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Immunohistochemistry of a Component Protein of the Breast Cystic Disease Fluid with Mol. Wt 15,000*

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Abstract—*A specific protein from the liquid of a mammary cyst with a molecular weight of* 15,000 *(GCDFP* 15) *was studied in normal and pathological mammary tissue using an immunohistochemical method (peroxidase-anti-peroxidase complex). An immunoreactivity of the GCDFP type was found in normal idrosadenoid glands having an apocrine secretion. Histologically normal mammary tissue was not immunoreactive. In benign breast tissue the GCDFP was found particularly in epithelium undergoing apocrine metaplasia* (55/55) *and in atypical lobular epithelial hyperplasia* (8/10). *Of the adenocarcinomas of the breast* 136/161 (84%) *were immunoreactive, especially lobular carcinoma* (13/13). *The proportion of tumors with a high percentage of immunoreactive cells* (76-100%) *was greater for Bloom's grade I* (1/29: 34%) *than for grade III* (10/66: 15%). *A significant correlation was found between the percentage of immunoreactive cells and the cytosolic concentration of progesterone receptors. The morphological intracellular identification of GCDFP (due to its greater sensitivity) and its correlation with progesterone receptors allowed a more precise evaluation of the functional state and the hormonal dependency of the breast cells by underlining the heterogeneity of the tumoral cell population.*

INTRODUCTION

FIBROCYSTIC disease of the breast is principally a pre-menopausal disease that is characterized by the presence in the mammary gland of cysts containing an apocrine type of secretion [1]. This pathology, which seems to increase the risk of neoplastic transformation [2], is associated with a hormonal prevalence of estrogens [3] and, according to some authors, with an increased secretion of androgens [4, 5], which stimulate the development and the activity of the sweat glands that, like the axillary glands, have an apocrine secretion [6].

A principal component of the breast cyst fluid with a molecular weight of 15,000 (GCDFP 15) was purified and identified in saliva and human milk [7]. The levels of GCDFP in the cytosol of normal epithelial mammary cells varied during the menstrual cycle and were correlated with progesterone receptors [8]; they were higher in differentiated mammary tumors [9] presenting a metaplastic apocrine transformation [10].

In this study the GCDFP 15 was evaluated using an immunohistochemical method in different benign and malignant breast lesions with the aim of establishing correlation: firstly between this protein and the histological classification of the tumor; secondly with the Bloom's grading histoprognostic with its three components (tissular differentiation, nuclear polymorphism and mitotic activity); and thirdly with the presence or absence of estrogen and progesterone receptors.

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MATERIALS AND METHODS

Tissue samples

Ten samples of axillary apocrine glands from non-menopausal women, 14 samples of morphologically normal mammary tissue from peritumoral zones or from the periphery of adenofibromas, 15 adenofibromas, 33 fibrocystic dystrophies and four intraductal papillomas were obtained from 42 menopausal and 24 non-menopausal women. In fibrocystic disease several types of lesions can coexist in the same sample (Table 1).

One hundred and sixty-one malignant tumors were randomly sampled during the partial or total mastectomies of 48 non-menopausal and 113 menopausal women. The histological classification is the one proposed by the OMS in 1981 for breast cancers [11], and the histoprognostic grading is that of Bloom *et al.* [12]. The histological types of these 161 tumors are described in Table 2.

Anti-GCDFP antiserum

The GCDFP 15 purified from the breast cyst fluid [13] was used for immunizing a rabbit according to the Vaitukaitis *et al.* method [14]. The specificity of this antiserum was established by radioimmunoassay and by immunoelectrophoresis. The antiserum was specifically directed against human GCDFP 15,000 and did not cross-react with other milk proteins (casein, lactalbumin, lactoferrine or the secretory fragment of IgA), protein extracted from the breast cyst fluid (GCDFP 24,000) or human serum proteins (in particular, human albumin). No cross-reaction was obtained with tumoral markers (HCG and its

