dies will answer this question.

It is of great interest to note that Sertoli cells did not respond to the antibody. Several investigators have localized albumin in Sertoli cells of mammalian testes such as human [6, 15], hamster [9] and rat [3]. It is not clear presently why the immunoreactivity of albumin was not observed in Sertoli cells of *X. laevis* testis. In the seminiferous tubules of mammalian testis, Sertoli cells form a barrier, so-called the blood testis-barrier, to retard or exclude many substances in the blood plasma from entrance into the lumen [19, 25]. Most germ cells, except for spermatogonia, reside within the barrier or the adluminal compartment. In anurans, on the other hand, spermatogenesis takes its course in the cysts of the testes. Germ cells develop within groups of "follicle" cells which are thought to be comparable to the Sertoli cells in the mammalian testis. According to Bergmann *et al.* [1], substances like nutrients and hormones in the blood in this species is probably accessible to most developing germ cells. Taking all these findings into consideration, it is not surprising that Sertoli cells had no response to the antibody of albumin. Perhaps, albumin is not associated with Sertoli cells. T may be transported to germ cells from the interstitial space without going via Sertoli cells.

Finally, the HPLC sample consisted of two proteins, as judged from the result of SDS-PAGE analysis. One with Mr=74 kD was a very strong band and the other with Mr=68 kD was a very faint band (see Fig. 4A; lane g). Both bands immunoreacted with the antibody of albumin (data not shown). This is not unusual. The frog, *X. laevis*, has two albumin genes that code for a 74 kD and a 68 kD serum albumin [11, 18]. In addition, two molecular forms of proteasome [7], calreticulin (a Ca^{2+}-binding protein) [24] and prolactin [26] have also been reported in this animal. Two forms of these proteins may have occurred from a duplication of the entire genome in the genus *Xenopus* [2]. In view of these findings, we must have purified two albumins together, but could not separate one from another by the methods used in this study. One explanation for this may be as follows; albumin exists in two forms that migrate on SDS-PAGE with relative molecular weights of 74 kD and 68 kD, respectively. As the number of amino acids of the two albumins is equivalent (608 residues), the anomalous behaviour on SDS-PAGE may be due to the glycosylation, which is specific for the 74 kD albumin [18]. It might be possible to separate one from another by changing the range of NaCl concentrations on HPLC.

As to which albumins are more closely associated with immunoreacted cells remains unclear at the present time. In the serum of *X. laevis*, the 74 kD albumin exists to a much greater extent than the 68 kD albumin (data not shown). It seems, therefore, very likely that the former is more closely associated with the surface of testicular cells. We do not know yet how spermatogenesis in *X. laevis* is controlled by T. Nevertheless, it is extremely interesting to note that the developing germ cells and the interstitial tissue (probably SH-secreting cells) are associated with albumin. Considering that serum albumin can bind T, spermatogenesis may be influenced under T with the aid of serum albumin in this species as well as in others.

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Spatio-Temporal Pattern of DNA Synthesis Detected by Bromodeoxyuridine Labeling in the Mouse Endometrial Stroma during Decidualization

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ABSTRACT—In order to examine the patterns of proliferation and differentiation of endometrial stromal cells before and during decidualization in pseudopregnant mice, the rate of DNA synthesis was immunocytochemically determined by means of bromodeoxyuridine (BrdU) labeling. On day 4 of pseudopregnancy induced by mating with vasectomized male, both uterine horns were traumatized to induce deciduoma. On day 5 of pseudopregnancy (one day after traumatization), BrdU-labeling index was markedly increased, and the labeled cells were found in almost all parts of endometrial stroma. From day 6 to day 8 of pseudopregnancy (2–4 days after traumatization), the labeling index remained high in the stromal cells of all parts except for the periluminal region. In the endometrial stromal cells in the peripheral region of myometrium, however, the labeling index was maximum on day 8 and decreased remarkably on day 9. In the stromal cells in the periluminal region where deciduomal cells developed, the labeling index was high on day 5 and low on day 6, no labeled cells being found on day 8. There results clearly show that each region of uterine endometrial stroma has a different responsiveness to traumatization, and each region plays a different role in the formation of deciduoma.

