**HEAT-STABLE ALKALINE PHOSPHATASE IN THE NORMAL
FEMALE GENITAL ORGAN
—WITH SPECIAL REFERENCE TO THE HISTOCHEMICAL
HEAT-STABILITY TEST AND
L-PHENYLALANINE INHIBITION TEST—**

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The Regan isoenzyme, a heat-stable alkaline phosphatase, which has been thought to be derived only from cancer or the placenta, has been reported in non-cancer, non-pregnant patients. We performed histochemical studies to determine whether the heat-stable alkaline phosphatase exists in normal cells of the female reproductive organ. For this purpose, the heat-stability test and the L-phenylalanine inhibition test were used. Heat-stable and L-phenylalanine-sensitive alkaline phosphatase was found in normal cells of Muellierian duct origin, e.g., uterine cervical reserve cells, cervical columnar cells and epithelial cells of the endometrial surface. Our findings suggest that heat-stable alkaline phosphatase, such as the Regan enzyme, may be produced by normal cells of Muellierian duct origin.

Electrophoresis and specific inhibition tests resulted in the identification of several alkaline phosphatase (ALP) isoenzymes, and it is now known that the liver, intestine, bone, and placenta, for example have specific ALP. Fishman *et al.* (3) identified the Regan isoenzyme which is heat-stable and L-phenylalanine (L-p)-sensitive and thought to be ectopically produced in tumors. As the Regan isoenzyme cannot be biochemically differentiated from placenta ALP, it has attracted attention as a carcino-placental antigen (4). However, Stolbach *et al.* (16) and Nathanson *et al.* (13) reported the detection of the Regan isoenzyme in the serum of non-cancer, non-pregnant patients which gave rise to the speculation that it may not only be produced in cancer or in the placenta. In a preliminary investigation, Nozawa *et al.* (15) found that the Regan isoenzyme was present in some uterine cervical reserve cells of non-cancer, non-pregnant women in 1975.

The present study was designed to determine whether heat-stable ALP, such as the Regan isoenzyme, exists in normal cells of the female reproductive organs. Therefore, we investigated the ALP activity in these cells and studied the isoenzyme patterns, using the histochemical heat-stability test and the L-phenylalanine inhibition test.

MATERIALS AND METHODS

Biopsy materials and surgical specimens obtained from 454 patients seen at the Department of Obstetrics and Gynecology of Keio University Hospital, were used in this study. For examination of reserve cell hyperplasia, epithelial specimens, obtained during the healing stage after cryosurgery, were obtained.

Light microscopic studies

Tissues were fixed for 9 hr at 4°C in Baker's 10% formol calcium (1) and washed overnight at 4°C in Holt's gum sucrose (6). Then, serial sections, 6 μ m in thickness, were cut with a cryostat and mounted on glass slides.

ALP staining

The modified method of Burstone (2) was used. The substrate was naphthol AS-BI phosphate (1.1 mM), the azo dye was fast-red-violet LB salt (0.75%), and the buffer consisted of 0.05 M propandiol (pH 9.8). The pH of the incubation medium was 9.3–9.5, and incubation was for 15 min at room temperature (20). Simultaneously, as a combined heat-stability test, ALP staining was performed with or without heat-inactivation at 65°C for 30 min in a 0.005 M MgCl_2 solution (7).

For the L-phenylalanine inhibition test, the method described by Watanabe and Fishman (18) was used. ALP staining was performed after incubation for 15 min at room temperature in a medium containing 0.03 M L-phenylalanine. For the control, the incubation medium contained 0.03 M D-phenylalanine, which is stereo-specific for L-phenylalanine.

Following these procedures, the tissues were dehydrated in a graded series of alcohol.

Nuclear staining

This was by the Azur-A-Schiff method as described by Watanabe *et al.* (20).

