Requirement for Progesterone Priming and Its Long-Term Effects on Implantation in the Mouse (43032)

YVETTE M. HUET-HUDSON[1] AND S. K. DEY[2]

Department of Obstetrics-Gynecology and Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66103

Abstract. **At least 48 hr of progesterone (P_4) priming has been documented to be essential for P_4 and estrogen to initiate implantation in the rat. However, the length of this P_4 priming requirement for implantation in the mouse has not been experimentally defined. Therefore, our first objective was to determine the length of P_4-priming requirement for implantation in the mouse. Day 4 blastocysts were transferred into the uteri of Day 5 or Day 6 pseudopregnant mice that were ovariectomized on Day 1 (=vaginal plug) and treated with a single injection of P_4 and 17β-estradiol (E_2) only on Day 5, or a single injection of P_4 on Day 5 followed by a second injection of P_4 plus E_2 on Day 6, respectively. Although none of the transferred blastocysts implanted in the uteri of P_4-unprimed recipients, 46% of the transferred blastocysts implanted into the uteri of all recipients that were first primed with P_4 24 hour prior to a second injection of P_4 and E_2. These results suggest that in contrast to the rat, the mouse uterus requires at most 24 hr of P_4 priming before P_4 and estrogen can initiate implantation. Our second objective was to determine whether P_4 priming has a long-term effect on implantation in the mouse. Our present results and those of others suggest that the mouse uterus is exposed to rising P_4 levels for 24 hr prior to implantation on Day 4 of pregnancy. Therefore, in the present investigation, induction of implantation by an injection of P_4 and E_2 following 5 days of ovariectomy performed on Day 4 of pregnancy clearly suggests that once exposed to P_4 for 24 hr, the mouse uterus retains a long-term effect, i.e., following P_4 withdrawal for several days, 24 hr of initial P_4 priming is no longer required for P_4 and estrogen to initiate implantation. Our next objective was to explore whether this long-term effect of P_4 priming on implantation can be prolonged and potentiated by increasing the length of initial P_4 priming. Thus, when the mice were ovariectomized on Day 4 of pregnancy and treated with P_4 beginning on Day 5 for 4 days, the long-term effect on implantation was prolonged (8 days vs 5 days following P_4 withdrawal) and potentiated (94% vs 0% mice with implantation following 8 days of P_4 withdrawal) as compared with those with no P_4 priming after ovariectomy. Taken together, the results suggest that 24 hr of P_4 priming is not only adequate for implantation in the mouse, but this priming has a long-term effect on implantation in this species.** [P.S.E.B.M. 1990, Vol 193]

In the mouse and rat, uterine preparation for embryo implantation depends upon a temporal sequence of progesterone (P_4) and estrogen treatment. In the rat, at least 48 hr of P_4 priming is essential for P_4 and estrogen to initiate implantation (1). Although several studies in the past dealt with the steroid hormonal control of implantation in the mouse (2–4), the length of P_4-priming requirement for implantation in the mouse has not been determined specifically. Based on work in the rat (1), at least 48 hr of P_4 priming is customarily used to study implantation in the mouse (5–7). Using experimentally induced decidualization as a model for implantation, Finn (2) stated that the induction of decidualization was better in the estrogen-primed mouse uterus that was exposed to P_4 for 48 hr instead of 28 hr. Implantation in the mouse occurs on the evening of Day 4, whereas in the rat it occurs on the evening of Day 5. Thus, there is a difference of 24 hr in the timing of implantation in the mouse and rat,

[1] Present address: Monsanto Company, 700 Chesterfield Village Parkway, AA4C, St. Louis, MO 63198.

[2] To whom requests for reprints should be addressed.

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although plasma P_4 levels begin to rise in these two species from Day 3 of pregnancy (8–10). This implies a different requirement of P_4 priming in the mouse. Recent reports document that in certain instances once the target tissues are transiently exposed to estrogen, they can retain a memory for estrogen, i.e., they show long-term estrogen-specific responses without further exposure to the hormone or enhanced responses to the subsequent suboptimal hormonal stimulation. This has particularly been demonstrated both *in vivo* and *in vitro* on hepatic gene expression in egg-laying vertebrates (11–14) and on the synthesis of apolipoproteins by an established human hepatocarcinoma cell line (15). The exhibition of long-term responses by target tissues is probably due to an establishment of an autoregulatory loop permanently inducing estrogen receptor mRNA following transient estrogen administration (16). To our knowledge, no such long-term effect of P_4 in the target tissues of any species has been reported. Therefore, we have determined the length of P_4-priming requirement as well as long-term effects of P_4 on implantation in the mouse.