Table 1. GCDFP immunoperoxidase-normal and pathologic breast

Histology	No.	Immunoperoxidase Negative	Positive
Normal breast	14	14	0
Adenofibromas	15		
Without apocrine or features	10	6	4
With apocrine features	5	0	5
Fibrocystic dystrophy	33		
Cyst:			
cubic of flat epithelium	20	20	0
epithelium in simple idrosadenoid* metaplasia	30	0	30
epithelium in florid idrosadenoid* metaplasia	25	0	25
Atypical ductal epithelial hyperplasia	22	10	12
Atypical lobular epithelial hyperplasia	10	2	8
Intraductal papillomas			
Without idrosadenoid features	2	2	0
With idrosadenoid features	2	0	2

*Idrosadenoid cell = apocrine-like cell.

Table 2. GCDFP immunoperoxidase-malignant breast tumors

Histology	Total No.	GCDFP 15-like immunoreactive cells 0%	1-25%	26-75%	76-100%
Invasive lobular C*	13	0	2	7	4
Medullary C	8	2	3	2	1
Mucinous C	8	1	0	6	1
Papillary C	2	0	0	1	1
Invasive ductal C	107	15	29	42	21
Apocrine-like C†	11	2	2	5	2
Comedo-carcinomas	8	3	2	3	0
Hemangiosarcomas	2	2	0	0	0
Phyllode sarcomas	2	0	1	1	0
Total	161	25	39	67	30

*C = carcinomas.

†Apocrine-like C = invasive ductal carcinomas with various apocrine features.

sub-units alpha and beta, and none with the carcino-embryonic antigen) nor with a mammotropic hormone such as prolactin. By electrophoresis the anti-GCDFP antiserum reacted with the purified protein with a single precipitation line in the region of alpha-globulins, and did not react with human serum proteins.

The control serum was prepared by filtration of 1 ml of specific antiserum on a Sepharose CNBr 4B column (2.5 × 2 cm, Pharmacia, Uppsala, Sweden) on which was fixed [15] 2 mg of purified human GCDFP. The treated antiserum no longer bound the GCDFP 15,000 that was labelled with ^{125}I [16], and during electrophoresis no longer produced a precipitation line with the pure protein.

The specific gamma-globulins remaining on the GCDFP 15 bound on the immuno-absorbent were eluted with a 3-M sulfocyanic buffer in a Sorensen phosphate buffer, pH 7.4, 10 mM, dialyzed against distilled water and lyophilized.

Immunohistochemical method (peroxidase-anti-peroxidase according to Sternberger [17])

The specimens were first fixed in a 0.15-M NaCl solution containing 10% formaldehyde for 48 hr at room temperature. After embedding in paraffin, the tissues were cut into sections 4 μm thick. The sections were deparaffinized with xylene and graded alcohols and then rinsed in PBS for 30 min at room temperature. Endogenous peroxidase was blocked by a solution of methanol containing 0.3% H_2O_2 for 30 min at room temperature, and then by normal sheep serum for 5 min. The sheep serum was inactivated by heating at 60°C for 1 hr and diluted to 1/5.

The rabbit anti-GCDFP antiserum was applied in a humid chamber for 30 min at a dilution of 1/100. The sections were then rinsed three times in PBS, treated with sheep anti-rabbit serum proteins (Pasteur Institute), diluted to 1/10, rinsed again three times with PBS and finally treated with a rabbit gamma-globulin peroxidase-anti-peroxidase (Dako B 157) complex diluted to 1/50. The peroxidase was revealed by incubating the sections in diaminobenzidine tetrahydrochloride (D.A.B. Sigma, No. D 563), diluted to 0.05% in a 50 mM Tris buffer (pH 7.6), to which 0.01% H_2O_2 was added. The sections were then washed three times in PBS and dehydrated with ethanol and xylene.

In order to facilitate the histologic observation, a coloration with Harris hematoxylin was used.

