DNA synthesis and cell proliferation during the formation of deciduomata in mice

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After ovulation and fertilization of the ovum, several typical changes occur in the uterus of pregnant mammals that are related to the implantation of the blastocyst. The formation of a deciduoma, in which endometrial cells are transformed to decidual ones, is one of these changes (Shelesnyak, 1957, 1960). Apart from an interplay of hormones the formation of a deciduoma is determined by local effects. Loeb (1907) was the first to show this by traumatizing the uterine wall. Since then the role played by hormones, histamine and other agents in the production of deciduomata has been intensively studied (Rigler & Rosenkranz, 1955; Rosenkranz & Rigler, 1958; Shelesnyak, 1959\(a, b, c\); and many others.)

Using \(^3\)H-thymidine from the 7th day of pregnancy, Atlas, Bond & Cronkite (1960) revealed by means of autoradiography an intense proliferation of decidual cells followed by proliferation of the cells of the uterine epithelium. Shelesnyak & Tic (1963) also found an increase in the DNA synthesis in the uterus during pseudopregnancy. As a co-ordinated reaction of all the uterine tissues takes place before implantation, while the production of deciduomata is related not only to proliferation but to polyploidization and migration of the cells as well, an autoradiographic study of DNA synthesis using tritiated thymidine was undertaken to investigate these processes.

MATERIAL AND METHODS

The uptake of \(^3\)H-thymidine into the cells of the uterine endometrium of C57B1 mice was studied from the 7th day of pregnancy; the beginning of pregnancy was determined by the presence of a vaginal plug in females after

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mating. The day on which the plug was found was regarded as the first day of pregnancy. Two non-pregnant females in metoestrus whose hormonal background was close to that of pregnant animals during the first developmental stages were taken as the control. Tritiated thymidine was obtained from the Radiochemical Centre, Amersham, England. Two experimental series were carried out.

In the first series, $^3$H-thymidine (specific activity 2.5 c/mM) was dissolved in sterile distilled water and injected into the females at a concentration of 2 $\mu$g in 0.3 ml. Females from the 2nd to the 7th day of pregnancy were injected and sacrificed 2 h later. The numbers of labelled nuclei in the epithelium and connective tissue of the endometrium were counted on autoradiographs. In two additional instances, in order to study the process of cell migration, animals were killed on the 4th and 5th day of pregnancy 2 and 24 h after the injection of thymidine.

In the second series $^3$H-thymidine (specific activity 8.5 c/mM) was dissolved in the same manner and 0.5 ml of the final solution were injected (0.5 $\mu$g). On the 5th day of pregnancy the mitotic cycle was determined graphically by the percentage of labelled mitoses (Quastler & Sherman, 1959). One female on the 5th day of pregnancy was injected with $^3$H-thymidine three times at 6 h intervals, material being fixed 1 h after the last injection of the isotope.

In all the experimental series material was fixed in Carnoy fluid, dehydrated in an alcohol series and embedded in paraffin through chloroform. Sections 5 $\mu$m thick were covered with liquid emulsion of 'R' type (N.I.K.F.L., Moscow) by a method previously described (Zhinkin, Zavarzin, Lebedeva & Adreeva, 1961) and exposed for 10–20 days at +4 °C. After development the preparations were stained with hemalaun eosin or methyl green–pyronin.

RESULTS

Series 1. The uteri of mice in metoestrus served as controls. Two hours after the injection of $^3$H-thymidine about 60 % of nuclei of the epithelium in the uterine lumen and 40 % of epithelial gland cell nuclei were labelled, which corresponds to a high proliferative activity; however, only 0.62 % of connective tissue cells of the endometrium had labelled nuclei (Plate 1, fig. A). Labelled nuclei occurred but rarely in the smooth muscle cells, which is in agreement with the data of Messier & Leblond (1960) obtained with intestinal muscle.

