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Immunohistochemical Detection of Alphalactalbumin in Breast Lesions*

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Abstract—*Alphalactalbumin was investigated in breast cells using the immunoperoxidase technique with a specific anti-alphalactalbumin serum. In 50 benign tumors alphalactalbumin immunoreactivity was demonstrated in the epithelium of fibroadenomas and in fibrocystic disease showing apocrine metaplasia. Alphalactalbumin immunoreactivity, investigated in 124 breast cancers, was present in all cells of lobular tumors but in only 76% of other cancers where the pattern was heterogeneous. Perineoplastic mammary tissue of normal appearance was not labeled with the anti-alphalactalbumin antiserum except where lobular hyperplasia was present. The presence of alphalactalbumin-type immunoreactivity was not correlated with the histopathological staging of Bloom and Richardson, nor with menopausal status. The incidence of positivity was 90% when estrogen and progesterone receptors were present in the tumor. The demonstration of alphalactalbumin using immunoperoxidase could be used to complement the histological classification of breast cancers.*

INTRODUCTION

HUMAN alphalactalbumin is a major protein of human milk required for the conversion of glucose to lactose [1]. It is synthesised by the epithelial cells of the breast under the influence of a specific hormonal milieu during pregnancy and lactation and also during some normal menstrual cycles [2, 3].

Study of both benign and malignant breast disease is often based on tissue morphology. Alphalactalbumin, like casein, represents a final synthetic product of the breast. Its presence indicates that the sequence of receptor interaction–protein synthesis is intact.

Alphalactalbumin was studied by immunochemistry in various benign and malignant lesions of the breast with the aim of establishing the possible correlation between its presence or absence, histological type of tumor, the presence or absence of estrogen and progesterone receptors, and the prognostic grading of Bloom and Richardson with its three components: tissue differentiation, nuclear polymorphism and mitosis.

MATERIALS AND METHODS

Tissue samples

One hundred and twenty-four malignant tumors were removed by partial or total mastectomy in 45 premenopausal and 79 postmenopausal women. The 1981 WHO histological classification of breast cancer [4] was used together with the prognostic grading system of Bloom and Richardson [5]. Certain tumors such as *in situ* carcinoma, mucinous carcinoma and hemangiosarcomas were not graded. The distribution of the 124 tumors is described in Table 1. The surrounding histologically normal breast tissue was also examined.

Fifty benign lesions were removed by partial mastectomy in 35 premenopausal and 15 postmenopausal women: 15 fibroadenomas, 3 intracanalicular papillomas, 2 tubular adenomas and 30 specimens of fibrocystic disease.

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Table 1. Tumors studied and heterogeneity of alphalactalbumin at the cellular level

Malignant tumors: histological type (WHO, 1981)	Total No.	Percentage of cells positive for alphalactalbumin 0%	1–25%	26–70%	71–100%
Invasive ductal carcinoma	88	22	13	48	5
Invasive lobular carcinoma	9	0	0	0	9
Comedocarcinoma	9	6	1	2	0
Tubular carcinoma	6	0	1	3	2
Medullary carcinoma	4	1	1	2	0
Mucinous carcinoma	4	1	1	2	0
Papillary carcinoma	2	0	0	2	0
In situ ductal carcinoma	1	0	1	0	0
Hemangiosarcoma	1	1	0	0	0
Total No.	124	31	18	59	16