INTRODUCTION

Immunohistochemical detection of bromodeoxyuridine (BrdU), which is a uridine analogue and incorporated selectively into the cellular DNA at S-phase of the cell cycle, has been proven useful for the analysis of cell proliferation in place of ^{3}H-thymidine incorporation into replicating cells [5].

Differentiation of the endometrial stromal cells into decidual cells occurs soon after the implantation of blastocysts. In mice and rats, however, decidual reaction can be induced artificially in the uteri without blastocysts by mechanically scratching the luminal surface [17]. Changes in the structure and function of uterine tissue during decidualization are mainly controlled by the ovarian estrogen and progesterone [2, 11, 13, 14]. Decidualization is a highly regulated process characterized by a variety of events including increase in DNA synthesis [1, 8, 15], changes in vascular permeability [7], and polyploidization and hypertrophy of stromal cells [12, 16]. Therefore, formation of deciduoma has been widely applied as a useful experimental model for the study not only of implantation but also of the mechanisms of cell proliferation and differentiation. BrdU labeling patterns of the cells in uterine tissue were reported in normal cycling and prepubertal mice [6].

Because the changes of cell proliferation during decidualization as a function of time have not been reported, the present study was designed to examine the spatial and temporal patterns of DNA synthesis in the mouse uterine stromal cells during decidualization after traumatization.

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MATERIALS AND METHODS

Animals

Female mice of the ICR strain purchased from Japan CLEA Inc. (Tokyo, Japan) were used in the present study. They were housed in plastic cages (3–7 mice per cage) under controlled lighting (12–hr light and 12–hr darkness; lights on at 06:00) and temperature (25 ± 0.5°C), and were provided with a commercial diet (CE-7: Japan CLEA) and tap water *ad libitum*.

Induction of deciduoma

Virgin female mice at 50–60 days of age were mated with vasectomized males to induce pseudopregnancy. The day when a vaginal plug was found was designated as day 1 of pseudopregnancy. On day 4 of pseudopregnancy, the anti-mesometrial luminal surface in both uterine horns was traumatized by a single scratch with a bent needle. The needle was inserted into the uterine lumen from a small incision made with a small scissors at the posterior end of uterine horn, adjacent to the uterine cervix, under light nembutal anesthesia [17]. The pseudopregnant mice were killed by cervical dislocation on various days after traumatization (Fig. 1). In order to check the effect of trauma, some pseudopregnant mice without traumatization were killed as controls between 2 and 10 days after mating with vasectomized males. Immediately after autopsy, the uterine horns were removed and weighed. The uterine weight was used as a parameter of decidual reaction.

In addition, virgin cycling mice at 50–60 days of age were also killed at varius phases of the estrous cycle and the uterine weights were recorded.

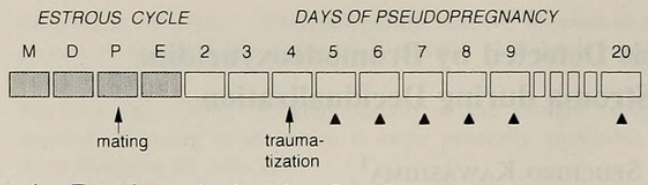

FIG. 1. Experimental schedule. Pseudopregnant mice received a deciduogenic stimulus (traumatization) on day 4 of pseudopregnancy and were given a single injection of BrdU on various days (▲) after traumatization. M: metestrus, D: diestrus, P: proestrus, E: estrus.

BrdU labeling and immunocytochemistry

The pseudopregnant mice received a single intravenous injection of bromodeoxyuridine (BrdU, 30 mg/kg body weight: Amersham, UK) at 24-hr intervals after traumatization. Four hr after BrdU injection, the uteri were fixed in ice cold 10% phosphate-buffered neutral formalin for 5 hr at room temperature. The uteri were dehydrated, embedded in paraffin, and the sections were cut at 4μm thickness.