Starting from the second day of pregnancy no morphological changes could be found in the epithelium but the proportion of labelled nuclei dropped sharply. No labelled nuclei were found in the epithelium of the lumen after 3 days. After 5 days labelled nuclei appeared; their number at the level of each deciduoma was small, being considerably greater in interdecidual areas. Changes in the percentage of labelled nuclei in the epithelium are shown in Text-fig. 1.

From the 2nd day onwards, connective tissue cells of the endometrium ad-
Fig. A. Autoradiograph of the uterine wall of a metoestral mouse 2 h after injection of $^3$H-thymidine. $\times 900$. a, Labelled epithelial cells; b, labelled connective tissue cell.

Fig. B. Autoradiograph of a cross-section through the centre of deciduoma on the 5th day of pregnancy. $\times 1500$. 1, Uterine lumen; 2, zone of decidual cells that have completed the cycle (corresponds to the zone 1 on Text-fig. 4); 3, zone of labelled decidual cells (corresponds to zone 2).

Fig. C. General view of the section through the centre of a deciduoma on the 5th–6th day of pregnancy after three injections of $^3$H-thymidine. $\times 140$. 1, Uterine lumen; 2, implanting embryo.

Fig. D. Autoradiograph of a deciduoma after three injections of $^3$H-thymidine on the 5th day of pregnancy. Zone 3 of decidual cells. $\times 900$. 

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joining the epithelium became larger, and their cytoplasm became more basophilic. Cells immediately next to uterine epithelium that were arranged more densely than the main mass could be distinguished. Labelled nuclei were 2% of the total. More pronounced changes occurred by the 3rd day, when the cells adjoining the uterine lumen increased greatly in number, and a zone consisting of 4–5 rows of cells could be distinguished. About 30% of these contained labelled nuclei (Text-fig. 2b). This zone was surrounded by a second, more distant one, of whose cells about 15% had labelled nuclei (Text-fig. 2c). The remaining mass of connective tissue cells contained the same proportion of labelled nuclei as on the 2nd day of pregnancy.

On the 4th day the endometrium at the level of a deciduoma could be divided into several areas: zone 1 having 5–6 rows of cells adjoining the developing crypt, in which about 30% of the nuclei were labelled; zone 2, whose cells contained 70% labelled nuclei; zone 3 with over 40% of labelled nuclei, and zone 4 of cells adjoining the smooth muscle, containing very few labelled nuclei.

On the 5th day especially marked changes occurred. The cells of zone 1 were already decidual, and seemed to correspond to the primary decidual cells of Krehbiel (1935, 1937). They were large, with basophilic cytoplasm and large
light nuclei. No labelled nuclei were found among them (Plate 1, fig. B). In zone 2 the cells were also rather large but somewhat smaller than those in the first zone. When compared with the 4th day, the number of labelled nuclei decreased. Due to the high proportion of labelled nuclei zone 3 was quite distinct. Its cells were smaller and contained 65–70% labelled nuclei. In zone 4 the percentage of labelled nuclei also increased, although their number was much less than in

Text-fig. 3. Schematic representation of the distribution and frequency of primarily labelled connective tissue cells in the uterine endometrium on the 5th day of pregnancy. a, Section that passed through the centre of a deciduoma with the designation of zones (1–4); b, inter-implantation area.

Text-fig. 4. Change in the percentage of primarily labelled nuclei of decidual cells in different zones of deciduomata at different stages of pregnancy. 1–4, Designations of zones of deciduoma The s.e. for each column is indicated by a vertical line.
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zone 3. The arrangement of these zones in a transverse section passing through the embryo is schematically presented in Text-fig. 3a, where the density of the points shows the distribution of primarily labelled nuclei. For the sake of comparison Text-fig. 3b presents a section that passed between deciduomata.

At later times the proportion of labelled nuclei in zone 2 and then in zone 3 decreased. The change in the percentage of primarily labelled nuclei at different stages of the formation of decidual cells can be seen in Text-fig. 4.