During each step of the reaction the following controls were established: (a) *positive control*: the specific immunoglobulins eluted from the affinity chromatography were diluted to 1/100 and used in place of the anti-GCDFP antiserum; and (b) *negative control*: replacement of the specific antiserum by human anti-GCDFP antiserum, with its specific antibodies extracted by the immunoabsorbent, or PBS buffer or normal rabbit serum, in order to control the absence of endogenous peroxidase.

The GCDFP content of malignant tumors was determined by the percentage of intracytoplasmic positive cells with respect to all the cells counted on an entire slide with a 250-fold magnification. The expressed percentage was calculated according to the mean values obtained after the cellular count of the most representative slides of each tumor. The variation coefficient from one slide to another for the same tumor was less than 5%. The tumors were then classified into four groups: one negative (0%) and three positive (1–25%; 26–75%; and 76–100%).

Hormonal receptors

The cytosol preparations and the assay for the estrogen and progesterone receptors were assessed according to the method described by the EORTC (European Organization for Research on Treatment of Cancer) [18]. The limit of detection was 10 fmol/mg of cytosolic protein determined by Bradford's method [19].

Statistical methods

The significance of deviation between the distribution of various groups was calculated by the chi-square test. An attempt to detect a linear or monotonic relationship between % of tumor cells positive and receptor activity was performed using a simple linear regression analysis and the Spearman rank correlation coefficient [20].

RESULTS

Normal apocrine glands

Immunoreactive-like GCDFP was found in normal apocrine glandular tissues, in particular in axillary apocrine glands, whose epithelium showed a florid character (Fig. 1).

Normal or dystrophic mammary tissue

As indicated in Table 1, epithelial cells of morphological normal mammary tissue that were peritumoral or peripheral to adenofibromas did not present an immunoreactive-like GCDFP in our series (0/14).

In adenofibromas, when there existed epithelial zones with an idrosadenoid or apocrine metaplasia were present, the immunoperoxidase reactions were positive; in the absence of these morphological characteristics, 4/10 of the adenofibromas were positive.

In fibrocystic disease the more the epithelium

was of a metaplastic nature with hyperplasia on its outer layer, the more it tended to exhibit an immunoreactive-like GCDFP positivity. However, when the epithelium was cubic or flat (20 cases), no cell was positive. When it underwent simple idrosadenoid metaplasia (unistratified epithelial lining layer) all the cells of the 30 cases were weakly positive; when the idrosadenoid metaplasia was florid (pluristratified epithelial lining layers) all the cells of the 25 cases were strongly positive. Atypical lobular epithelial hyperplasia presented an immunoreactivity in 8/10 cases. In the case of an intraductal papilloma, only the epithelium undergoing cylindric or idrosadenoid metaplasia (2/2 cases) was strongly positive. When apocrine metaplasia was absent, the immunoperoxidase reaction was negative.

These apocrine-like carcinomatous cells were large, columnar, eosinophilic and often granular. The two hemangiosarcomas, which are mesodermal tumors, were negative. The two sarcoma phyllodes were positive in their epithelial zones. The positivity of mammary cancers in non-menopausal women (37/46 cases: 80%) was not different from that found in menopausal women (96/112: 83%).

GCDFP and histoprognostic grading (*Table* 3). There was no correlation between the presence of GCDFP 15,000 in the tumors and the Bloom's grading. For grade I tumors, 26/29 (89%) for grade II tumors 50/58, and for grade III tumors 55/66 (83%) were positive for GCDFP. However, the proportion of tumors having an important cellular positivity (76–100%) was greater for grade I (10/29: 34%) than for grade III (10/66: 15%)

Table 3. *Relation between GCDFP-like immunoreactivity and histoprognostic grading*

Bloom's grading	Total No.	GCDFP 15-like immunoreactive cells 0%	1–25%	26–75%	76–100%
Grade I	29	3 (11%)	5 (17%)	11 (38%)	10 (34%)
Grade II	58	8 (14%)	13 (22%)	27 (47%)	10 (17%)
Grade III	66	11 (17%)	20 (30%)	25 (38%)	10 (15%)