Anti-alphalactalbumin antiserum

Human lactalbumin was purified from human milk using the method of Schultz and Ebner [6]. The purity of the protein was established by its elution in a single peak after chromatography on Sephadex G100, by its migration as a single band on 1% SDS polyacrylamide gel electrophoresis and by a single precipitation line in immunoelectrophoresis in the alphaglobulin zone in the presence of an anti-milk protein antiserum (Nordic Immunological Laboratories, Tilburg, Netherlands, RAHu/TM) and in the presence of an anti-human lactalbumin antiserum (Nordic Immunological Laboratories, Tilburg, Netherlands, RAHu,Al.alb). This preparation was used to prepare the affinity chromatography and to reach antiserum. The antiserum was raised in rabbits using the technique of Vaitukaitis *et al.* [7]. The antiserum specificity was established by radioimmunoassay and by immunoelectrophoresis. The antiserum was directed specifically against human alphalactalbumin and showed no cross-reaction in the two methods with other milk proteins (the caseins, lactoferrin and the secretory fragment of IgA), with the proteins from the breast cyst fluid, or with serum proteins, in particular human albumin and IgA; there was no cross-reaction with tumor markers (HCG and its alpha and beta subunits, carcinoembryomic antigen) or with mammotrophic hormones such as prolactin. In immunoelectrophoresis, the rabbit antiserum antilactalbumin reacts with the purified lactalbumin by a single precipiting line and does not react with human serum proteins; the anti-human serum protein antiserum does not react with human lactalbumin.

Control serum was prepared by filtering 1 ml of specific antiserum on a column (2.5 cm × 2 cm) of Sepharose CNBr 4B (Pharmacia Uppsala, Sweden) to which 5 mg of purified human alphalactalbumin had been bound [8]. The antiserum so treated no longer bound labeled human alphalactalbumin in RIA.

The gamma globulins specifically retained by the purified alphalactalbumin bound to the immunoadsorbant were eluted using Sorensen phosphate butter (Na_2HPO_4 0.5 mol/l, KH_2PO_4 0.05 mol/l pH 7.4) 10 mM containing 3 M sulphocyanate; the solution was dialyzed against distilled water and lyophilized.

Immunoperoxidase technique

The specimens were initially fixed in a solution of 0.15 mol/l NaCl containing 3% formaldehyde for 48 hr at room temperature. After embedding in paraffin, the tissues were cut into 4-μm sections and stained with hematein-eosin-safran for histological control. The paraffin was removed from the sections by incubation in 10 mM PBS (pH 7.2) for 30 min at the melting temperature of paraffin, then rinsed in xylene for 10 min at room temperature. Endogenous peroxidase was blocked using a solution of methanol containing 0.3% H_2O_2 for 30 min at room temperature, then subsequently with normal sheep serum, inactivated by heating at 60°C for 30 min and diluted 1:5.

The rabbit anti-alphalactalbumin antiserum was applied in a moist chamber for 15 min at 1:100 dilution, as based on previous experiments. The sections were rinsed three times in PBS, treated with sheep anti-rabbit serum proteins (Institut Pasteur), diluted 1:10, rinsed three more times with PBS and treated with anti-rabbit gamma globulin peroxidase antiperoxidase (Dako B 157) diluted 1:50. The bound peroxidase was demonstrated by incubating the sections in a 50-mM Tris buffer (pH 7.2) containing 0.01% H_2O_2 and 0.05% diaminobenzidine (DAB) tetrahydrochloride (Sigma No. D 563). The sections were washed three times in PBS, dried with ethanol and rinsed in xylene.

To facilitate examination, counter-staining with Harris hematoxylin was used [9]. At each stage in the reaction, control sections were made by incubating tissue slices with the following