Starting from the 4th day marked differences occurred in the number of labelled nuclei in the cells of the stroma located along the uterus. In the areas of implantation the number of labelled nuclei, and therefore the proliferative activity as well, was higher than in inter-implantational areas. The total count (without subdivision into zones) of the proportion of labelled nuclei in both areas on the 5th day was $55.4 \pm 1.4\%$ at the level of deciduomata, but only $7.5 \pm 1.22\%$ in interdecidual regions. These differences in the frequency of the occurrence of labelled nuclei can be seen in the Text-fig. 3. The numbers presented for the first and second areas were obtained on longitudinal sections of the same preparation and on cross-sections through the uterine wall on the same animal.

Table 1. Comparison of the percentage of labelled nuclei on the mesometrial and antimesometrial sides of the uterus

<table>
<thead>
<tr>
<th>Deciduoma</th>
<th>Days of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesometrial part</td>
<td>4</td>
</tr>
<tr>
<td>Antimesometrial part</td>
<td>49.2</td>
</tr>
<tr>
<td>Difference and error</td>
<td>6.0 ± 2.35</td>
</tr>
</tbody>
</table>

It should be noted that some difference in the percentage of labelled nuclei in different areas located along the uterus could be observed on the 3rd day, but this difference was not very striking. The problem of the time and site of the first appearance of intensely proliferating connective tissue cells requires further investigation, yet their presence on the 4th and especially on the 5th day is beyond doubt. The percentage of labelled nuclei in decidual cells following a single injection of $^{3}$H-thymidine showed a difference between the mesometrial and antimesometrial parts of deciduomata, as was shown earlier by Atlas et al. (1960).

The proportion of labelled nuclei found at different stages of pregnancy are presented in Table 1.

On the 4th day of development the difference is small and of little significance, yet on the 5th and subsequent days it is clear and statistically significant.

The presence of regional differences in the proportion of labelled nuclei points to an intense growth of the deciduoma, that may be provided not only by cell
reproduction in situ but by cell migration as well. To reveal possible migration of cells from one zone to the other, animals on the 4th and 5th day of pregnancy were killed 2 and 24 h after the injection of \(^{3}\text{H}\)-thymidine. (The isotope was always injected at the same time of day, between noon and 13.00 h).

The counts of labelled nuclei in the zones in question showed that on the 4th day there were about 30 % in zone 1, over 60 % in zone 2, and about 50 % in zone 3. Twenty-four hours after injection of \(^{3}\text{H}\)-thymidine their number in zone 1 was about 70 %, in zone 2 their number somewhat decreased to about 50 %, while zone 3 almost totally lacked labelled nuclei. Therefore the cells shifted from zone 3 to the lumen. An increase in the number of cells with labelled nuclei in zone 1 could have occurred by their division on the site and by migration from zone 2, where the percentage of labelled nuclei somewhat decreased (Text-fig. 5).

On the 5th day zone 1 lacked labelled nuclei, as mentioned above; after 24 h, however, their number increased to over 30 %, which suggests migration of nuclei from the periphery to the centre of deciduomata.

**Series 2.** Since the primary decidual cells ceased to synthesize DNA on the 5th day, while in the adjoining zones the proportion of labelled nuclei was very high, a detailed study of the kinetics of cell proliferation was carried out, and the mitotic cycle determined. To this end mice received a single injection of \(^{3}\text{H}\)-thymidine on the 5th day of pregnancy and were killed after 1, 2, 4, 6, 8, 10, 12, 15, 18 or 24 h. On autoradiographs obtained simultaneously not less than 100 mitoses were counted for each of these times and the percentage of labelled...
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The count was carried out on sections that had passed through the centre of a deciduoma or close to it. Mitoses were most common in zone 3. The count was carried out in all zones. Generation time and individual stages of the cycle were determined graphically by the method of Quastler & Sherman (1959).

The results of the counts at different times after injection are presented in Text-fig. 6. The first labelled mitoses (prophases only) were observed after an hour. After 2 h the number of labelled mitoses rose to over 30%, all stages of mitoses being found labelled. After 3–6 h the proportion of mitoses decreased.