Materials and Methods

Charles River (CD-1) female mice (48 days old, 20–25 g) were used. To determine the length of P_4-priming requirement for P_4 and estrogen to initiate implantation, mice were ovariectomized on the morning of Day 1 of pseudopregnancy produced by mating with vasectomized males (Day 1 = vaginal plug) and rested for 4 days. These mice served as recipients and were given a single injection of P_4 (2 mg/mouse, Sigma Chemical Co., St. Louis, MO) on Day 5 or Days 5 and 6 before embryo transfer. Blastocysts were recovered from uteri on Day 4 of pregnancy in Whitten's medium (17). They were transferred into the uteri of the recipients on Day 5 or Day 6 (18). Following blastocyst transfers, the recipients received an implantation-inducing dose of 17β-estradiol (E_2, 20 ng/mouse; Sigma) and were checked for implantation 24 hr later.

To determine whether P_4 exhibits any long-term effects on implantation, mice were ovariectomized on the morning of Day 4 of pregnancy to induce delayed implantation (19, 20). Animals were allowed to rest for various time periods and then given a combination of P_4 (2 or 0.5 mg/mouse) and E_2 (20 ng/mouse) to initiate implantation. In order to determine whether the long-term effect could be prolonged by increasing the initial length of uterine exposure to P_4, mice were ovariectomized on the morning of Day 4 of pregnancy and were injected daily with P_4 (2, 0.5, or 0.1 mg/mouse), beginning on Day 5 for 4 days. On a specific day following P_4 withdrawal, they were given an injection of E_2 (20 ng/mouse) or a combination of P_4 (2, 0.5, or 0.1 mg/mouse) plus E_2 (20 ng/mouse) and checked for implantation 24 hr later. To determine whether the effect of withdrawal of P_4 was on the embryo or the uterus, blastocysts recovered from P_4-withdrawn uteri were transferred to Day 4 pseudopregnant mice and checked for implantation the next day (18). Initiation of implantation was determined by an intravenous injection of 0.1 ml of 1% Chicago Blue B (Sigma) 5 min before killing (1). Discrete blue bands along the uterus indicated implantation sites. Mice with no implantation sites nor recoverable embryos were excluded from the experiment. Serum and uterine levels of P_4 were measured by radioimmunoassay (10). Uterine and blood samples for P_4 determination were collected between 0900 and 1000 hr. Serum was directly assayed. Uteri were trimmed of fat and homogenized in 2 ml of ethanol. The ethanolic extracts were dried down in a vortex evaporator and resuspended in assay buffer. The lowest detectable level of P_4 that could be distinguished from the 0 ng/ml standard was 0.10 ng/ml at the 95% confidence limit. The intraassay and interassay coefficients of variation did not exceed 8% and 6%, respectively.

Results

The first objective of the present study was to determine the length of P_4-priming requirement for implantation in the mouse. As shown in Table I, a total of 97 embryos, transferred into the uteri of six Day 5 pseudopregnant mice that were ovariectomized on Day 1 and received P_4 and E_2 only on Day 5 failed to implant. In contrast, implantation of 45 of 97 embryos (46%) occurred when they were transferred into the uteri of similarly prepared recipients (n = 6), but receiving a priming dose of P_4 on Day 5 followed by an injection of P_4 and E_2 on Day 6. These results clearly indicate that unlike in the rat, 24 hr of P_4 priming is adequate for embryo implantation in the mouse.

The second objective was to determine whether P_4 priming has a long-term effect on implantation in the mouse. As indicated in Table II, about 70% of the animals showed implantation if they received an injection of P_4 and E_2 on any specific day until 5 days following ovariectomy on Day 4. These animals had rising plasma and uterine P_4 levels from Day 3 of pregnancy (48.4 ± 6.2 ng/ml plasma and 117.6 ± 15.3 pg/mg uterus on Day 3, n = 5) and thus by the time of ovariectomy on Day 4 they were exposed to P_4 for 24 hr (9). However, the number of animals with implantation sites reduced to about 40% by 7 days and by 8 days after ovariectomy none of the animals had implantation following an injection of P_4 and E_2. Decreasing the dose of P_4 from 2 mg to 0.5 mg/mouse was still effective in inducing implantation in most of the animals after 5 days of ovariectomy. These results suggest that the mouse uterus can exhibit a long-term effect for several days after an exposure to P_4 for 24 hr and that

Table I. Requirement of P_4 Priming for P_4- and E_2-Initiated Implantation in the Mouse[a]

No. of recipients	Treatment	Day of treatment	Day of transfer	No. of embryos transferred	No. of embryos implanted	No. of mice with implantation sites
6	$P_4 + E_2$	5	5	97	0	0
6	P_4	5	6	97	45	6
	$P_4 + P_2$	6				

[a] Blastocysts for transfer were recovered from uteri of Day 4 pregnant mice. Recipient mice were ovariectomized on Day 1 of pseudopregnancy and rested for 4 days before P_4 and E_2 treatments and embryo transfer. P_4 (2 mg/mouse) and E_2 (20 ng/mouse) were dissolved in sesame seed oil (Sigma) and injected subcutaneously. Recipients were examined for implantation sites 24 hr after embryo transfer.

