HISTOCHEMICAL LOCALIZATION OF ACID UTERINE AMINOACYLNAPHTHYLAMIDASES IN EARLY PREGNANCY AND IN DIFFERENT HORMONAL STATES OF THE MOUSE

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Summary. Two different aminoacylnaphthylamidase reactions in the mouse uterus are described, a particulate one in the glandular epithelium and a vacuolar one in the luminal epithelium and at the implantation sites. Maximum epithelial enzyme activity was observed on Day 5 of pregnancy or after 6 days' progesterone treatment of the spayed mouse. No further increase was achieved after oestradiol superimposed on progesterone treatment. At implantation sites on Day 8, the activity was mainly localized antimesometrially to the embryo. It is suggested that the uterine epithelial activity contributes to the breakdown of uterine secretion before the attachment reaction.

INTRODUCTION

In pregnant mice, a faint proteolysis occurs at the implantation sites and a slightly stronger one in the adjacent luminal epithelium (Bergström, 1970). These results suggest that the uterine luminal epithelium may have an autolytic capacity during early pregnancy in the mouse. To test this hypothesis, a modification of a histochemical technique designed for acid naphthylamidases was used in the present study. This technique is claimed to demonstrate early cell injury, e.g. in autolysis (Niemi & Sylvén, 1969).

MATERIALS AND METHODS

Virgin albino mice (NMRI, Bethesda) weighing about 20 g were used throughout this study. They were divided in two main groups: mice in the first group were mated and used during various stages of early pregnancy; mice in the second group were spayed 3 weeks before use.

The first group of mice was allowed to proceed to Day 2, 4, 5, 6 or 8 of pregnancy, Day 1 being the day on which a vaginal plug was found. The mice were killed by cervical dislocation at 09.00 hours on each of these days.

Mice in the second group were treated with combinations of progesterone and oestrogen according to Table 1 and subsequently killed in the same way as those in the first group.

At autopsy, the uteri were immediately removed in small pieces and freshly

frozen in isopentane cooled to -79°C with dry-ice. Sections ($10\text{ }\mu\text{m}$ thick) were then cut in a cryostat at about -20°C . For comparison, some other mice from both groups were given Nembutal® intraperitoneally and perfused through the abdominal aorta with 2.5% glutaraldehyde buffered with phosphate buffer (pH 7.4). Their uteri were removed after perfusion for 5 min and were subsequently embedded in polyethylene glycol (PEG) with a melting point of 37 to 38°C (Sandström & Westman, 1969).

Sections of both freshly frozen and PEG-embedded uterine tissue were then processed along three lines.

(1) This procedure was designed to test the presence of cathepsin B (Sylvén, 1968) and was performed in seven steps (a to g) before mounting: (a) incubation in 0.05 M-acetate buffer at pH 5.5 for 30 min; (b) incubation in cysteine (0.004 M) in 0.05 M-EDTA dissolved in the above buffer, pH 5.5 at room temperature for 30 min; (c) rinsing in fresh buffer as in (a) for several minutes; (d) incubation at 37°C in the substrate solution (Nachlas, Crawford & Seligman, 1957) containing L-leucyl- β -naphthylamide (LNA) and tetrazotized diorthoanisidine (Diaz Blue B), for 30 to 40 min (cryostat sections) or for 3 hr (PEG-embedded sections). The substrate, LNA, is split by a wide variety of enzymes irrespective of its L-leucyl component (Sylvén, 1968) and LNA-splitting enzymes are thus most correctly called aminoacylnaphthylamidases (AANA-ases). Substrate was dissolved in the 0.05 M-acetate buffer; (e) rinsing in saline for 2 min; (f) incubation in 0.1 M-cupric sulphate for 5 min to allow chelation of copper to the azo dye; (g) dehydration in graded concentrations of alcohol to xylol and then mounting in Caedax.

