THE RAT BLASTOCYST DURING NORMAL PREGNANCY AND DURING DELAYED IMPLANTATION, INCLUDING AN OBSERVATION ON THE SHEDDING OF THE ZONA PELLUCIDA

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Summary. It has been reported in the literature that blastocysts during delayed implantation (here referred to as 'dormant blastocysts') differ from blastocysts on Day 5 (spermatozoa in vagina = Day 1) of regular pregnancy (here referred to as 'active blastocysts') as regards size, mitotic activity and fine structure. In the present study, it was attempted to determine whether these two types of blastocysts also differ in their physiological potential, i.e. whether they can survive under the same environmental conditions. The survivability was tested as follows: 'active blastocysts' were transferred into 'dormant uteri' (uteri during delayed implantation) and 'dormant blastocysts' were transferred into 'sensitive uteri' (uteri on Day 5 of pregnancy). In both types of transfers, the blastocysts developed into normal, live foetuses. The compatibility between the uterus and the blastocyst, if one is 'active' and the other is 'dormant', is discussed.

INTRODUCTION

Normally, fertilized rat eggs take about 3 days to pass through the oviduct, and on the 4th day of pregnancy they enter the uterus. From Day 4 to Day 5 (spermatozoa in vagina = Day 1), the eggs lie free, unattached, in the uterine cavity, and during this period they undergo transformation from morulae to blastocysts. In the afternoon of Day 5, attachment of the blastocyst to the uterine epithelium starts, and this we consider the beginning of implantation.

If a female mates during post-partum oestrus and suckles six or more pups, the blastocysts usually do not implant on Day 5, but implant 2 to 14 days later (Krehbiel, 1941; Brumley & De Feo, 1964). When such a delay occurs, one speaks of 'delayed implantation'. During the period of delay, the blastocysts lie free in the uterine lumen where they attain a slightly larger size (Schlafke & Enders, 1963; Baevsky, 1963), and where their development and mitotic

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activity cease (Baevsky, 1963). Schlafke & Enders (1963) also noted quantitative changes in some of the fine structures of both the inner cell mass and the trophoblast cells. Delayed implantation can be experimentally induced in pregnant, non-suckling animals, if they are ovariectomized on either Day 2, Day 3 or on the morning of Day 4 of pregnancy and are given daily injections of progesterone. The delay period can be terminated, i.e. implantation can be induced, by injecting oestrogen. The technique of experimental delay and subsequent induction of implantation is one of the tools currently used to study the process of implantation (see reviews by Mayer, 1959, 1963a, b).

In this paper, during the period of delayed implantation, blastocysts are referred to as 'dormant blastocysts' and uteri as 'dormant uteri'. During Day 5 of pregnancy, blastocysts are termed 'active blastocysts', since they are ready to implant, and uteri are termed 'sensitive uteri', since they are competent to produce a decidua as a result of blastocyst stimulation.

The basic problem we have been interested in is, in terms of both the blastocyst and the uterus, what is dormancy, and how does a system change from a dormant to an active stage and vice versa? In the present experiments, attention was focused on some aspects of this problem as it relates to the blastocyst. As already mentioned above, the 'dormant blastocyst' differs from the 'active blastocyst' as regards size, mitotic activity and fine structure. However, it has not been shown whether these different characteristics also reflect physiological differences; for example, whether the two types of blastocysts can survive under the same environmental conditions. As a working hypothesis, it was assumed that there was a physiological difference between the two types of blastocysts. It was further postulated that this difference develops when both the blastocyst and the uterus simultaneously undergo a gradual change to become 'dormant'. Similarly, as a result of oestrogen stimulation, the 'dormant uterus' gradually changes to become 'sensitive', and at the same time the 'dormant blastocyst' changes to become 'active'. In order to test this hypothesis, we set out to answer the following questions: (1) Can 'active blastocysts', recovered from donor animals, survive if they are transferred directly (without undergoing a gradual change) into a 'dormant uterus'? And conversely (2) can 'dormant blastocysts' implant within a few hours after transfer into a 'sensitive uterus'? The rationale for asking these questions is further shown if we consider an additional difference between the 'dormant blastocyst' and the 'active blastocyst', namely, that the former is chronologically older than the latter. Previous studies (Dickmann & Noyes, 1960; Noyes & Dickmann, 1960) established that the uterus on a certain day of pregnancy is a favourable milieu for embryos of a certain age, but at the same time it is a hostile milieu to embryos of a different age. For example: Day 5 eggs, i.e. blastocysts, survive and develop normally if transferred into a Day 5 uterus. By contrast, Day 4 eggs, i.e. morulae, die subsequent to their transfer into a Day 5 uterus.