After deparaffinization, the sections were washed in 0.01M phosphate-buffered saline (PBS, pH 7.4) three times and digested with 0.1% trypsin (Sigma) in 0.1% $CaCl_2$ (pH 7.8) for 20 min at 37°C. After washing in PBS (15 min, 3 times), endogenous peroxidase activity was blocked by immersing the sections in 0.3% H_2O_2 in methanol for 20 min, followed by washing in PBS (15 min, 3 times). Thereafter, the sections were incubated with monoclonal anti-BrdU antibody containing 10 units/ml nuclease (Amersham) for 1 hr at room temperature and then rinsed in PBS (15 min, 3 times). Finally, the sections were incubated with peroxidase-conjugated rabbit anti-mouse IgG for 30 min at room temperature. After washing in PBS (15 min, 3 times), the antibody binding sites were visualized by 0.05% 3, 3′-diaminobenzidine tetrahydrochloride solution. Each incubation was conducted in a moist chamber at room temperature. After immunostaining, the sections were counterstained with 0.1% Kernechtrot in 5% $Al_2(SO_4)_3$, dehydrated through an ethanol series, cleared in xylene, and mounted. The immunocytochemistry was controlled by sections overlaid with PBS instead of anti-BrdU antibody, which showed no immunoreactivity.

Measurement of labeling index

In order to examine the BrdU labeling index, two sections which were separated by at least 40μm apart were randomly chosen from the middle part of the uterine horn or the middle part of deciduoma in each mouse. Cell counting was carried out in the four regions; anti-mesometrial side of the endometrial stroma (AME), periluminal endometrial stroma (PLE), peripheral endometrium adjacent to the myometrium (PPE), and mesometrial side of the endometrial stroma (MME) (Fig. 2). Total number of BrdU labeled cells was counted out of 1,000 cells each in two sections from the four regions by using

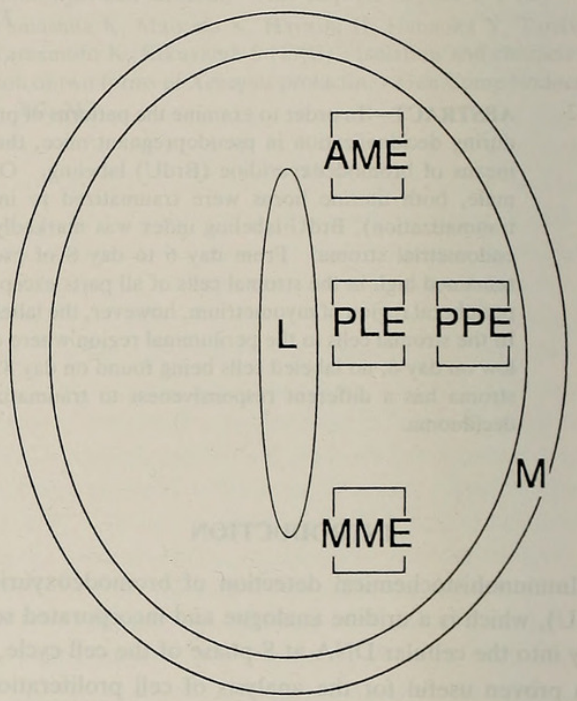

FIG. 2. Four regions of uterus for examining BrdU labeling index. L: lumen, M: myometrium, AME: antimesometrial side of endometrial stroma, PLE: periluminal endometrial stroma, PPE: peripheral endometrial stroma adjacent to myometrium, MME: mesometrial side of endometrial stroma.