Electron Microscopic studies

Fresh tissues were cut into $0.5 \times 0.5 \times 5$ mm rectangles and prefixed by the double fixation method. First they were fixed for 4 hr at 4°C in 4.5% paraformaldehyde, followed by 1 hr fixation at 4°C in 2.5% glutaraldehyde. Then the tissues were washed overnight at 4°C in 0.05 M tris-maleate buffer (pH 7.4) and 40 μ m sections were cut with a tissue sectioner. The sections were incubated for 30 min at room temperature according to the method of Mayahara *et al.* (10) in 40 ml of a medium consisting of 7.6 ml 0.1 M Na, β -glycerophosphate (350 mg/10 ml), 6.4 ml 0.2 M tris-HCl buffer (pH 7.5), 10 ml 0.015 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 16 ml 0.5% lead citrate (pH 10.0–11.5). The sections were dehydrated in a graded series of alcohol, embedded in epoxy-resin, thick and thin sections were prepared and double stained with uranyl acetate and lead citrate.

Sections exhibiting any non-specific ALP activity in the plasma membranes of a circumscribed area within the epithelium were designated as ALP-positive. Only if there was no ALP activity at all, was the section designated ALP-negative. ALP-positivity was classified into 4 grades ranging from $\pm$ to 3+. The enzyme activity in the capillary endothelial cells of the same section was used as the standard of comparison.

With respect to heat stability, we established 4 categories by comparing the

TABLE 1. *Criteria of heat stability and L-phenylalanine sensitivity*

	Category 1			Category 2	Category 3			Category 4		
Heat Stability	none			slight	moderate			marked		
non-specific ALP	(+)	(++)	(+++)	(+++)	(++)	(++)		(+)	(++)	(+++)
Heat Inactivation		↓		↓	↓	↓		↓	↓	↓
	(-)			(+)	(+)	(++)		(+)	(++)	(+++)
L-p Sensitivity	none			slight	moderate			marked		
D-p Inhibition	(+)	(++)	(+++)	(±)	(+)	(++)	(+++)	(++)		(+++)
L-p Inhibition	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
	(+)	(++)	(+++)	(-)	(±)	(-)	(+)	(-)	(±)	(-)

intensity of ALP-staining in sections that had, or had not, been heat-inactivated. In category 1 sections, which exhibited no heat-stability, no ALP-activity was noted after heat-inactivation. Category 2 was applied when there was a considerable difference between the heat-inactivated and non-inactivated sections. If there was little or no difference between the sections, they were categorized as heat-stability 3 and 4, respectively (Fig. 1).

With respect to L-phenylalanine sensitivity, we established 4 categories; the higher the assigned number, the greater the L-phenylalanine sensitivity. Sections were classified according to the difference in ALP-staining after incubation in the presence of L-phenylalanine or D-phenylalanine (Table 1).

RESULTS

The number of specimens examined, their origin and non-specific ALP-activity are shown in Table 2.

Reserve-Cell Hyperplasia

Of 92 specimens examined, 82 (89.1%) were ALP-positive (Table 2). Of the

TABLE 2. *Non-specific ALP activity*

		No. of Total Cases	Non-specific ALP	
			No.	%
Reserve Cell Hyperplasia		92	82	89. 1
Metaplasia	Immature	36	24	66. 7
	Mature	15	0	0
Squamous Epithelium		24	1	4. 2
Cervical Columnar Epith.		132	83	62. 9
Endometrium	Surface	48	40	83. 3
	Gland	92	87	94. 6
Total		439		

82 ALP-positive sections, 52 were serially sectioned and further examined for heat-stability and L-phenylalanine sensitivity (Table 3). All of the sections were either moderately or markedly heat-stable (categories 3 and 4), and with respect to L-phenylalanine-sensitivity, almost half of the sections exhibited moderate (category 3) sensitivity (Table 2, Figs. 1-4).

Electron microscopically, the nuclei of hyperplastic reserve-cells were comparatively large and some tonofilaments were observed in their cytoplasm. Desmosomes were present between the plasma membranes. ALP-activity was recognized as black dots in microvilli and plasma membranes (Figs. 13-14).