Malignant tumors

As indicated in Table 2, 136/161 cases (84%) of malignant tumors contain immunoreactive GCDFP-like material. All lobular carcinomas were positive (13/13). The other histological types contained GCDFP-like material in various proportions and this variability was randomly distributed. In positive cells the cytoplasmic distribution was homogeneous. Invasive ductal carcinomas with apocrine-like cells were no more positive than the usual invasive ductal carcinomas. tumors. No significant difference appeared for the three separately studied components (tissue differentiation, nuclear polymorphism or mitosis). However, the incidence of tumors having an important proportion of positive cells (76–100%) was greater for the well-differentiated tumors (4/10: 40%) than for the poorly differentiated tumors (7/43: 16%) (Table 4).

GCDFP and estrogen and progesterone receptors. Of tumors that had positive estrogen and progesterone receptors 60/66 (90%) showed a

Table 4. *Relation between GCDFP* 15,000 *and tumor differentiation*

Degree of tissue differentiation*	Total No.	GCDFP 15-like immunoreactive cells 0%	1–25%	26–75%	76–100%
d1	10	1 (10%)	1 (10%)	4 (40%)	4 (40%)
d2	100	15 (15%)	21 (21%)	45 (45%)	19 (19%)
d3	43	6 (14%)	14 (33%)	16 (37%)	7 (16%)

*The degree of differentiation is from d1 to d3 according to Bloom's grading.

Table 5. *Relation between GCDFP* 15-*like immunoreactivity and estrogen and progesterone receptors*

RO	RP	Total No.	GCDFP 15-like immunoreactive cells 0%	1–25%	26–75%	76–100%
-	-	58	14 (24%)	13 (22.5%)	20 (34.5%)	11 (19%)
+	-	35	5 (14%)	10 (29%)	15 (43%)	5 (14%)
-	+	2	0 (0%)	1 (50%)	1 (50%)	0 (0%)
+	+	66	6 (9%)	15 (23%)	31 (47%)	14 (21%)