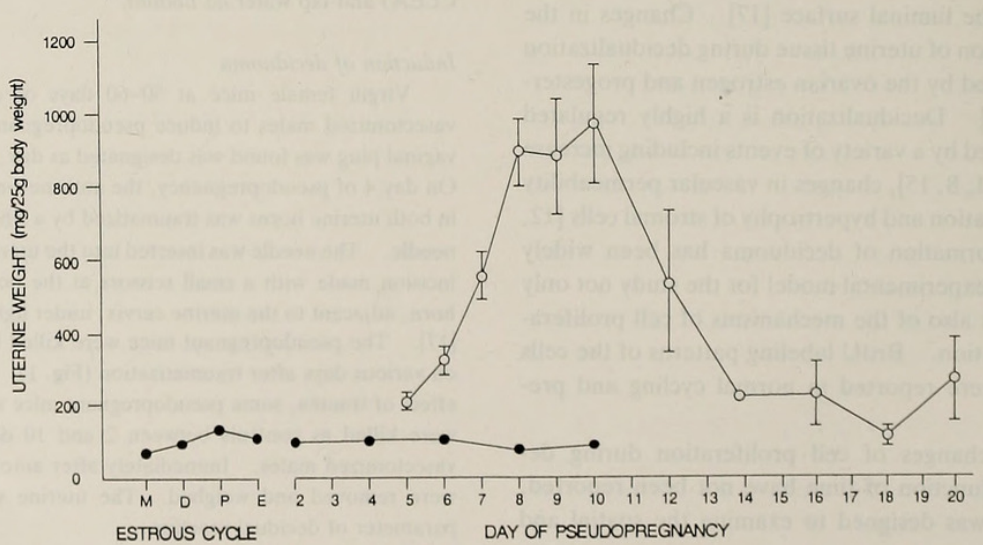

FIG. 3. Changes in uterine weights in mice during estrous cycle and pseudopregnancy with (-○-) or without (-●-) traumatization. Deciduoma were induced on the next day of traumatization given on day 4 of pseudopregnancy. Each point depicts the mean and SEM of 3–5 mice. M: metestrus, D: diestrus, P: proestrus, E: estrus.

an image processor-analyzer (LUZEX; NIRECO Co. Ltd, Tokyo). The labeled indices were expressed as percentages of labeled cells per 1,000 cells.

Statistical analysis

The statistical significance of the difference between groups were evaluated by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for the uterine weights and labeled indices.

RESULTS

Changes in uterine weight during decidualization

Changes in uterine weights during the estrous cycle and pseudopregnancy before and after traumatization are shown in Figure 3. In normal cycling mice, the uterine weight was lowest at metestrus, followed by an increase during diestrus ($P<0.01$). The weight reached maximum at proestrus, the value being significantly higher than those in the other phases of estrous cycle ($P<0.01$).

The uterine weight of pseudopregnant mice without traumatization between 2 and 10 days after the mating was almost the same as that of diestrous mice. Traumatization of the uteri on day 4 of pseudopregnancy resulted in the development of deciduoma in the next day. The weight increased rapidly after traumatization, reaching maximum on day 8 of pseudopregnancy, and decreased on day 12 and onward. The weights were significantly higher between days 6 and 12 than the other stages of pseudopregnancy with traumatization ($P<0.01$, in all comparisons).

BrdU labeling index

On day 2 of pseudopregnancy before traumatization, BrdU labeled cells were observed in the luminal and glandular epithelia but not in the endometrial stroma. On days 3 and 5 of pseudopregnancy before and one day after traumatization, a large number of labeled cells were observed in the endometiral stroma but very rarely in the luminal epithelium (Fig. 4a). On days 7 and 8 of pseudopregnancy given no traumatization, labeled cells were not found in the stroma and appeared again in the luminal epithelium.

The BrdU-positive cells on day 5 of pseudopregnancy in mice given traumatization (Fig. 4b) were more numerous compared to those on day 3 of pseudopregnancy (Fig. 4a) and on day 5 of pseudopregnancy without traumatization (data not shown, but regardless of the regions, the indices were less than 1% in mice given no traumatization).

Detailed spatio-temporal patterns of BrdU labeling index during decidualization are shown in Figure 5. On day 5 through 8 of pseudopregnancy with traumatization, the indices were significantly higher in the endometrial stroma than