Metaplastic Epithelium

The metaplastic epithelium was subdivided into immature and mature epithelium. None of the mature metaplastic epithelium specimens exhibited ALP activity (Table 2). Of the 36 immature metaplastic epithelium specimens, 24 (66.7%) were ALP positive (Table 2). Of the 24 ALP positive specimens, 20 underwent serial sectioning and they were evenly divided between heat-stability categories 3 and 4. With respect to L-phenylalanine sensitivity, half of the sections were classified as either category 2 or 3, while the other 10 sections showed marked (category 4) sensitivity (Table 2).

Electron microscopically, ALP activity was localized in the microvilli and plasma membranes. Cells exhibiting few tonofilaments or desmosomes constituted the majority of the immature metaplastic cells in which ALP activity was observed.

Squamous Epithelium

In only one of 24 specimens (4.2%) was there weak ALP-activity in the horny layer of the superficial epithelium (Table 1). However, this activity disappeared with heat-inactivation.

Electron-microscopically, no ALP activity was localized in the plasma membrane.

Cervical Columnar Epithelium

Of 132 cervical columnar epithelium specimens, 83 (62.9%) were ALP-positive (Table 1) and of these, 48 were serially sectioned. The majority of the sections were

FIG. 1. Red-colored precipitate in columnar cells and reserve cells of uterine cervix shows ALP activity.

FIG. 2. After heat inactivation, heat-stable ALP activity persisted.

FIG. 3. After D-Phenylalanine inhibition test (control).

FIG. 4. After L-Phenylalanine inhibition test. An obvious difference in inhibition is seen. The ALP in cervical columnar cells and reserve cells resembles placental ALP or the Regan isoenzyme produced heterotopically by tumors.

FIG. 5. Strong ALP activity is seen on the glandular epithelial cells of endometrium.

FIG. 6. Heat inactivation of ALP activity.

FIG. 7. After D-P inhibition test (control).

FIG. 8. After L-P inhibition test. As compared with FIG. 7, little inhibition is recognized.

FIG. 9. Strong ALP activity is recognized in endometrial luminal lining epithelial cells.

FIG. 10. ALP activity persisted after heat inactivation.

FIG. 11. After D-P inhibition test (control).

FIG. 12. After L-P inhibition test. Comparison with FIG. 11 shows this ALP on lining epithelial cells to resemble placental ALP or the Regan isoenzyme.

classified as category 4 with respect to heat-stability and all of them exhibited some degree of L-phenylalanine sensitivity (Table 2, Figs. 1-4).

Electron-microscopically, ALP activity was located in microvilli at the free margin and in intercellular plasma membranes of cervical columnar cells. (Figs. 13-14)

Endometrium

The endometrium was subdivided into the superficial epithelium lining the endometrial luminal surface (48 specimens) and the glandular epithelium of the functional and basal layer (92 specimens). Of the former 40 (83.3%), and of the latter, 87 (94.6%) were ALP-positive (Table 1).

Serial sectioning was performed on 32 specimens from the endometrial surface and 7 of these (21.9%) exhibited some degree of heat-stability (Table 2). However, the areas in which heat-stable ALP-activity was noted were very limited and the intensity of the stain was weak. More than half of the serial sections were in category 2 with respect to L-phenylalanine sensitivity (Figs. 9-12), however, all of the 7 sections exhibiting heat-stable ALP, showed moderate or marked (Categories 3 or 4) L-phenylalanine sensitivity.

Of the 46 serially sectioned specimens from the glandular epithelium, all but 2 were heat-sensitive, and all but 6 showed L-phenylalanine sensitivity of either category 2 or 3 (Table 2, Figs. 5-8).

In the 2 cases with slightly heat-stable ALP, the glandular epithelial cells with ALP heat-stability continued from the ALP heat-stable superficial luminal epithelial cells.

Electron-microscopically, ALP activity was localized in microvilli and plasma membranes of endometrial epithelial cells.

DISCUSSION

Serum ALP can be divided into several kinds of isoenzymes by electrophoresis, heat-inactivation or the use of inhibitors such as L-phenylalanine.