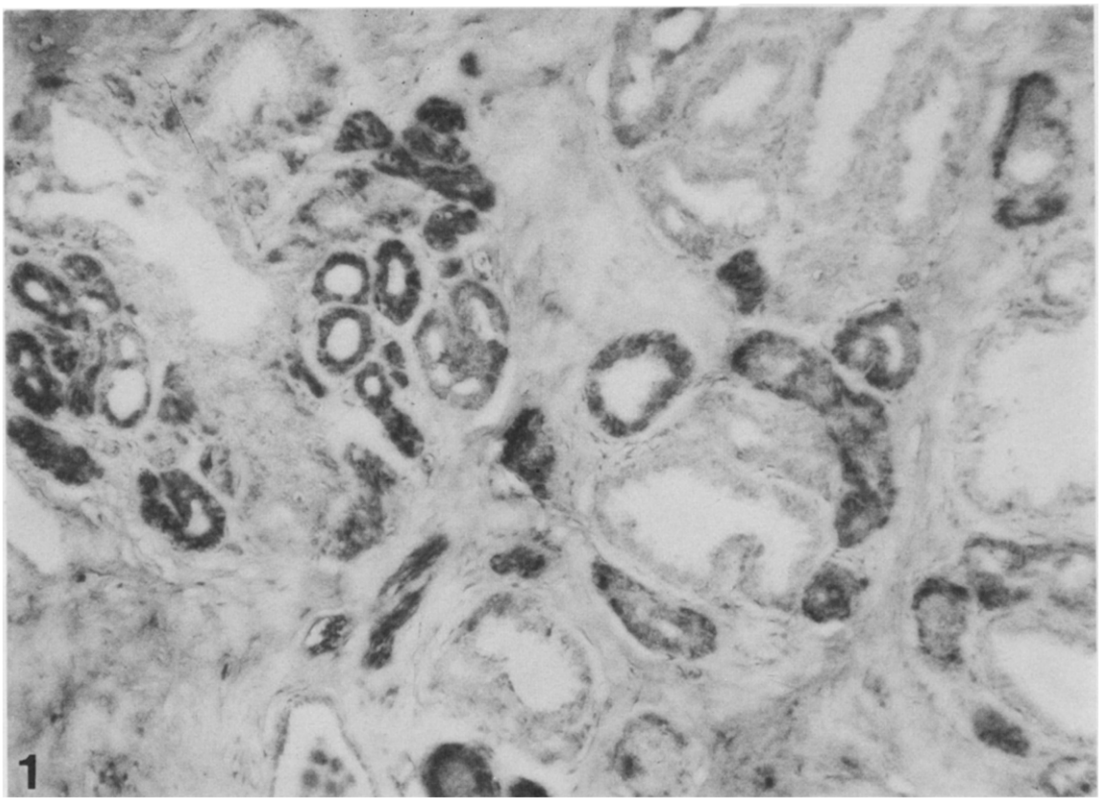

Fig. 1. Adenosis: *the intra and extra-lobular ducts are alphalactalbumin-negative, but the ductules are alphalactalbumin-positive* (×160).