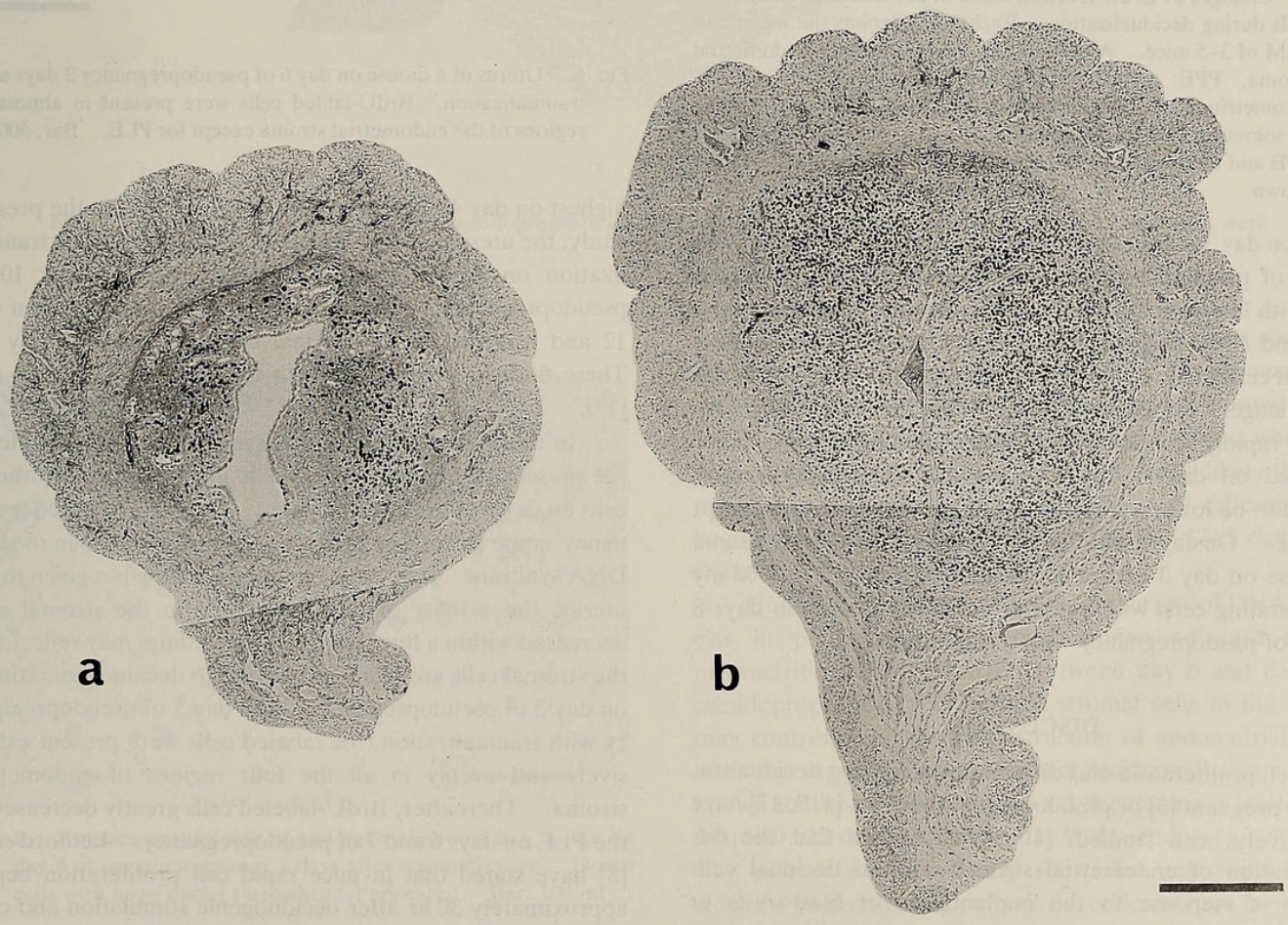

FIG. 4. Uteri of mice on day 3 (a) and day 5 (b) of pseudopregnancy, one day before and after traumatization, respectively. BrdU-labeled cells (black dots) were visible in the endometrial stroma. Bar: 500μm