MATERIALS AND METHODS

Egg transfer technique

Adult virgin female rats of the Holzman strain, weighing 180 to 240 g, were used. The egg transfer technique was a simplification of the one described by

Noyes & Dickmann (1960). To recover blastocysts, donors were killed on the morning of Day 5 of pregnancy, and their uteri were excised and flushed with a medium consisting of two parts saline (0.9% NaCl) to one part rat plasma. To prevent clotting of the plasma, one drop of heparin (1000 USP units/1 ml) was added to every 5 ml of whole blood before it was centrifuged. Until the time of flushing of the uteri, the medium was kept at 37° C, but thereafter no further heating was applied. The blastocysts to be transferred were drawn up into a micropipette in 0.0001 to 0.0005 ml of medium. Recipients were anaesthetized with ether, and their uteri were exposed via a mid-ventral incision. The ovarian end of the uterus was gently held between the thumb and the index finger and the antimesometrial wall of the uterus was punctured with a fine cutting-edge surgical needle so as to create a passage into the uterine lumen. Then, the micropipette containing the blastocysts was inserted through this passage and the blastocysts were deposited in the uterine lumen. After transfers were made to both uterine horns, the abdominal wall was closed with silk sutures, and the skin was closed with wound clips.

TEXT-FIG. 1. Treatment schedule of recipients in Experiment 1.

Outline of experiments

Experiment 1: Transfer of 'active blastocysts' into 'dormant uteri'. The schedule of Experiment 1 is shown in Text-fig. 1. On the day of pro-oestrus (Day 0) and again on the following day (Day 1), recipient rats were cervically stimulated with a vibrating rod. Almost 100% of rats so treated become pseudopregnant (De Feo, 1966). On Day 2, the recipients were ovariectomized. From Day 2 to Day 9, the recipients were given, daily, subcutaneous injections of 2 mg progesterone dissolved in 0.1 ml of sesame oil. On Day 7, 'active blastocysts' (blastocysts recovered from the uteri of donors on Day 5 of pregnancy) were transferred into the uteri of recipients. On Day 10, the recipients were killed, their uteri were excised and flushed with saline, and the recovered blastocysts were examined in the fresh state with the light microscope.

Experiment 2: Induction of implantation and survival of the foetus. The same procedure as in Experiment 1 was followed, but the recipients were not killed on Day 10. Starting on Day 10 and continuing to Day 23, the recipients were injected daily with 2 mg progesterone + 1 µg oestrone in 0.1 ml of sesame oil.

This hormonal combination was previously found to be effective in: (1) terminating delayed implantation in lactating rats (Brumley & De Feo, 1964) and (2) maintaining pregnancy in ovariectomized rats (De Feo, unpublished). On Day 24, the recipients were killed, and their foetuses were counted and weighed.

Experiment 3: Transfer of 'dormant blastocysts' into 'sensitive uteri'. The same procedure as in Experiment 1 was followed, but the 'dormant blastocysts' which were recovered on Day 10 were re-transferred to the 'sensitive uteri' of a second recipient, referred to as the definitive recipient, which was in its 5th day of pseudopregnancy. On Day 18 or Day 22 of the anticipated pregnancy, the definitive recipients were killed, and their foetuses were counted and weighed.

RESULTS AND DISCUSSION

Experiment 1: Transfer of 'active blastocysts' into 'dormant uteri'

One hundred and nineteen 'active blastocysts' were transferred into the 'dormant uteri' of sixteen recipients. At the termination of the experiments, a total of seventy-four blastocysts was recovered, sixty-nine of which looked normal (Pl. 1, Figs. 1 and 2), while the remaining five blastocysts appeared degenerate. These results demonstrated that 'active blastocysts' can be rendered 'dormant' when placed into 'dormant uteri'. However, it remained to be proved conclusively that these normal-looking 'dormant blastocysts' were indeed viable. The proof was provided in Experiment 2.