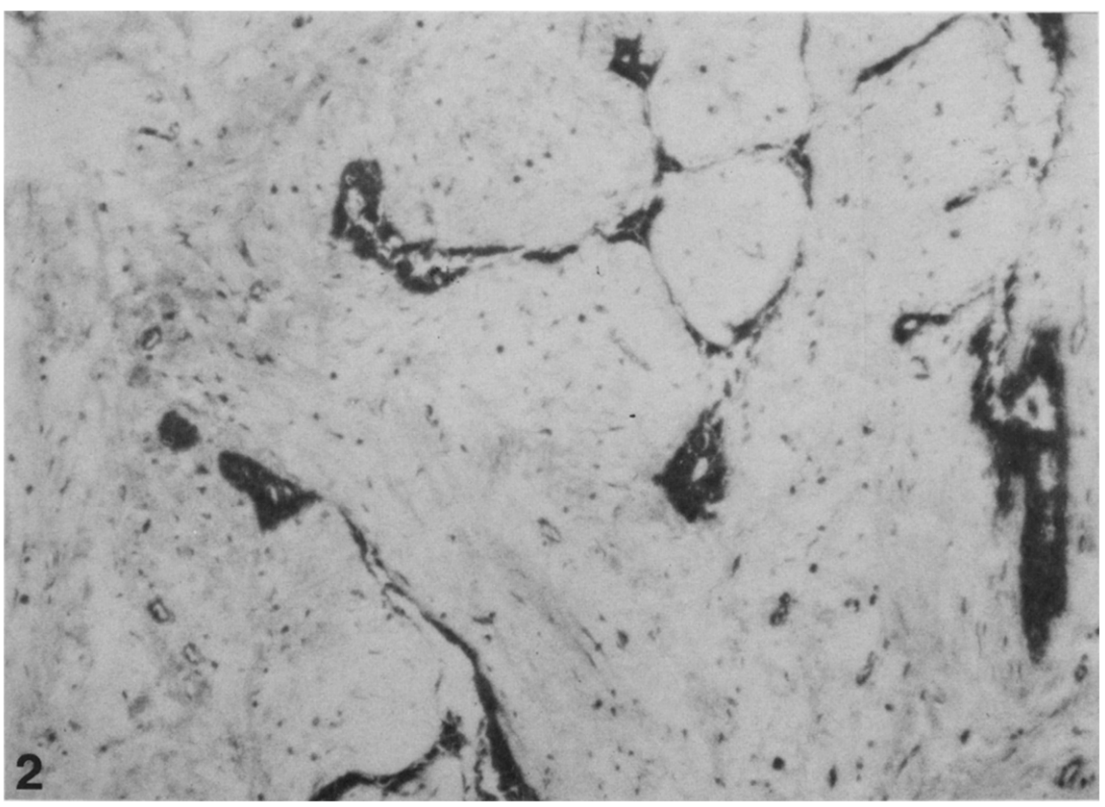

Fig. 2. Fibroadenoma: *all the epithelial elements are alphalactalbumin-positive, but the connective tissue is alphalactalbumin-negative* (×100).

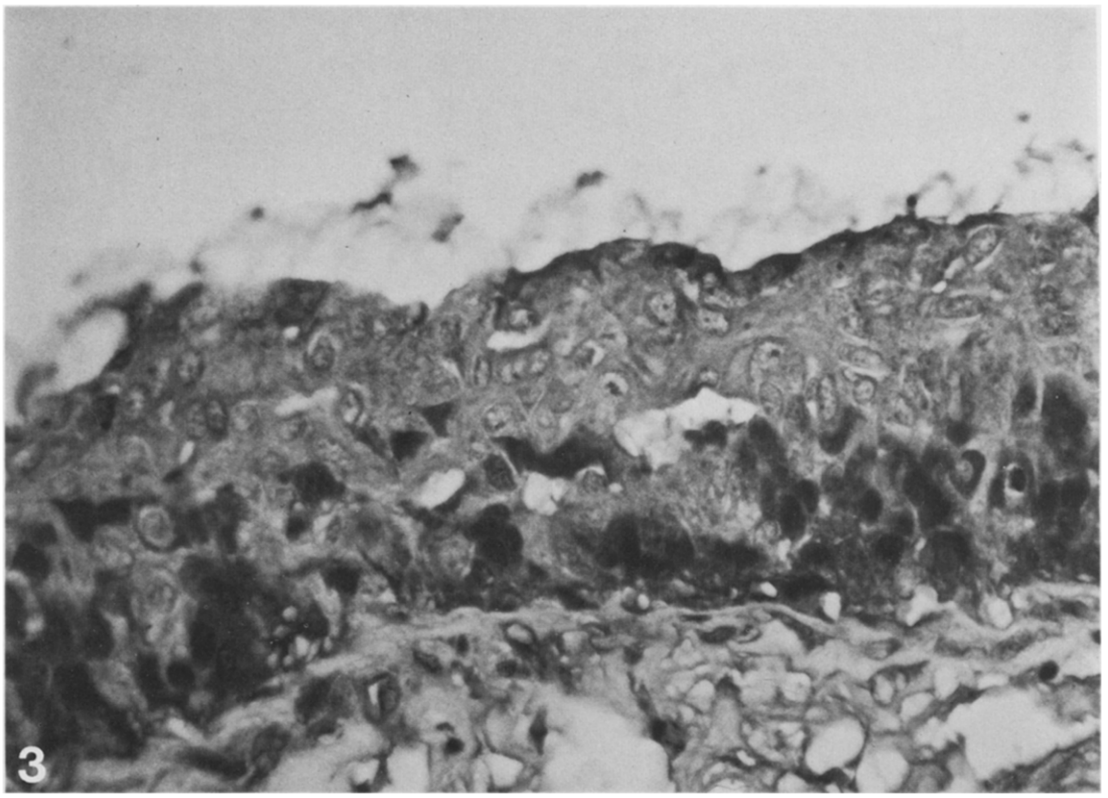

Fig. 3. Atypical ductal hyperplasia: *only some basal epithelial cells are alphalactalbumin-positive* (×400).