Jensen *et al.* (7) incubated placental tissue for 30 min at 56°C or 70°C in a 0.01 M MgCl₂ solution and Kameya *et al.* (8) used 65°C, 15 or 35 min incubation in a 0.005 M MgCl₂ solution. In other biochemical studies, the incubation temperature ranged between 55°C (11) and 70°C (14). As the length of incubation may affect the degree of heat-stability, in our study only those sections that exhibited ALP-activity after 30 min incubation at 65°C were considered to be heat-stable ALP-positive.

For their L-phenylalanine inhibition tests, Watanabe *et al.* (18) and Kameya *et al.* (8) used an incubation medium containing 0.05 M L-phenylalanine. However, at that molecular weight, our preliminary studies revealed the occurrence of

FIG. 13. Localizations of ALP activity are seen as black dots (→) in microvilli of cervical columnar cells. (×7600)

FIG. 14. ALP localizations (→) are observed only at the lateral plasma membrane of reserve cells of uterine cervix. (×6300)

Arrow-ALP activity, columnal cell (cc), secretory granule (sg), reserve cell (rs), basement membrane (bm), desmosome (dm).

nonspecific inhibition, even in the presence of D-phenylalanine which should induce no inhibitory reaction. Therefore, we chose a molecular weight of 0.03 M as being most adequate for the purpose of obtaining comparatively strong inhibition with L-phenylalanine, while there was little reaction in the presence of D-phenylalanine.

When the histochemical method described in the present report was used in preliminary studies to examine placental syncytio-trophoblasts which possess the heat-stable, L-phenylalanine-sensitive ALP isoenzyme, ALP activity was retained after heat-inactivation, and disappeared after L-phenylalanine inhibition.

The heat-stable ALP has been thought to exist only in tumors or in placental trophoblasts, therefore, it has drawn attention for possibly representing a carcino-fetal protein and has been designated carcino-placental antigen (4).

The Regan isoenzyme, a heat-stable ALP first reported by Fishman *et al.* (3) in the serum, tumor effusion and tumor tissue of lung cancer patients, was a trigger in this particular area of cancer research. Other heat-stable ALP are the Nagao isoenzyme (12) and the Kasahara isoenzyme (5). While those enzymes were initially thought to exist only in the serum or in the tissues of some malignant tumors, Stolbach *et al.* (16) and Nathanson and Fishman (13) reported such enzymes in the sera of a few non-cancer patients. Furthermore, Usategui-Gomez *et al.* (17) who used immunological methods, found the Regan isoenzyme in normal human sera. These findings have awakened a special interest in discovering the histologic origin, other than trophoblasts or placenta, of such heat-stable ALP in normal human sera.

The origin of the heat-stable ALP in normal human serum has eluded discovery in the past because it is difficult to identify cells that produce this ALP by the previously used biochemical methods. However, the method described in the present report revealed a heat-stable, L-phenylalanine-sensitive ALP similar to the Regan isoenzyme in uterine cervical reserve cells, cervical columnar epithelial cells, and superficial epithelium lining the endometrial luminal surface. These cells were normal human cells which are thought to derive from the Muellerian duct. Our data coincide with those of Malkin *et al.* (9) who used the biochemical method and reported the presence of placental ALP in normal human cervical tissue and to a lesser degree, in human endometrium.

Our data suggest that cells of Muellerian duct origin may represent one of the production sites of heat-stable ALP detected in normal human serum.

Although both reserve cells of the uterine cervix and endometrial glandular cells derive from the Muellerian duct, the former exhibited the heat-stable, L-phenylalanine-sensitive ALP, while in most of the latter, heat-labile, L-phenylalanine-sensitive ALP was detected. This finding is particularly interesting in view of the fact that these two types of cell are thought to represent the origins of uterine cervical cancers and endometrial cancers, respectively. The present study has provided basic data with reference to the question of whether a new type of isoenzymes emerges in the course of carcinogenesis of the uterus by applying an enzyme histochemical method which facilitates the application of biochemical concepts to cell morphology.

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