After 6 and 10 h the number of labelled mitoses was low, then the second rise of the curve (the second division) occurred so that after 15 h they were about 60% of the total. After 18 and 21 h the percentage of labelled mitoses dropped again. We succeeded thus in obtaining a clear-cut two-peak curve, the second peak of which was lower and flatter than the first. Using the 50% level of the curve to calculate the parameters of the mitotic cycle the generation time $T = 11$ hr. If one measures from the middle of the first peak to the middle of the second one, $T'$ becomes about 10 h. The $S$ phase, when based upon the 50% level of both the ascending and descending parts of the curve, lasts about 5 h. Subtracting 1 h for the circulation of $^3$H-thymidine, the $S$ phase can be assumed to last 4 h. The $G_2$ phase varies from 1 h, which can be taken as the minimum time, up to 2.5 h (maximum time), which includes the duration of half of the mitosis. From this, $G_1 + m$ lasts 3.5–4 h. Attention is drawn to the fact that the second peak of the curve is much lower than the first one. This seems to be related to withdrawal of some of the cells from the mitotic cycle. It is likely that the generation time in interdecidual areas is somewhat greater than that determined for the

Text-fig. 6. Change of the percentage of labelled mitoses after a single injection of $^3$H-thymidine on the 5th–6th day of embryonic development.
deciduomata. However, the percentage of labelled mitoses turned out to be the same at all the points of the curve for both zones 3 and 4; the latter zone is similar to interdecidual areas, which suggests that even if there is a difference in the generation time it is not very large.

In order to reveal the proliferative pool, simultaneously with the experiments on the determination of the generation time, $^3$H-thymidine was injected three times at intervals of 6 h to animals from the same group. The intervals between the injections were longer than the $S$ phase; therefore the data presented give only an approximate measure of the proliferation pool. However, as in the case of a single injection of $^3$H-thymidine the whole deciduoma could be subdivided in terms of the proportion of labelled nuclei into the four zones shown in Text-fig. 3a. A section that passed through the embryo (Plate 1, fig. C) shows it at small magnification. Zone 3 is especially clear-cut since the majority of the nuclei are labelled (Plate 1, fig. D). The proportion of labelled nuclei in different zones at the level of a deciduoma is presented in Table 2.

Table 2. Percentage of labelled nuclei on the 5th–6th day of development in different zones of deciduomata after a single or triple injection of $^3$H-thymidine

<table>
<thead>
<tr>
<th>No of injections of $^3$H-thymidine</th>
<th>Zones of deciduoma</th>
<th>Space between deciduomata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>29.0 ± 1.5</td>
<td>74.1 ± 1.3</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>43.5 ± 1.8</td>
</tr>
</tbody>
</table>

Table 3. Percentage of labelled nuclei in the epithelium of the uterine lumen on the 5th day of pregnancy after one or three injections of $^3$H-thymidine

<table>
<thead>
<tr>
<th>Centre of deciduoma</th>
<th>Space between deciduomata</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 injection</td>
<td>Space between deciduomata</td>
</tr>
<tr>
<td>3 injections</td>
<td></td>
</tr>
<tr>
<td>3.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>7.9 ± 1.62</td>
<td></td>
</tr>
<tr>
<td>7.3 ± 0.2</td>
<td>29.5 ± 1.2</td>
</tr>
</tbody>
</table>

No difference was found in the proportion of labelled nuclei between the mesometrial and antimesometrial regions. No significant differences in the proportion of labelled nuclei could be found between the epithelium and the mesometrium.

Labelled nuclei were also found in this experiment in the uterine epithelium, their number being different at the level of deciduomata and in the space between deciduomata (Table 3).

It should be noticed that in the region of implantation epithelial cells with labelled nuclei are lacking as a rule; at the level of deciduomata they are found at some distance from the embryo.