(2) This procedure was chosen to show whether or not acid cytoplasmic aminopeptidases were present in the sections (Sylvén, 1968) and was performed in almost the same way as was the 'cathepsin B' procedure above. The only deviation from this schedule concerned step (b), where preincubation was carried out in 0.01 M- $\text{Mn}(\text{NO}_3)_2$ dissolved in acetate buffer of pH 5.5 as above instead of the cysteine-EDTA-incubation.

(3) This procedure served as a control with the exclusion of any of the previous preincubation baths. The purpose was to test the enzyme independence of Mn^{2+} and cysteine-EDTA.

RESULTS

The findings are summarized in Table 1.

Two different types of enzyme reaction were distinguished: a particulate one in the glandular epithelium (Pl. 1, Figs. 1 and 2) and a vacuolar one in the luminal epithelium (Pl. 1, Figs. 3 and 4). The latter type was also observed around the implantation sites (Pl. 2, Figs. 5 and 6).

The structure was generally better preserved in fixed than in unfixed sections in spite of the six times longer incubation time required for the fixed sections.

Fixed tissues showed a lower activity than unfixed ones. The former had a yellowish background, absent from the latter, which was probably due to a denaturation effect on the proteins in the fixed tissue (Sylvén, personal communication). Localization of the enzyme activity in the fixed tissues was always

PLATE 1

FIG. 1. Mouse uterine glands on Day 5 of pregnancy. Cryostat section showing AANA-ase activity. $\times 550$.

FIG. 2. Mouse uterine glands on Day 5 of pregnancy. Toluidine blue-stained section of Epon-embedded material. $\times 550$. (By courtesy of Dr S. Reinius.)

FIG. 3. Mouse uterine lumen on Day 5 of pregnancy. Cryostat section showing AANA-ase activity. $\times 470$.

FIG. 4. Mouse uterine lumen on Day 5 of pregnancy. Toluidine blue-stained section of Epon-embedded material. $\times 470$. (By courtesy of Dr S. Reinius.)

(Facing p. 178)

PLATE 2

FIG. 5. Mouse implantation site on Day 8 of pregnancy. Cryostat section showing AANA-ase activity. No preincubation. M = mesometrial direction; A = antimesometrial region. Note active region antimesometrial to the embryo, fading off in the mesometrial direction. The embryonic tissue is inactive. $\times 40$.

FIG. 6. Region mid-way between mesometrial and antimesometrial end of the embryo (junction of embryonic and maternal tissues). E = embryonic trophoblast; D = maternal decidua. Note AANA-ase activity in vacuoles adjacent to invading trophoblast tissue. $\times 800$.

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diffusely cytoplasmic and bright red. In unfixed tissues, the enzyme activities were always of the particulate type (glandular epithelium) or of the vacuolar type (luminal epithelium) and the stain was always bluish. Corresponding reaction intensities were obtained at an incubation time of 30 to 40 min for unfixed sections and about 3 hr for fixed sections.

TABLE 1
RELATIVE ACTIVITIES OF AANA-ASE IN GLANDULAR AND LUMINAL
EPITHELIA OF THE MOUSE UTERUS IN DIFFERENT STAGES OF PREGNANCY
AND DIFFERENT HORMONAL STATES

Type of uterus	No. of animals	Preincubation	Glandular epithelium*	Luminal epithelium*
Day 2/9†	8	Cysteine + EDTA	—	—
		Mn ²⁺	++	+
		None	+(+)	+
Day 4/9	2	Cysteine + EDTA	—	—
		Mn ²⁺	++(+)	+
		None	++(+)	+
Day 5/9	7	Cysteine + EDTA	—	—
		Mn ²⁺	+++	+(+)
		None	++(+)	+(+)
Day 6/9	3‡	Cysteine + EDTA	—	—
		Mn ²⁺	++	+
		None	+(+)	+
Day 8/9	6	Cysteine + EDTA	—	—
		Mn ²⁺	++	(+)
		None	+(+)	(+)
Spayed	8	Cysteine + EDTA	—	—
		Mn ²⁺	(+)	(+)
		None	(+)	(+)
Spayed + A	3	Cysteine + EDTA	—	—
		Mn ²⁺	+	(+)
		None	+	(+)
Spayed + B	5	Cysteine + EDTA	—	—
		Mn ²⁺	+++	+(+)
		None	++(+)	+(+)
Spayed + C	5	Cysteine + EDTA	—	—
		Mn ²⁺	+++	+(+)
		None	++(+)	+(+)

A = 1 mg progesterone subcutaneously daily for 2 days; B = 1 mg progesterone subcutaneously daily for 6 days; C = 1 mg progesterone subcutaneously daily for 6 days + 0.1 μ g oestradiol-17 β after 5 days' progesterone treatment.

