 $\pm$ 2.3	15	4.4 $\pm$ 0.7	15
	44	11.0 $\pm$ 4.2	10	—	—
	48	14.6 $\pm$ 4.5	20	5.4 $\pm$ 2.6	20
50 ng	0	6.9 $\pm$ 1.2	40	6.3 $\pm$ 1.2	15
	3	5.3 $\pm$ 0.2	10	6.5 $\pm$ 2.4	10
	6	5.4 $\pm$ 0.2	10	3.6 $\pm$ 1.0	10
	12	10.9 $\pm$ 1.1	10	2.3 $\pm$ 0.7	10
	24	4.3	5	4.4 $\pm$ 1.1	10
	40	10.1 $\pm$ 4.3	10	4.2 $\pm$ 0.5	10
	48	13.3 $\pm$ 7.5	10	5.4 $\pm$ 2.0	10

Qualitative changes

In the following section, the proteins are described as α - or β -globulins (Cons & Glass, 1963), or according to their relationship with serum transferrin and albumin (Text-fig. 1).

Transferrin, albumin and haemoglobin. Serum transferrin (transferrin 3, genotype Trf^b/Trf^b; Renfree & McLaren, 1974) and albumin were observed in all the uterine flushings examined. Albumin appeared as a single quantitatively dominant band or occasionally as two bands running in close proximity. A double albumin band has also been observed following the electrophoresis of rat blood plasma (Beaton, Selby & Wright, 1961) and its appearance in the present study did not appear to be related to the physiological state of the animals. Since the flushing medium used was hypotonic to serum, most of the flushings examined also contained prominent haemoglobin bands. The Q strain segregates for both Hbs and Hbd forms of mouse haemoglobin, giving a maximum of 4 protein bands in the pooled samples. Two dominant proteins (*Ra* 0.60, 0.63) corresponded with the monomers designated Hb₂ and Hb₃ by Morton (1966), while two minor components (*Ra* 0.57, 0.68) resembled Hb₁ and Hb₅ of Morton's classification.

Days 2, 3 and 4 of pregnancy

With the exception of a major, non-serum, slow α -globulin (*Ra* 0.06) observed on Day 2, transferrin, haemoglobin and albumin dominated the protein profiles on Days 2 and 3. On Day 4, however, a large number of intense protein bands were observed, of which the most significant were 2 slow α -globulins (*Ra* 0.16, 0.20) and a fast α -globulin (*Ra* 0.74) of serum origin, and 22 non-serum components comprising 8 slow α -globulins (major proteins having *Ra* values of 0.06, 0.10, 0.25, 0.32, 0.34), 4 β -globulins, 3 fast α -globulins (major proteins having *Ra* values of 0.82, 0.89), 6 prealbumins (major proteins having *Ra* values of 1.19, 1.31) and a large quantity of proteinaceous material migrating near the origin of the gels behind the major serum lipoprotein band. The uterine proteins

Text-fig. 1. Electrophoresis of mouse blood plasma to demonstrate the classification system used for describing proteins in this study. Proteins are characterized by their mobility relative to the centre of the albumin band (*Ra* value; *Ra* albumin = 1.0). Regions of the gel are designated as slow α-globulin (*Ra* 0–0.34), β-globulin (*Ra* 0.35–0.70) and fast α-globulin (*Ra* 0.70–1.00). Alternatively, the gel may be divided into post-transferrin (*Ra* 0–0.43), pre-transferrin (*Ra* 0.43–0.70), post-albumin (*Ra* 0.70–1.00) and pre-albumin (*Ra* > 1.00) zones. T = transferrin; A = albumin.

recovered on Day 4 were also characterized by the absence of one serum (*Ra* 0.47) and 2 minor non-serum (*Ra* 0.92, 0.94) components observed on Day 3 (Text-fig. 2).

Delayed implantation

During experimentally induced delayed implantation, 29–30 protein bands were consistently observed; 16 were not of serum origin. The protein profiles were dominated by transferrin, haemoglobin and albumin, the remaining proteins being present in trace amounts only. Qualitatively the delay pattern resembled that observed on Day 4 of normal pregnancy, although the concentration of high molecular weight proteins in the delay samples was seriously diminished. The protein profile observed during experimental delayed implantation did, however, feature one serum (*Ra* 0.47) and 2 non-serum (*Ra* 0.92, 0.95) bands not visible on Day 4 (Text-fig. 2; Plate 1).

Lactational delayed implantation was also characterized by a lack of proteins in the high molecular weight region of the gels. A maximum of 37 bands were observed at this time, including 22 non-serum components, but once again transferrin, haemoglobin and albumin were the major proteins present (Text-fig. 2; Plate 1).