To get a more precise idea of the proliferative activity of endometrial cells,
mitoses were counted in 1500–2000 cells on preparations stained with haematoxylin. At the level of deciduomata the mitotic index found on the 5th day was 6% (from 5 to 7% in individual areas). The mitotic index between deciduomata was 1.8%. Correspondingly, the sectional area in the centre of the deciduomata, determined planimetrically, was three times that between deciduomata, which was usually seen as a thickness of the uterine wall. However, this difference was due both to a difference in the number of cells and to a difference in cell size. On the 5th day the proliferation of smooth muscle cells markedly increased, while on the 7th day the proportion of labelled nuclei reached 5–6%.

DISCUSSION

Starting with the first days of pregnancy several regular successive changes occur in the uterine endometrium, preceding the production of a deciduoma. These changes are both of a general and of a local character. From the 2nd day of pregnancy the proliferative activity of the epithelium decreases sharply, reaching zero, but recovers after the implantation of the embryo, first of all in the interdecidual areas. As development proceeds the proliferation of the epithelium becomes more active. Atlas et al. (1960) demonstrated that from the 7th day of development the proportion of labelled nuclei in the epithelium increased, while that in the connective tissue decreased. On the 8th day these authors found 14% labelled nuclei in the epithelium and 40% in decidual cells. On the 12th day no decidual cells with labelled nuclei could be found, their proportion in the epithelium being 50%.

The data obtained in this investigation show that proliferation of the epithelium and of the decidual cells in development are to some extent antagonistic processes. It seems that during the period of increased proliferation and production of the decidual cells, epithelial cells pass to the Go phase (Quastler, 1963) in the same manner as connective tissue cells do at the end of pregnancy.

In the endometrium an increased proliferation begins around the epithelium and gradually shifts towards the periphery. On the 4th day the highest proportion of the labelled nuclei was found in zone 2; on the 5th day in zone 3. The displacement of the zone of intensely proliferating cells correlates with the formation and cessation of DNA synthesis in already differentiated decidual cells.

However, especially marked regional differences in the proportion of labelled nuclei were found at the level of deciduomata and intermediate areas. Regional peculiarities, once established, comprised not only the formation of decidual cells but also different proportions of labelled nuclei. Interdecidual areas remained at the proliferation level of the 3rd day, while proliferation around the embryo increased sharply. Unfortunately, the experiment with the triple injection of 3H-thymidine ('saturation') was not very accurate so that the numerical data obtained can only be regarded as demonstrating the proliferative
pool approximately. The proportion of labelled nuclei increased about threefold in interdecidual areas of 'saturation' in both epithelial and connective tissue. The mitotic index at the level of a deciduoma was also three times that in intermediate areas. This suggests that regional differences in proliferative activity depend mainly on differences in the proliferation pool.

If we assume that the major role is played by the change in the proliferation pool, the high proportion labelled nuclei on the 4th and 5th day of development needs explanation. Thus, on the 5th day about 70% of primarily labelled nuclei were found in zone 3, and 63.7% in zone 2.

If the pool reached 100%, then at $T = 10$ h and $S = 4$ h the labelled nuclei should have comprised only 40% of the total. However, if we assume that $Pc/Js = T/ts$ (where $Js$ = percentage of labelled nuclei; $Pc$ = proliferative pool; $T$ = duration of mitotic cycle; $ts$ = duration of the S phase) and that on the 5th day in zone 3 $Pc = 88.6$, $T = 10$ and $ts = 4$, then $Js = 63.7%$. Therefore $28.3\%$ of cells with labelled nuclei turn out to be 'excessive'. If we assume the ideal case when $Pc = 100$, 'excessive' cells with labelled nuclei are 23.7% of the total.

In zone 2 $Js = 43.5$, but since after triple injection of $^{3}H$-thymidine $Pc = 74$, then, on the basis of the same calculation $Js$ should have equalled 29.6 ($Js = 74.4/10$), i.e. $13.5\%$ of cells were 'excessive'. This discrepancy between the data obtained and these calculations can be explained by a migration of cells that have already synthesized DNA from the region of high proliferation to zone 1 of differentiated decidual cells, which was shown in experimental series 1. If our speculations are correct, this suggests that polyploid cells (Sachs & Shelesnyak, 1955), have undergone a final replication, migrate to zone 1. Therefore, on the 4th and 5th days when migration of cells was found, zones 2 and 3 contain a heterogeneous population of cells including both mitotically dividing cells and also polyploid cells that have undergone endo-reduplication and subsequent migration.