* The two kinds of epithelia should not be reciprocally compared (see text).

† Day 2/9 = Day 2 at 09.00 hours, etc.

‡ One of these animals had very low glandular activity.

The glandular activity was high in relation to the activity of any other uterine structure but different degrees of glandular activity were observed in adjacent glands.

The implantation sites (Day 8) showed a diffuse activity, most pronounced antimesometrially to the embryo, the activity fading off in the mesometrial direction (Pl. 2, Fig. 5). The embryonic tissues proper, the endoderm and ectoderm, were inactive (Pl. 2, Fig. 6). On the mesometrial side of the deciduomal

swelling, the glandular epithelium showed some activity, though this was less than that on Day 5.

DISCUSSION

The basis for the present histochemical reactions is the hydrolysis by tissue enzymes of aminoacid naphthylamides and the simultaneous coupling of the liberated naphthylamine with a tetrazonium salt to produce an azo-dye (Nachlas *et al.*, 1957). The stability of this compound is rather low but can be increased by its chelation of copper.

A further development of this technique has indicated that a selective demonstration of cathepsin B (Keilová & Keil, 1969) is possible under special conditions (Sylvén, 1968). Biochemical evidence has demonstrated that cathepsin B cleaves certain aminoacid naphthylamides at an acid pH, where aminopeptidases are virtually inactive (Sylvén & Snellman, 1968). Interestingly, the histochemical application of this method showed cathepsin B to be localized in autolytic vacuoles and enlarged lysosomes, suggesting a relation to cell injury at an early stage (Niemi & Sylvén, 1969).

The specificity of the present method has been a matter of controversy. Cysteine plus EDTA is claimed to stimulate cathepsin B but to inhibit aminopeptidases, while the reverse is the case for Mn^{2+} (Sylvén, 1968).

The present results demonstrate (cf. Table 1) that, in the mouse uterus at pH 5.5, any LNA-splitting enzyme activity is abolished by preincubation in cysteine plus EDTA, while only a little stimulation is attained by Mn^{2+} . This indicates the presence of LNA-splitting enzyme(-s) at pH 5.5 other than cathepsin B, a finding which is contrary to the statement by Sylvén (1968) that the present histochemical procedure will exclude all LNA-splitting enzymatic activity but cathepsin B if carried out at pH 5.5. The uterine activity found is most correctly ascribed to aminoacylnaphthylamidase (-s) (AANA-ase) since LNA is split by a wide variety of enzymes irrespective of its L-leucyl component (Sylvén, 1968).

The possibility that the stain observed merely represents non-specific aggregates of substrate is contradicted firstly by the fact that no Diazo Blue B precipitate whatsoever was found in the first histochemical procedure, where all AANA-ase activity was inhibited, secondly by the different colours of Cu-chelating azo-dye (bluish) and the tetrazonium salt (brownish) and thirdly by the distinct variation of azo-dye in animals of different hormonal states (cf. McCabe & Chayen, 1965).

The particulate and vacuolar appearances of the AANA-ase localizations seem to be a constant feature of glandular and luminal epithelium respectively. In Epon-embedded sections, lipid inclusions of the luminal epithelium correspond very closely to the vacuoles in the cryostat sections (Pl. 1, Figs. 3 and 4) while—at the light microscope level—no clear corresponding structure could be demonstrated concerning the glands (Pl. 1, Figs. 1 and 2). There are some lipid inclusions also in the glandular epithelium but, obviously, these cannot account for the activity in the glands (cf. Pl. 1, Figs. 1 and 3). The apparent localization of AANA-ase in the walls of these luminal vacuoles is in accordance

with the general statement by Felgenhauer & Glenner (1966) that peptidases in general are membrane-bound.