Ovariectomy

The weak protein pattern observed in ovariectomized non-pregnant mice was dominated by transferrin, haemoglobin and albumin bands, although traces of non-serum proteins were observed near the origin of the gels and in the prealbumin region (Text-fig. 2; Plate 1).

Administration of oestradiol-17β during delayed implantation

The elevated protein levels observed 4–6 h and 12 h after the administration of 1 μg and 50 ng oestradiol-17β, respectively, were associated with a marked increase in the intensity of several non-

Polyacrylamide gel electrophoresis of the uterine flushings of ovariectomized pregnant (Fig. 1) or pseudo-pregnant (Fig. 2) mice after administration of 1 μ g oestradiol-17 β (i.e. delayed 'implantation'). OX = ovariectomized animals; ED = experimental delayed implantation; S = serum; 1, 2, 4, 6, 12, 24 and 40 = h after oestradiol administration. Note the pronounced increase in the number and intensity of bands 4–6 h after injection, the general decline in protein concentration at 12 h and the subsequent increase in non-serum proteins at 40 h (Fig. 1) and 24 and 40 h (Fig. 2).

Fig. 1. At S, 4 and 6 h, open arrows = serum slow α -globulins (R_{α} 0.16 and 0.20); at 40 h, closed arrow = major non-serum slow α -globulin at R_{α} 0.32.

Fig. 2. At S, 4 and 6 h, open arrows = serum slow α -globulins (R_{α} 0.16 and 0.20), closed arrows = proteinaceous material at the origin; at 40 h, closed arrows = major non-serum slow α -globulins at R_{α} 0.06 and 0.32.

Text-fig. 2. Polyacrylamide gel electrophoresis of mouse uterine flushings during pregnancy, experimental and lactational delayed implantation, and following ovariectomy. S = mouse plasma; D2 = Day 2 of pregnancy—arrow = non-serum slow α -globulin (Ra 0.06); D3 = Day 3 of pregnancy; D4 = Day 4 of pregnancy—open arrows = major serum protein at Ra 0.16, 0.20, 0.74, closed arrows = major non-serum proteins at Ra 0.06, 0.10, 0.25, 0.32, 0.34, 0.82, 0.89, 1.19, 1.31; ED5 = Day 5 of experimental delayed implantation—closed arrows = non-serum proteins at Ra 0.92, 0.95 which were not present on the day of implantation; ED7 = Day 7 of experimental delayed implantation; ED9 = Day 9 of experimental delayed implantation; LD = lactational delayed implantation; OX = ovariectomized mice not receiving hormone treatment. i = major serum lipoprotein band; t = transferrin; h = haemoglobin bands; a = albumin. Proteins identified by closed circles are presumed to be of serum origin.

serum bands (Ra 0.06, 0.10, 0.25, 0.32, 0.34, 0.72, 1.06, 1.19, 1.32) and the appearance of a large quantity of proteinaceous material near the origin of the gels. An increase in the concentration of many serum proteins was also particularly evident at this time, especially in the high molecular weight region of the gels (Ra 0.16, 0.20). From 12 to 24 h after oestradiol administration there was a general reduction in the intensity of all protein bands, followed at 40–48 h by an increase in the concentration of certain non-serum components. The most dominant of these bands was a slow α -globulin (Ra 0.32), while the other major proteins present included slow and fast α -globulins (Ra 0.06, 0.10, 0.25, 0.34, 0.84, 0.89) and a maximum of 10 prealbumins (Ra 1.06, 1.09, 1.16, 1.19, 1.24, 1.27, 1.32, 1.34, 1.38, 1.42). The intensity of these non-serum proteins was greater in those animals receiving the higher dose of oestradiol-17 β . With the exception of albumin and transferrin, serum proteins were only present in trace amounts 40–48 h after oestradiol injection (Pl. 1, Fig. 1).

Although the uterine response to oestradiol was quantitatively different in pregnant and pseudo-pregnant mice, the qualitative changes induced by this hormone were similar in both groups of animals (Pl. 1, Fig. 2).