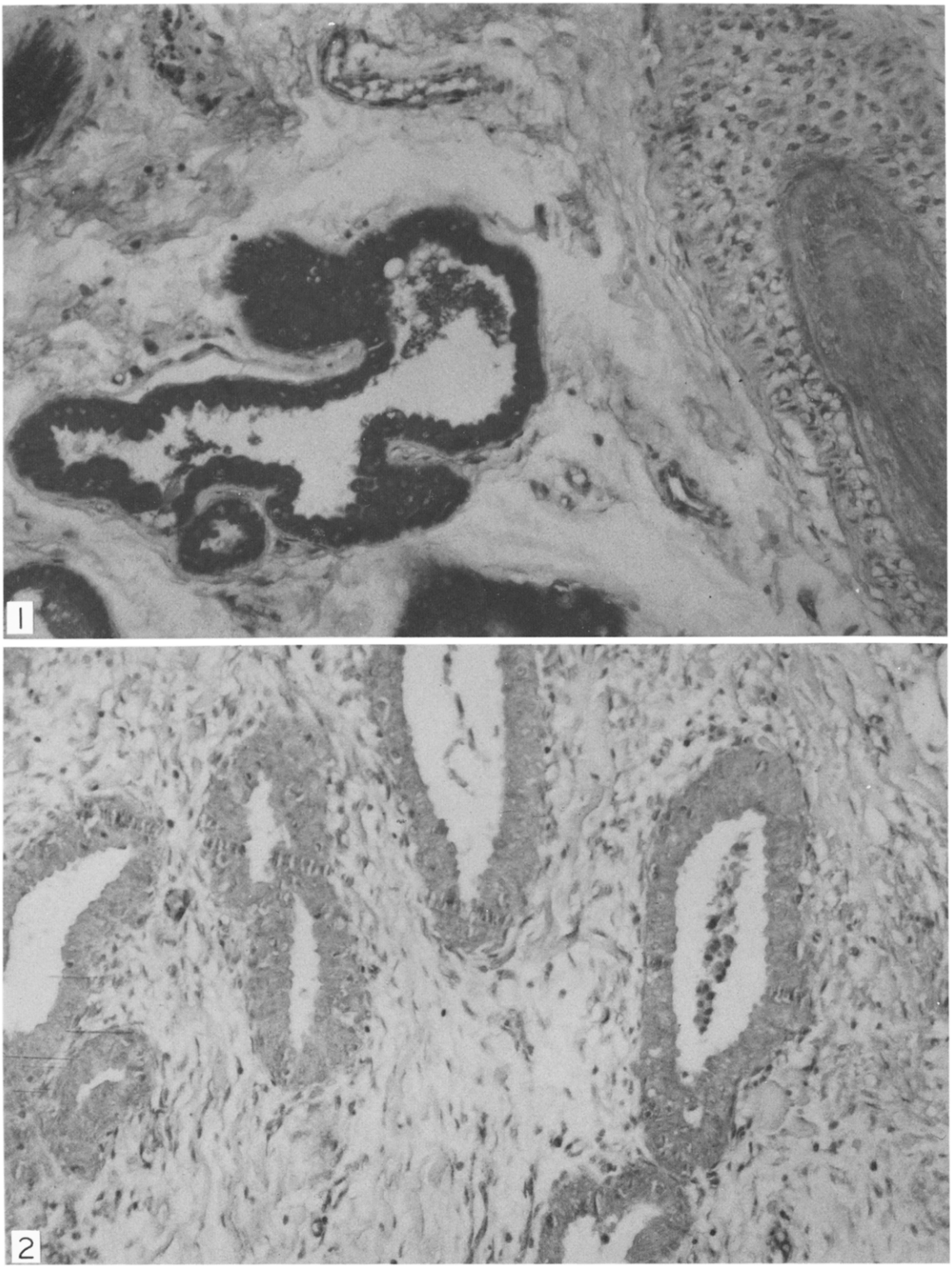

Fig. 1. *H.E.S.*, ×160. *Axillary apocrine glands with GCDFP+. Hair follicle is GCDFP-.*
Fig. 2. *H.E.S.*, ×160. *Adenofibroma: epithelial zones are GCDFP-, without idrosadenoid metaplasia.*