Table II. Effect of P_4 Withdrawal on P_4- and E_2-Initiated Implantation in the Ovariectomized Delayed Implanting Mouse[a]

Days after ovariectomy	Treatment	No. of mice	No. of mice with implantation sites (%)	No. of implantation sites
3	P_4 (2 mg) + E_2 (20 ng)	6	4 (67)	7.8 ± 2.0
5	↓	13	9 (69)	4.8 ± 0.9
6	↓	17	7 (41)	4.7 ± 0.6
7	↓	8	3 (38)	3.7 ± 2.0
8	↓	7	0 (0)	0
5	P_4 (0.5 mg) + E_2 (20 ng)	6	5 (83)	6.4 ± 1.4

[a] Mice were ovariectomized on Day 4 of pregnancy. They received a single injection of P_4 and E_2 on a specific day after ovariectomy and were checked for implantation sites 24 hr later. The vehicle and route of administration for P_4 and E_2 were as in Table I. Mean (±SE) numbers of implantation sites were calculated only for animals with implantation sites. Numbers in parentheses indicate percentage of animals with implantation sites.

following this initial exposure, 24 hr of P_4 priming is no longer required for implantation.

Our next objective was to see whether this long-term effect of P_4 on implantation could be prolonged by increasing the initial duration of P_4 priming. As shown in Table III, implantation was induced in 94% of the animals exposed to P_4 (2 mg/mouse) for 4 days following ovariectomy on Day 4 and given a single injection of P_4 plus E_2 8 days after the last injection of P_4. In contrast, animals without daily exposure to P_4 for 4 days showed no implantation 8 days later after an injection of P_4 and E_2 (see Table II). A sharp decline in the number of animals with implantation (27%) was recorded with an injection of P_4 and E_2 following 9 days of P_4 withdrawal (Table III). These results clearly show that 4 days of initial exposure to P_4 not only prolonged (8 days vs 5 days), but also potentiated (94% vs 0% animals with implantation 8 days later) the long-

Table III. Effect of P_4 Withdrawal on E_2, or P_4- and E_2-Initiated Implantation in the Ovariectomized Delayed Implanting Mouse following Initial Exposure to Exogenous P_4[a]

Days after P_4 treatment	Treatment	No. of mice	No. of mice with implantation sites (%)	No. of implantation sites
1	E_2 (20 ng)	8	8 (100)	11.8 ± 0.7
2	↓	12	5 (42)	7.0 ± 2.4
3	↓	4	0 (0)	0
4	P_4 (2 mg) + E_2 (20 ng)	10	10 (100)	6.9 ± 1.0
5	↓	6	5 (83)	8.0 ± 1.2
8	↓	17	16 (94)	7.6 ± 0.8
9	↓	11	3 (27)	6.7 ± 2.9
8	P_4 (0.5 mg) + E_2 (20 ng)	10	4 (40)	7.3 ± 1.9
8	P_4 (0.1 mg) + E_2 (20 ng)	8	0 (0)	0

[a] Mice were ovariectomized on Day 4 of pregnancy. They were then treated with P_4 beginning on Day 5 for 4 days. On a specific day after the last P_4 injection (P_4 withdrawal), these animals were treated with a single injection of either E_2 or P_4 plus E_2, and checked for implantation sites 24 hr later. The same doses of P_4 were used for initial exposure and initiation of implantation. The vehicle and route of administration for P_4 and E_2 were as in Table I. Mean (±SE) numbers of implantation sites were calculated only for animals with implantation sites. Numbers in parentheses indicate percentage of animals with implantation sites.

term effect of P_4 as compared with no initial exposure to exogenous P_4 following ovariectomy on Day 4 of pregnancy (compare Table II versus Table III). Although P_4 at a reduced dose of 0.5 mg with E_2 was still effective in inducing implantation in 40% of the animals following 8 days of P_4 withdrawal, the further reduction of the dose to 0.1 mg was no longer effective (Table III). E_2 alone induced implantation in several animals only up to 48 hr after 4 days of initial P_4 exposure (Table III). The induction of implantation by E_2 in the absence of P_4 could be due to residual progesterone (133.8 ± 48.0 pg/mg uterus, $n = 5$) still present in the uterus 48 hr after 4 days of P_4 treatment. On the other hand, induction of implantation by P_4 plus E_2 following 8 days of P_4 withdrawal is suggestive of a long-term effect because P_4 levels in these uteri were extremely low and not different from those that did not show implantation 9 days following P_4 withdrawal after 4 days of initial exposure to P_4 (2.5 ± 0.2 pg/mg uterus, $n = 5$ vs 3.7 ± 0.8 pg/mg uterus, $n = 6$). Indeed, these P_4 values were insignificant as compared with the uterine levels (58.4 ± 6.8 pg/mg uterus, $n = 7$) of intact Day 4 pregnant mice prior to implantation. A total of 28 of 38 embryos (74%) recovered from 10 mice 9 days after P_4 withdrawal implanted in all mice ($n = 4$) after transfer into the uteri of Day 4 pseudopregnant mice. The results of this experiment indicate that implantation failure in most of the animals 9 days following P_4 withdrawal was not due to an adverse effect on the embryo.