Experiment 2: Induction of implantation and survival of the foetus

Fifty-six 'active blastocysts' were made 'dormant' by transferring them into the 'dormant uteri' of six recipients (see Experiment 1). Three days after the blastocysts had been transferred, oestrogen, in addition to progesterone, was injected into the recipients to induce implantation. Daily injections of the two hormones were continued to maintain pregnancy. Of the fifty-six blastocysts that had been transferred, twenty-seven (48%) developed into live foetuses of normal appearance and weight by Day 24. The hormonal treatment which was started on Day 10 initiated implantation on Day 11, and therefore made Day 11 the equivalent of Day 5 of regular pregnancy. Day 24 thus became the equivalent of Day 18 of regular pregnancy. There was no significant difference between foetal weights on Day 24 of the present experiment and foetal weights on Day 18 of regular pregnancy.* These results clearly demonstrate that 'active blastocysts' which are rendered 'dormant' remain viable. Our results confirm and extend the observation of Psychoyos (1961) who, for the purpose of studying the mechanism of implantation, transferred 'active blastocysts' into 'dormant uteri' and found that blastocysts were able to implant only after oestrogen had been administered.

Experiment 3: Transfer of 'dormant blastocysts' into 'sensitive uteri'

In order to render them 'dormant', 144 'active blastocysts' were transferred into the 'dormant uteri' of seventeen temporary recipients. Of the 144 blasto-

* When the hormonal combination of 2 mg progesterone + 1 μ g oestrone was used to maintain pregnancy beyond Day 18, both foetuses and placentae were heavier than normal (De Feo, unpublished).

PLATE 1

FIG. 1. A blastocyst after 3 days residence in a 'dormant uterus'. The zona pellucida is intact. $\times 520$.

FIG. 2. A blastocyst after 3 days residence in a 'dormant uterus'. The zona pellucida had been shed. $\times 520$.

(Facing p. 6)

cysts transferred, seventy-seven were recovered 3 days later, seventy-three of which were retransferred into the 'sensitive uteri' of seventeen definitive recipients. Nine of these recipients did not show evidence of pregnancy, i.e. neither foetuses nor resorption sites were observed at autopsy. The eight recipients that became pregnant, received thirty-three blastocysts, fifteen of which developed into normal, live foetuses.

Fifteen foetuses resulting from the transfer of seventy-three blastocysts represent a 20.6% 'take', which is a rather poor result when compared with over 70% 'takes' that we obtain routinely when we transfer 'active blastocysts' into the uteri of Day 4 or Day 5 pseudopregnant recipients. Why is it that the 'takes' were relatively low in the present experiment? The following explanations may be offered: (1) the double transfer, and thus the two-time exposure to the in-vitro conditions, may have deleteriously affected an appreciable percentage of the blastocysts; and (2) the abrupt change from a 'dormant uterus' to a 'sensitive uterus' may have been harmful to some of the blastocysts.

In the recipients that had foetuses, implantation must have occurred during the afternoon of Day 5 of pseudopregnancy, since this is the only time that a pseudopregnant (or pregnant) uterus will permit the initiation of implantation (Dickmann & Noyes, 1960). Therefore, it can be stated categorically that 'dormant blastocysts' can initiate implantation within a few hours after being placed in a 'sensitive uterus'.

OBSERVATIONS ON THE FAILURE OF BLASTOCYSTS TO SHED THEIR ZONAE PELLUCIDAE

Of the sixty-nine normal-looking blastocysts recovered in Experiment 1, fifty-three (77%) were still zona-encased (Pl. 1, Fig. 1), whereas only sixteen (23%) had shed their zonae (Pl. 1, Fig. 2). The retention of the zona in so large a proportion of the blastocysts was an unexpected result in view of reports by others that shedding of the zona is not postponed during delayed implantation (Psychoyos, 1961; Mayer, 1963b; Schlafke & Enders, 1963).