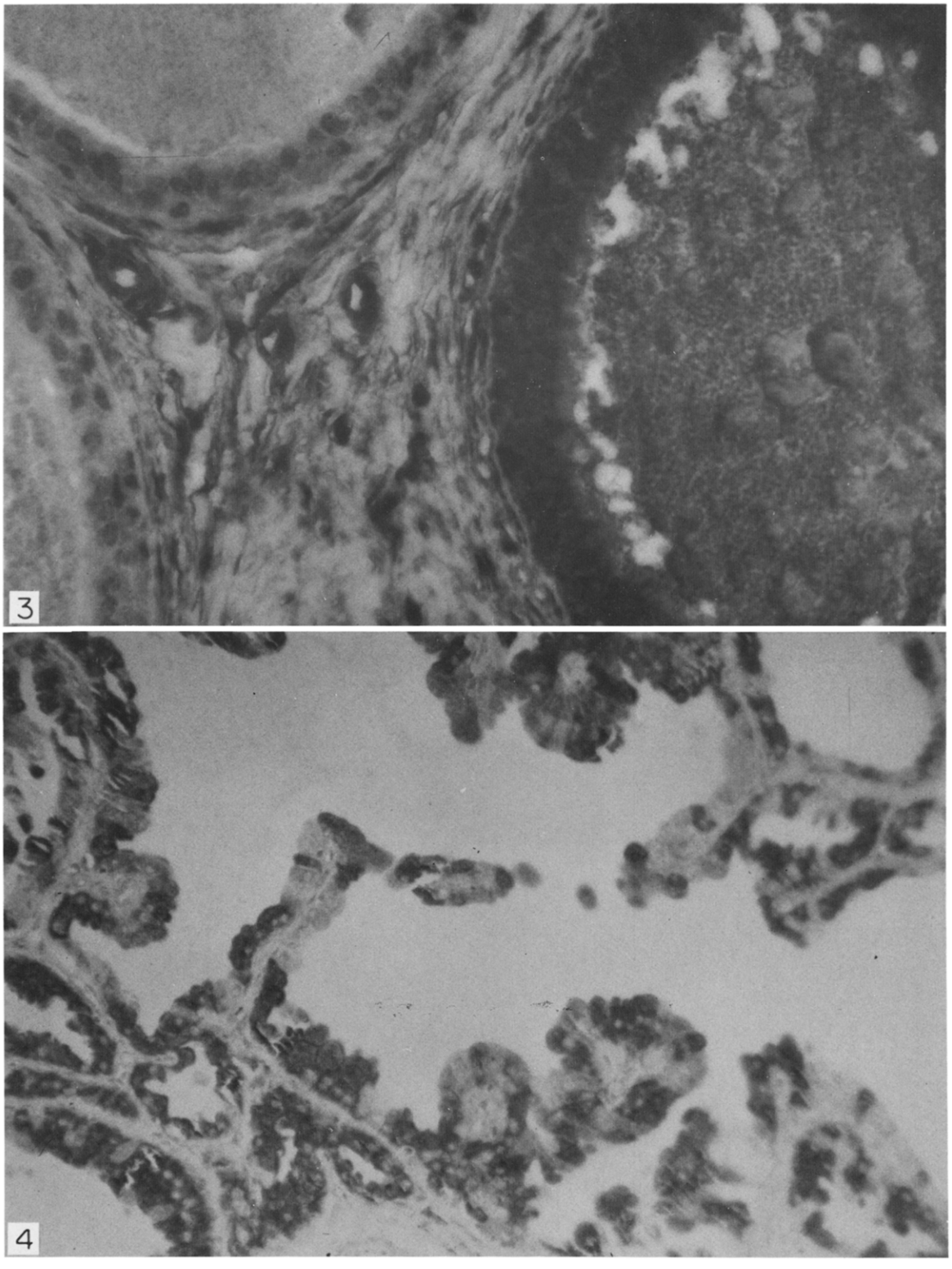

Fig. 3. H.E.S., ×400. Cyst with cylindric metaplasia GCDFP+ and cyst with flat epithelium GCDFP- (the high background is probably secondary to the leakage into the tissue of fluid from the adjacent cyst).
Fig. 4. H.E.S., ×160. Florid idrosadenoid metaplasia with GCDFP+ and GCDFP- cells.