Discussion

Our results clearly demonstrate that 24 hr of P_4 priming is adequate for estrogen to induce implantation in the mouse. This finding is in contrast to the requirement of 48 hr of P_4 priming in the rat (1). Because embryonic development must be synchronized with the hormonal preparation of the uterus for successful implantation and because blastocyst formation and implantation in the mouse occur about 24 hr earlier than in the rat, it is possible that the temporal requirement for P_4 is different in these two species. Interestingly, Dickmann (21) has claimed that even in the rat, 24 hr of P_4 priming is adequate for estrogen to initiate implantation. In this study, long-term ovariectomized rats, primed with P_4 for 1 day or 4 days as well as with no P_4 priming, received embryo transfers and then were treated with P_4 and estrone daily for 14–18 days and examined for fetuses and resorption sites 24 hr after the last injection of steroids. Fetal weights were not recorded and compared among various treatment groups. Thus, the results of these experiments do not elucidate as to when initiation of implantation occurred and fail to establish the claim for the requirement of 24 hr of P_4 priming in the rat. Furthermore, 40% of the transferred embryos implanted in 90% of the rats apparently not primed with P_4. This would then suggest that P_4 priming is not an absolute requirement for implantation in the rat. Although surprising, this is a serious issue, and further investigation is warranted to resolve this problem.

A broad spectrum of actions of P_4 in the mammalian uterus have been described (22). However, the mechanism by which P_4 priming prepares the uterus for P_4 and E_2 to initiate implantation is unknown. We also do not know how P_4 initiates a long-term effect in the mouse uterus. Two major manifestations of P_4 action in the rodent uterus during the peri-implantation period are (i) the differentiation of luminal epithelial cells and closure of the lumen and (ii) proliferation and differentiation of stromal cells to decidual cells (1, 23). Whether the induction of this long-term effect is cell type specific or not cannot be determined from the present investigation. Because P_4 can cause some stromal cell proliferation, it could be postulated that once induced, the long-term effect is propagated through cell division as documented for long-term effect of estrogen in hepatocarcinoma cell line *in vitro* (15). However, it is not expected that cellular proliferation will continue in the uterus in the absence of steroid hormones. In our opinion, the long-term effect of P_4 perhaps is induced via the differentiation of specific cell types that persist in the uterus for a period of time. The disappearance of these differentiated cells from the uterus could be responsible for the loss of responsiveness of the uterus for implantation 9 days following P_4 withdrawal. Moulton and Koenig (24) have shown that stromal cells that synthesized DNA during the preimplantation period or following progesterone and estrogen treatment persisted for several days and later differentiated into decidual cells. A cell-specific marker for P_4-induced differentiation will be required to address this issue.

Continuous presence of P_4 is required for maximal decidual response *in vivo* (25). However, stromal cells, exposed to P_4 *in vivo* prior to implantation, spontaneously decidualize in culture in the absence of P_4 (26, 27). We could perhaps suggest that P_4-exposed stromal cells retained a long-term effect of P_4 for decidualization *in vitro*. Alternatively, culture conditions could be responsible for spontaneous decidualization of stromal cells *in vitro*. The differences between the *in vivo* and *in vitro* responses may be due to modulation of P_4 action by other ovarian hormones *in vivo*, especially estrogen which has an antagonistic/synergistic relationship with P_4. Indeed, estrogen can transform P_4-induced uterine neutral phase to nonreceptive phase for blastocyst implantation after 24 hr (1). In the present experiments, the source of endogenous gonadal steroids was removed by ovariectomy, allowing us to show the long-term effect of P_4 *in vivo*. Although, we cannot rule out the possible contribution of steroids from adrenal

glands in ovariectomized mice, the low levels of P_4 detected in uteri following 8 or 9 days of P_4 withdrawal in ovariectomized mice suggest a minimal contribution by the adrenals. Our experiments have clearly shown for the first time that in the mouse, 24 hr of P_4 priming is adequate for implantation and that P_4 in the uterus of this species can induce a long-term effect which facilitates subsequent P_4 action. This work also establishes a model for the study of the mechanism(s) involved in this process.

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