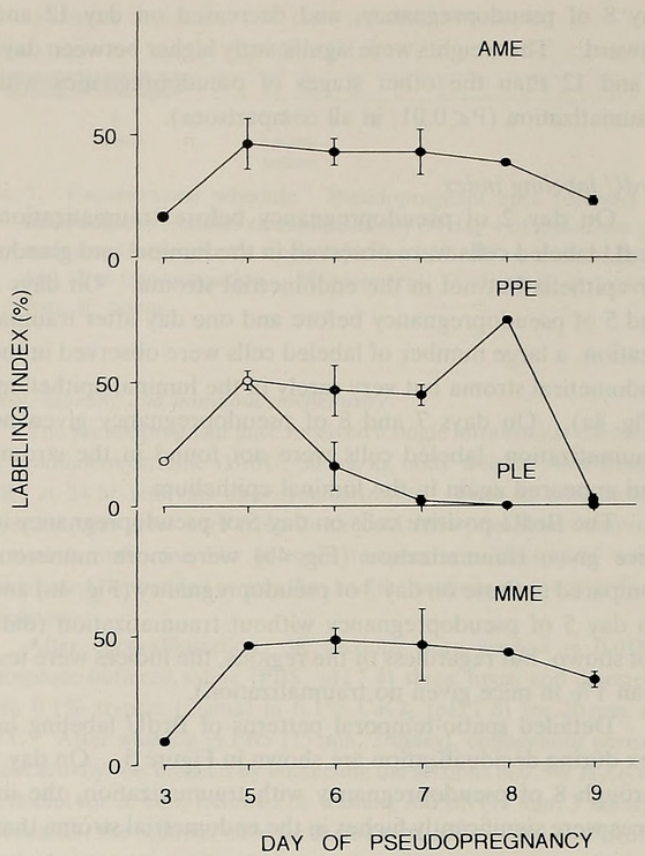

Fig. 5. Changes of BrdU lebeling index in the endometrial stromal cells during decidualization. Each point depicts the mean and SEM of 3–5 mice. AME: antimesometrial side of endometrial stroma, PPE: peripheral endometrial stroma adjacent to myometrium, PLE: periluminal endometrial stroma, MME: mesometrial side of endometrial stroma. On days 3 and 5, as PPE and PLE could not be counted separately, pooled data are shown.

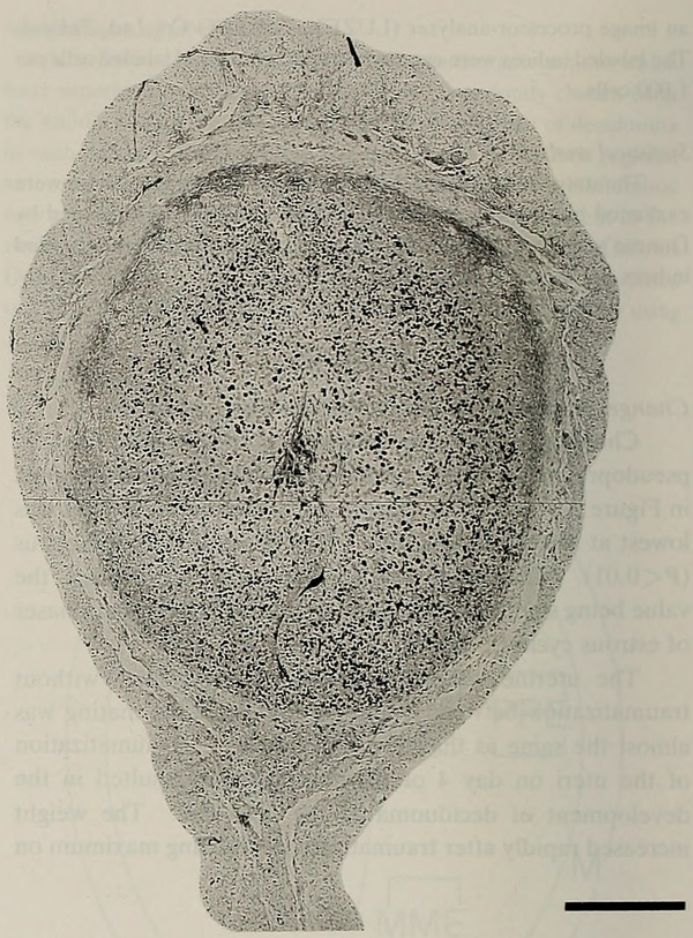

Fig. 6. Uterus of a mouse on day 6 of pseudopregnancy 2 days after traumatization. BrdU-labled cells were present in almost all regions of the endometrial stroma except for PLE. Bar: 500μm