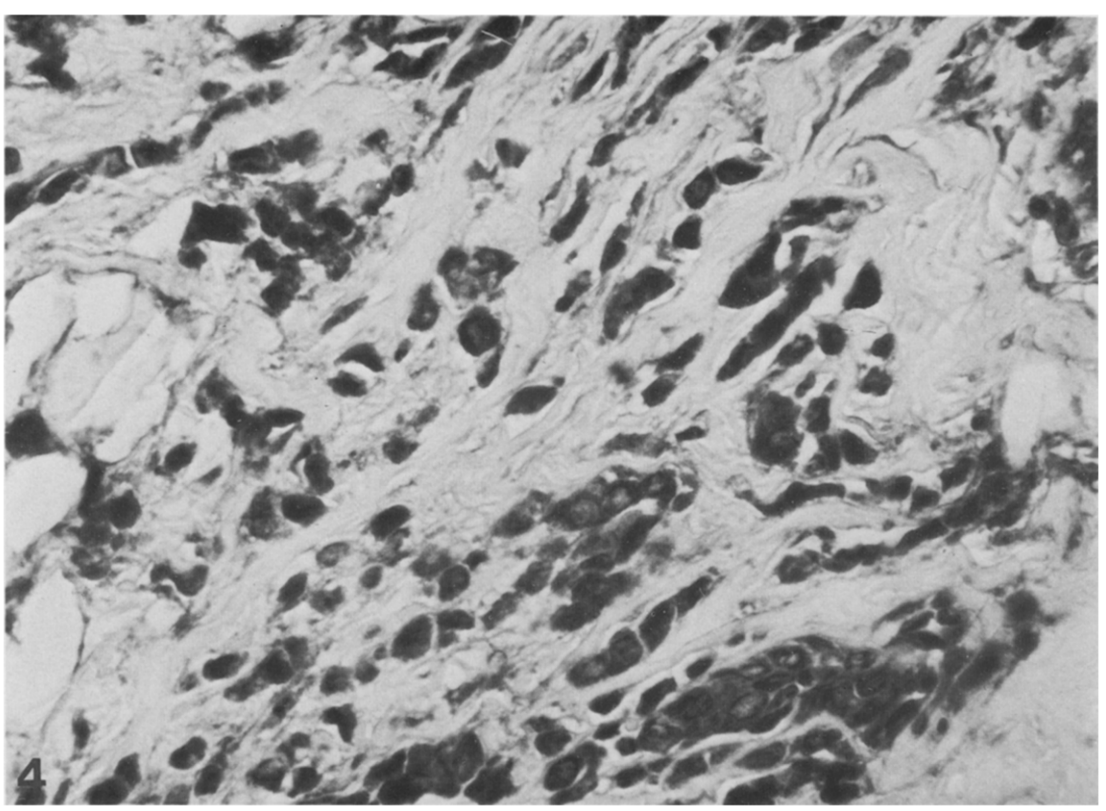

Fig. 4. Invasive lobular carcinoma: *each carcinomatous cell is alphalactalbumin-positive* (×400).