The thesis that the mean generation time does not significantly alter at the stages investigated is supported by the data. If one uses the method of calculation applied above, the proportion of primarily labelled nuclei between deciduomata $Js = 7.5$, the value taken approximately for the pool (on the basis of the triple injections), $Pc = 22.2$, from which $T = (22.2 \times 4)/7.5 = 11.8$ h. The generation time calculated was close to that found at the level of deciduomata.

It should be noticed that in zone 1, formed in the absence of migration and from cells reproducing $in situ$, the proportion of labelled nuclei did not exceed 40%; therefore the generation time should also have been close to that obtained for the 5th day of development. Such a high proportion of primarily labelled nuclei could depend upon some synchronization related to hormonal effect, but in this case a change in the proliferative pool seems to have a decisive role as well.

The mitotic cycle of the vaginal epithelium was determined and its course and
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Changes under the action of diethylstilboestrol were studied in detail by Peckham & Kiekofer (1962), Peckham et al. (1963) and Ladinsky & Peckham (1965). They demonstrated that the population of basal cells was homogeneous and that the mitotic cycle changed proportionally to the dose of the hormone, shortening from 10 to 4 h after hormone treatment. Bertalanffy & Lau (1963) demonstrated intense mitotic activity in the uterine epithelium. Bresciani (1965) observed a sharp reduction of the S phase in cells of the mammary gland after administration of β-oestradiol. Epifanova (1964, 1965) showed that injection of oestradiol to castrated mice reduced generation time in the uterine epithelium and caused the proliferation pool to increase threefold.

It is likely that individual hormone-dependent tissues respond to the action of hormones in a different manner according to their structure and function. The changes include the reduction of generation time, a sharp increase in the proliferation pool and, finally, differentiation. At pregnancy these changes develop in a certain sequence which seems to be determined by the effect of the embryo and the interaction of cell elements of different uterine tissues.

The formation of deciduomata is thus an integral response of the mass of cell located round the developing embryo, involving the stimulation of DN synthesis, proliferation, polyploidization and differentiation of decidual cells.

SUMMARY

1. On the 2nd day after fertilization DNA synthesis and proliferation of the uterine epithelium decrease sharply. Proliferation is resumed on the 5th day and increases with the fall of proliferative activity of the developing decidual cells.

2. The production of decidual cells is preceded by increased proliferation of connective tissue cells. The formed decidual cells complete DNA synthesis on the 5th day, i.e. by the time of implantation of the embryo.

3. The population of decidual cells increases by means of cell migration from adjacent areas which maintain high proliferative activity.

4. On the 4th–5th day of development differences appear in the number of cells synthesizing DNA at the anatomical level of the deciduomata and in interdecidual areas. These differences seem to be related to differences in the proliferation pool rather than to differences in generation time.

Выводы

1. Уже на 2-е сутки после оплодотворения резко падает синтез ДНК и пролиферация эпителия матки. Пролиферация возобновляется на 5-й день и возрастает далее по мере падения пролиферативной активности образующихся десидуальных клеток.

2. Образованию десидуальных клеток предшествует усиленная пролиферация соединительотканых клеток. Образовавшиеся десидуальные клетки на 5-й день, т.е. к моменту имплантации зародыша, прекращают синтез ДНК.
3. Увеличение массы децидуальных клеток происходит путем миграции клеток из прилежащих участков, сохраняющих высокую пролиферативную активность.

4. На 4-5-й день развития возникают различия в количестве клеток, синтезирующих ДНК на уровне децидуомы и в междецидуальных участках. Вероятно, эти особенности связаны, главным образом, с различием пролиферационного пульса, а не с разницей генерационного времени.

REFERENCES


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