those on day 3 at 0.01 level, except for PLE (Figs. 5–7). On day 6 of pseudopregnancy with traumatization, the plump cells with large nuclei over 25μm in diameter appeared in the PLE and AME (Fig. 8). In PLE, the percentage of labeled stromal cells on day 5 was significantly higher than that in the other stages, respectively ($P<0.01$). The percentage reduced rapidly on day 6 and BrdU labeling was no more detected on day 8 (Fig. 5). On day 8, labeling indices tended to be lower in the regions of the endometrium except for PPE. On day 9, all labeling indices were almost the same as those on day 3 except for MME (Figs. 5 and 9). Many degenerating cells with pyknotic nuclei appeared on days 8 and 9 of psudopregnancy with traumatization.

DISCUSSION

Cell proliferation and differentiation during decidualization in pregnant [3] or pseudopregnant rodents [4, 9, 17] have extensively been studied. It is well known that the differentiation of endometrial stromal cells to decidual cells occurs in response to the implantation of blastocysts or traumatization of artificial stimuli. In mice, the sensitivity of the uterus to a deciduogenic stimulus is known to be the highest on day 4 of pseudopregnancy [2, 10]. In the present study, the uterine weight increased immediately after traumatization on day 4 and reached maximum on day 10 of pseudopregnancy. The weight markedly decreased on day 12 and returned to the normal diestrous level on day 18. These findings accord well with the previous results in rats [17].

In the present study, DNA synthesis was detectable by the presence of BrdU-labeled cells in the luminal epithelial cells on day 2 of pseudopregnancy. On day 3 of pseudopregnancy, some stromal cells in the endometrium began to show DNA synhesis. If deciduogenic stimuli were not given to the uterus, the activity of DNA synthesis in the stromal cells decreased within a few days. These findings may reflect that the stromal cells are ready to respond to deciduogenic stimuli on day 3 of pseudopregnancy. On day 5 of pseudopregnancy with traumatization, the labeled cells were present extensively and evenly in all the four regions of endometrial stroma. Thereafter, BrdU-labeled cells greatly decreased in the PLE on days 6 and 7 of pseudopregnancy. Ledford *et al.* [8] have stated that in mice rapid cell proliferation begins approximately 30 hr after deciduogenic stimulation and continued for 72 hr in the endometrial stroma. After the initiation of decidualization, however, a population of stromal cells