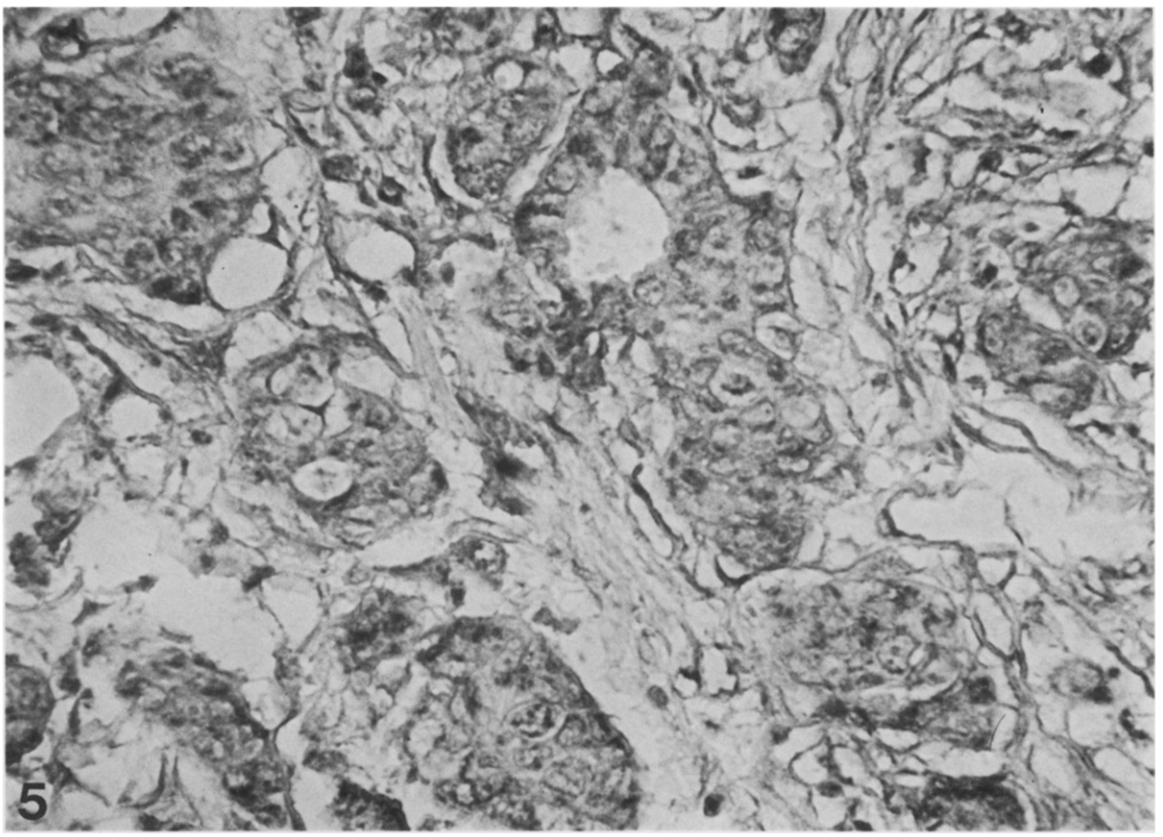

Fig. 5. Invasive ductal carcinoma: *only some cells are alphalactalbumin-positive.*