Discussion

The results obtained in this study support observations made on the fur seal (Daniel, 1971), rat (Surani, 1975), roe deer (Aitken, 1974a, b, 1975) and wallaby (Renfree, 1973) in associating embryonic diapause with a lack of proteinaceous material within the uterine lumen. A causal relationship

between this lack of secretory material and the induction of delayed implantation is suggested by the fact that mouse blastocysts enter a state of diapause *in vitro* when either whole serum or certain amino acids are omitted from the incubation medium (Gwatkin, 1966a, b; McLaren, 1973). Since the subsequent addition of serum to the medium results in a rapid resumption of blastocyst activity (McLaren, 1973), a causal relationship may also exist between the increase in luminal protein concentration, observed on Day 4 of pregnancy and following oestradiol administration, and blastocyst activation. The features of embryonic activation, whether induced by the addition of serum *in vitro* or the administration of oestradiol *in vivo*, certainly appear to be identical. In both instances the blastocyst responds with a marked increase in the rate of DNA and RNA synthesis (Ellem & Gwatkin, 1968; Prasad, Dass & Mohla, 1968; Psychoyos & Bitton-Casimiri, 1969), carbon dioxide production (McLaren & Menke, 1971) and cell division (Gwatkin & Meckley, 1966; Bowman & McLaren, 1970) and the appearance of trophoblastic giant cells (Gwatkin, 1966a, b; Spindle & Pedersen, 1974).

No evidence could be found in the present study for the existence of inhibitory proteins in the uterine fluids during delayed implantation. Although traces of two fast α -globulins (R_f 0.90, 0.94) were detected during experimental delay and not at the time of implantation, these proteins were absent during lactation and following ovariectomy, despite the fact that both conditions are also associated with embryonic dormancy. However, it is possible that the proposed inhibitor is either not a protein (Psychoyos, 1973) or not resolved on polyacrylamide gels at pH 8.9 because of its charge or molecular weight.

The sudden increase in luminal protein concentration observed on Day 4 of pregnancy appeared to be induced by oestrogen. Plasma oestrogen levels are maximal 1 day before implantation (McCormack & Greenwald, 1974) and, in the present study, oestradiol-17 β induced a marked rise in the protein content of the uterine fluids when administered during diapause. Examination of the uterine flushings obtained at different times after the injection of oestradiol showed that the response to this hormone was biphasic, involving increases in luminal protein concentration within 12 h and at 40–48 h after injection. Qualitatively, the first phase involved the release of high molecular weight proteins into the uterine lumen, some of the most dominant proteins being of serum origin. The presence of serum slow α -globulins in the uterine flushings may have been the result of a transient increase in capillary permeability, since a general increase in endometrial vascularity and oedema was observed at this time. Surani (1975) also observed the passage of high molecular weight proteins into the rat uterine lumen 13–20 h after oestradiol injection, and Bergström & Nilsson (1975) have presented ultrastructural evidence confirming the release of a uterine secretion within 8 h of oestrogen administration in the mouse. The fact that ultrastructural signs of blastocyst activation are evident within 8 h of an injection of oestradiol (Nilsson, 1974) suggests that this early phase of uterine secretory activity is responsible for terminating diapause.

The second phase of the uterine response to oestradiol occurred 40–48 h after the injection of hormone and involved a marked increase in the concentration of certain non-serum proteins. The most prominent of these had an R_f value of 0.32 and closely resembled proteins identified in the uterine flushings of women (Wolf & Mastroianni, 1975; H. Maathius & R. J. Aitken, unpublished), baboons (Peplow, Breed, Jones & Eckstein, 1973) and rats, 18–24 h after the administration of oestradiol, maintained in delay with progesterone (Surani, 1975).

The quantitative aspects of the uterine response to oestradiol appeared to depend upon the presence of blastocysts in the uterine lumen. Within 12 h of oestradiol administration during diapause a quantitative increase in luminal protein concentration was observed in pregnant but not pseudopregnant mice and the increase was apparent several hours before the initiation of blastocyst attachment. It is therefore possible that the activated blastocysts induced a local increase in uterine secretory activity and capillary permeability through the release of, for example, steroids (Perry, Heap & Amoroso, 1973) or specific proteins (Jones, Heap & Perry, 1976).

In most eutherian mammals the induction of uterine secretory activity (Urzua, Stambaugh, Flickinger & Mastroianni, 1970; Arthur & Daniel, 1972; Knight, Bazer & Wallace, 1973) and the initiation of implantation (Nalbandov, 1971; Cumming, Baxter & Lawson, 1974; Murphy & Mead, 1976) are under the control of progesterone. In the mouse (Yoshinaga & Adams, 1966) and rat

(Canivenc & Laffargue, 1956; Yoshinaga, 1961; Psychoyos, 1973; Surani, 1975), however, these events require the presence of oestrogen as well as progesterone. It may be significant that the other two species for which an oestrogen requirement for implantation has been suggested, the roe deer (Aitken, 1974a, b, 1975) and fur seal (Daniel, 1974), also exhibit the phenomenon of delayed implantation.

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