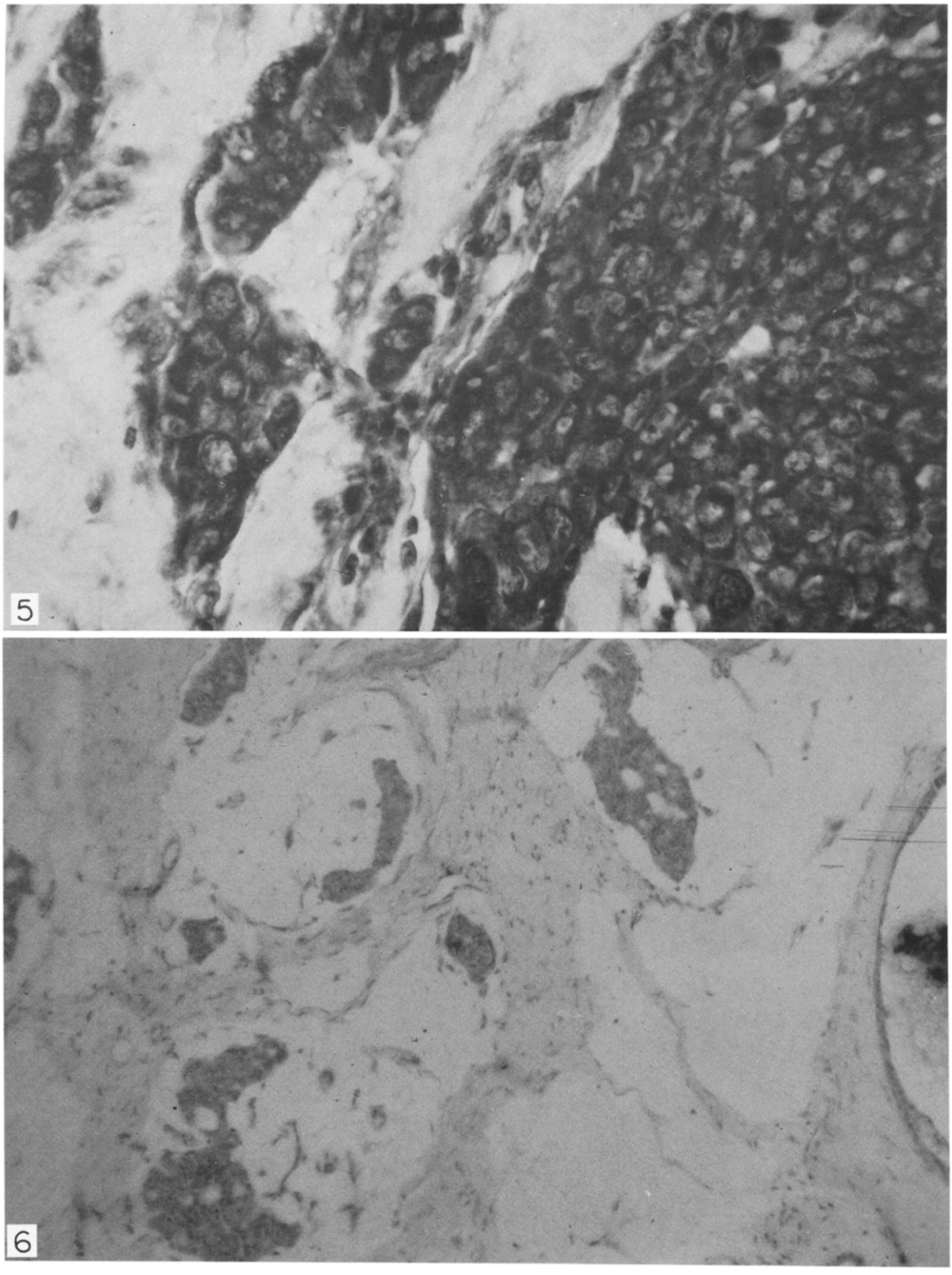

Fig. 5. *H.E.S., ×400. Invasive ductal carcinoma with idrosadenoid cells GCDFP+.*
Fig. 6. *H.E.S., ×100. Mucinous carcinoma GCDFP-.*

GCDFP-like immunoreactivity. When these same receptors were negative, 44/58 (75%) of the tumors showed positivity in GCDFP (Table 5).

However, a significant correlation existed between absolute values of the progesterone receptors and the percentage of positive GCDFP cells (Spearman's correlation coefficient = 0.1584, $P = 0.0135$).

DISCUSSION

The localization of an antigenic material using immunohistochemical methods depends primarily on the specificity of the immunological reaction which has been established by adequate controls: absence of reaction with non-immunized rabbit serum; with the anti-GCDFP antiserum from which the specific antibodies were extracted by chromatography; and by identical immunoreactivity between the total anti-GCDFP antiserum and the specific gammaglobulins eluted from the GCDFP affinity column.