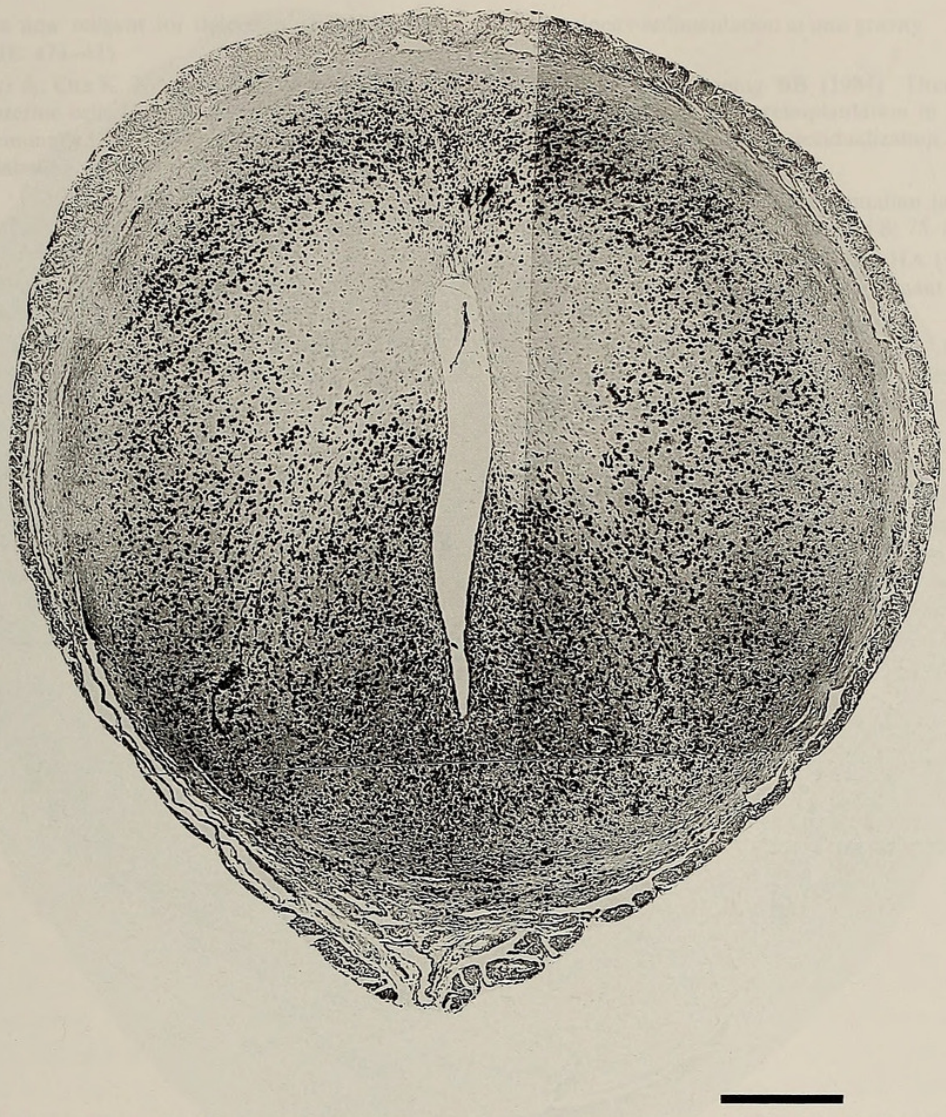

FIG. 7. Uterus of a mouse on day 7 of pseudopregnancy 3 days after traumatization. Only a few BrdU-labeled cells were visible in PLE. Bar: 500μm

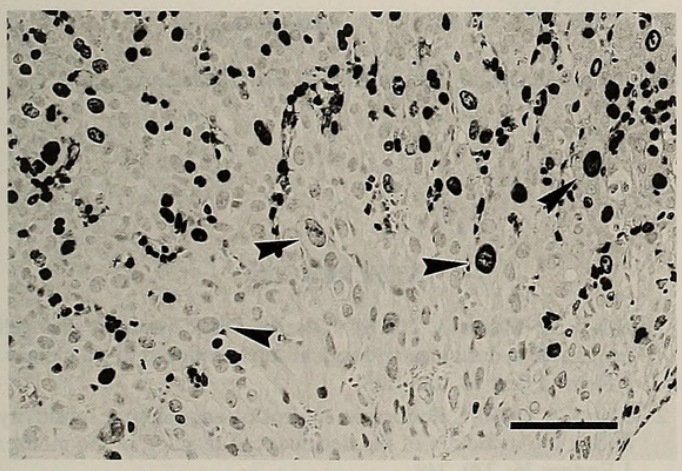

FIG. 8. Antimesometrial side of endometrial stroma in a mouse on day 6 of pseudopregnancy 2 days after traumatization. Plump cells with large nuclei (arrowheads) appeared. Bar: 100μm

is known to synthesize DNA and differentiate into polyploid decidual cells without cell division [1]. Deciduomal cells called plump cells in the present study are distributed exclusively in the periluminal part of endometrial stroma where the implantation normally occurs. Thus, it seems likely that the proliferation of deciduomal cells ceases and differentiation begins on day 6 or 7 of pseudopregnancy (2–3 days after traumatization/implantation).

During decidualization, a remarkable rise of DNA synthesis in peripheral endometrial stroma adjacent to the myometrium (PPE) occurred between day 6 and day 8 of pseudopregnancy. Proliferated stromal cells in this region may contribute to the reconstruction of endometrial tissue after the regression of preformed deciduomal tissue. It is known that the life span of the rat deciduoma is limited and frequent cell death occurs on day 9 of pseudopregnancy [14]. The present findings clearly show that regression of deciduoma begins in most parts of the endometrium from day 8 of pseudopregnancy, because many degenerated cells were encountered on days 8 and 9 of pseudopregnancy.