In the spayed and hormone-treated mice, the AANA-ase pattern that approaches the glandular appearance on Day 5 most closely is the one found in mice treated for 6 days with progesterone. The addition of oestradiol on the 5th day of progesterone treatment did not augment the intensity any further. Lammes (1963) reported a peak intensity on Day 6 of pseudopregnancy in mice in comparison with Days 3 and 9, using LNA and Garnet GBC in the substrate solution. He noted a strong apical reaction in the glandular epithelium at this time. A similar localization could not be demonstrated in the present work. Different histochemical methods may account for this discrepancy.

In a biochemical study of the uterus of the pseudopregnant rat, Rich (1965) found a maximum on Day 6 of an acid proteolytic enzyme using denatured haemoglobin as substrate. Only the organ as a whole was examined. Similar results have been reached by Wood & Psychoyos (1967) in the pseudopregnant rat. Their findings are in agreement with those of Rich and are analogous to the present histochemical results in the mouse.

The functional significance of the observed AANA-ase activity in the mouse uterus is unclear. Generally, the two effects ascribed to these types of enzymes concern protein breakdown or synthesis (Burstone, 1962). Of these, at least the former process can be expected to occur in the mouse uterus during implantation: firstly, in connection with the disappearance of the zona pellucida, where a uterine zona 'lysin' has been postulated and secondly, in connection with the disappearance of the progestational uterine secretion as the epithelial attachment reaction occurs.

The disappearance of the zona pellucida is thought to be in some way related to a 'lysin' which is evidently oestrogen-dependent and possibly of uterine origin (McLaren, 1970). The time of maximum AANA-ase activity and that of zona loss seem to coincide in normal pregnancy. Some animals examined on Day 4 and on Day 6 were found to have irregularly the pattern of Day-5 animals. However, the appearance of the Day-5 AANA-ase pattern after 6 days of treatment with progesterone alone, during which shedding delay is to occur, makes AANA-ase an unlikely 'zona lysin'.

The uterine secretion of early pregnancy disappears before the attachment reaction in the mouse on Day 5. A similar picture regarding the attachment reaction can be achieved by progesterone alone (Martin, Finn & Carter, 1970). Thus, there seems to be a parallelism between maximum AANA-ase activity and disappearance of the uterine secretion in the mouse. This finding suggests a rôle for AANA-ase in the absorption process of uterine secretion which is believed to occur before the attachment reaction.

The present findings do not lend any specific support to the hypothesis of an autolytic capacity in the luminal epithelium. No cathepsin B activity could be demonstrated at places with established proteolytic activity. However, the luminal epithelium might show this capacity in other states where catabolism is believed to occur (Thiery & Willighagen, 1963).

The implantation sites are active mainly in the antimesometrial region, which, unlike the mesometrial region, is known in rodents to be a lipid-rich

zone (Krehbiel, 1937). The vacuolar AANA-ase activity found here is similar to that found in the lipid inclusions of the luminal epithelium but the vacuoles are of greater variety. This periembryonic activity is similar to that described in the area of invading tumours (Glennner, Burstone & Meyer, 1959; Hess, 1960a, b). Thus, the invading trophoblast tissue appears to elicit the breakdown of the host tissue in the same way as an invasive tumour and there seems to be an enzymatic analogy between invading trophoblastic tissue and tumour growth. This periembryonic AANA-ase activity corresponds closely to the proteolytic activity earlier demonstrated by means of the gelatin digestion method (Bergström, 1970).

The conclusion of the present findings is that there are two different epithelial AANA-ase reactions in the mouse uterus, a particulate reaction in the glandular epithelium and a vacuolar one in the luminal epithelium, both of which seem to be in some way related to the implantation process, possibly to the breakdown of a secretory product.

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