Therefore, breast epithelial cells used in our study contained an immunological material that had an antigenic character that was similar to the protein 15,000 extracted from the breast cyst fluid and recognized by the rabbit serum immunized with this protein. The fixation of the samples by formol and their embedding in paraffin did not reduce this immunoreactivity, and did not destroy the antigenic determinants when the De Lellis *et al.* [21] deparaffining technique was used.

We can confirm the studies of Mazoujian *et al.* [10] that demonstrated the presence of a GCDFP-like immunoreactivity in normal apocrine gland cells (such as the apocrine axillary glands whose activity and secretion are androgen-dependent [6]. We can also confirm the absence of immunoreactivity in histologically normal mammary tissue taken from the periphery of benign or malignant mammary tissue.

In addition, our results showed a GCDFP-like immunoreactivity in epithelial cells when these cells underwent apocrine metaplasia. Thus the epithelium of cysts was more positive when it presented a florid metaplasia: 0/20 when the epithelium was flat, without secretory activity, and 55/55 when it was undergoing idrosadenoid metaplasia. However, in the absence of a morphological character of the idrosadenoid type, some glandular cells showed a positive immunoreactivity. Such was the case in 4/10 adenofibromas and in 8/10 atypical lobular epithelial hyperplasias considered by some authors as being a possible preliminary stage of evolution towards an *in-situ* lobular cancer [22].

In mammary carcinomas we studied the incidence of tumors showing a GCDFP-like immunoreactivity and the percentage of positive cells. The incidence of immunoreactivity of cancer cells was greater in our study (84%) than found by Mazoujian *et al.* [10] (46%). However, contrary to these authors, we did not find a greater incidence in apocrine-like carcinomas (9/11: 81%). This difference between the results of the two studies could be explained by the utilization of different anti-GCDFP antisera, or simply on the basis of the differences in subjective criteria in the frequency of apocrine features, or differences in fixation and staining technique.

Our histochemical study demonstrated a heterogeneity of the GCDFP identified within the same tumor, a larger proportion of immunoreactive cells in differentiated cancers (grade I) and, in addition, a correlation between the absolute values of progesterone receptors and the proportion of immunoreactive cells. These correlations parallel to those described by Sylva *et al.* [9] between the GCDFP 15,000 concentration in the cytosol of tumors with the tumor degree of differentiation and with the value of the progesterone receptors.

Thus the GCDFP was the first biochemical marker of apocrine-type glandular secretion. It was found in the normal apocrine glandular cells. In the breast, the normal epithelium did not show an apocrine-type of morphology nor of biochemical secretion. In apocrine metaplasia, particularly in the cystic dystrophy, the mammary epithelial cells took on not only the morphological aspect but also the functional character of an apocrine gland. This confirmed the similarity of these two epithelia which have the same embryological origin.

Eighty-four percent of breast adenocarcinomas secreted GCDFP without presenting an idrosadenoid-type morphology; the undifferentiated tumors were GCDFP-negative. There appeared to be a common biochemical relation between the following different tissues: normal apocrine gland, which is of the same embryological origin as the mammary gland; and breast cystic dystrophy, which increased the risk of neoplastic transformation [2]. Since androgens stimulate normal apocrine secretion [6] and are increased in cystic dystrophy [23] and breast cancer [4, 5], it is possible to postulate that they played a role in the genesis of these different pathologies.

At this time, the hormonodependence of a breast tumor is estimated by the presence of estrogen and progesterone receptors. The prognosis of these tumors appeared, in part, to be related to the presence of progesterone receptors [24–26]. The dosage of these receptors was obtained using a tumor homogenate. The number of receptors per cell was small and the

sensitivity of the method was limited by the number of positive cells. In addition, this method did not take into account the heterogeneity of tumor cells. The intracellular morphological identification of the GCDFP, due to its greater sensitivity and its correlation with the progesterone receptors, allowed a more precise evaluation of the functional state and of the hormonal dependence of breast cells by underlining the variability of the tumor cell population.

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