In order to explain the present results, it will be necessary to consider the concept proposed by Dickmann & Noyes (1961): in order for the blastocyst to shed its zona, the trophoblast cells have to reach a certain stage of maturation. Once maturation has been attained, trophoblast cells can then penetrate through the zona. This results in the eventual escape of the entire blastocyst from the zona. Normally, trophoblast maturation may take place any time from the morning to the afternoon of Day 5 of pregnancy. Using the egg transfer technique, it was shown that trophoblast maturation followed by shedding of the zona can also take place in a Day 4 pseudopregnant uterus and in a Day 1 pseudopregnant oviduct.

In the present experiments, the blastocysts, all of which were zonae-encased, were transferred between 09.00 hours and 11.00 hours. It is postulated that the majority of these blastocysts did not shed their zonae, because they had not attained trophoblast maturation at the time they were transferred, and could not achieve this maturation in the milieu of the 'dormant uterus'. The sixteen blastocysts that did shed their zonae in the 'dormant uteri' had already attained

trophoblast maturation in the uteri of the donors. In other words, the 'dormant uterus' inhibits trophoblast maturation, but it does not interfere with the actual act of shedding of the zona by the blastocyst.

In order to test the above hypothesis, Experiment 1 was repeated, though the blastocysts used were 4 to 7 hr older than those used in Experiment 1. Thus blastocysts were recovered and then transferred between 15.00 hours and 16.00 hours instead of 09.00 hours to 11.00 hours. The rationale for doing this experiment was based on the supposition that the older blastocysts might already possess mature trophoblast at the time of transfer, and therefore would shed their zonae in the 'dormant uteri'.

One hundred and eighteen zona-encased blastocysts were transferred into the 'dormant uteri' of thirteen recipients. Three days after the transfer, the recipients were killed and their uteri were flushed to recover the blastocysts. Sixty blastocysts were recovered: eight (13%) were zona-encased, whereas fifty-two (87%) were zona-free.

The fact that 87% of the older blastocysts as against only 23% of the younger blastocysts shed their zonae in the 'dormant uteri' clearly shows that, the blastocyst's ability to shed its zona is age dependent, which, in turn, may be interpreted as being dependent on trophoblast maturation.

CONCLUSION

In our working hypothesis, we assumed that the transfer of either 'active blastocysts' into 'dormant uteri' or the transfer of 'dormant blastocysts' into 'sensitive uteri' or both would result in the death of all the blastocysts due to blastocyst-uterine incompatibility. We postulated that such incompatibility does not exist during natural or experimentally-induced delayed implantation (which do not involve transfers), because in these situations the transition from 'active' to 'dormant' and vice versa, is gradual, and it occurs simultaneously in both the uterus and the blastocyst. In view of the present results, namely that both types of transfers did yield live fetuses, our original hypothesis has to be modified. Thus, the results may be interpreted in one of the following ways.

(1) There is an incompatibility between the uterus and the blastocyst if one is 'active' and the other is 'dormant'. However, upon transfer, the blastocyst can survive in the new (hostile?) milieu for a short period (e.g. a few hours) during which time it changes to become compatible with its new environment.

(2) Although the 'dormant uterus' and the 'sensitive uterus' have a different endocrinological basis, they are sufficiently similar, in permitting survival of blastocysts, so that blastocysts are not exposed to an antagonistic milieu when they are transferred from one type of uterus to another.

(3) In the event of lactation delayed implantation or experimentally-induced delayed implantation, or when 'active blastocysts' are transferred into 'dormant uteri', the blastocyst does not undergo any transformation to make it compatible with the 'dormant uterus'. Instead, the blastocyst of regular pregnancy is normally compatible with the 'dormant uterus'. Thus, it may be said that during regular pregnancy, the blastocyst stage constitutes a 'dormant' phase in embryo-

genesis. At present we do not have any indication as to which of the three interpretations is more likely to be correct.

The results of the morning compared with the afternoon transfers of blastocysts into 'dormant uteri' support our hypothesis that trophoblast maturation is a precursor for the shedding of the zona pellucida by the blastocyst, and that the 'dormant uterus' is inhibitory to such trophoblast maturation.

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