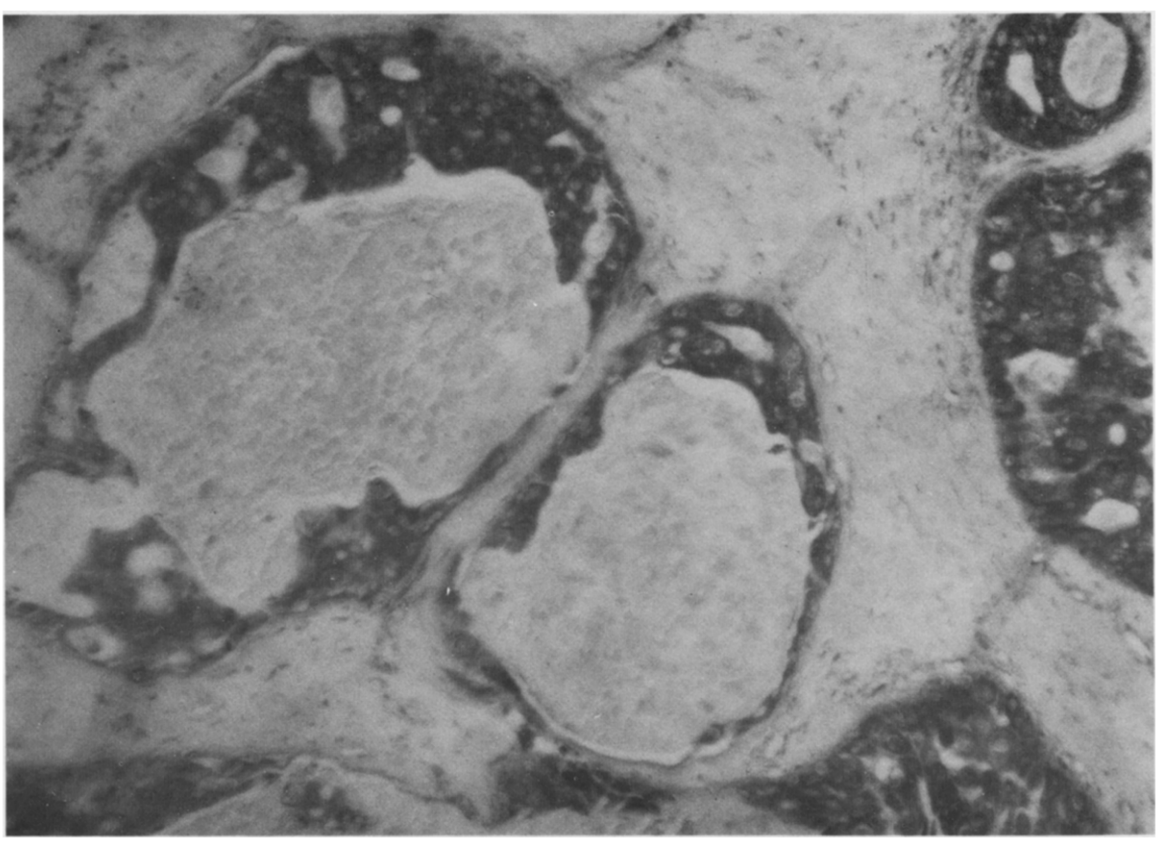

Fig. 6. Intra-epithelial carcinoma with roman bridges: *the epithelial cells are alphalactalbumin-positive but the secretory polarity has disappeared* (×160).

preparations: (a) positive control: replacement of the anti-alphalactalbumin antiserum by the specific immunoglobulins eluted by affinity chromatography, at a dilution of 1:100; and (b) negative controls: replacing the specific antiserum with human anti-alphalactalbumin antiserum from which the specific antibodies had been removed by immunoabsorption; PBS buffer or normal rabbit serum; or a solution of 50 mM Tris-HCl, pH 7.2, 0.01% H_2O_2, 0.005% DAB for 10 min at room temperature to control for the presence of endogenous peroxidase.

The alphalactalbumin content of the malignant tumors was expressed as the percentage of positive cells in relation to the total number of cells examined on the different microscope fields from a same section at an enlargement of ×250.

The percentage of positive cells was expressed as the mean value of the different percentage found in five of the most representative sections examinated for each tumor with a coefficient of variation from one section to the others of less than 5%. Tumors were classified into four groups: negative (0%) and positive (1–25%; 26–70%; and 71–100%).

Hormone receptors

Cytosol preparations and the assay of estrogen and progesterone receptors were carried out using the method described by the EORTC group [10]. The detection limit was set at 10 fmol/mg of cytosol protein, as measured by the technique of Bradford [11].

RESULTS

Normal or breast benign lesion tissue

Normal tissue. The histologically normal cells of the breast surrounding the tumors were not labeled with alphalactalbumin. However, the closer to the tumor one approached, whether the tumor was intra-epithelial or invasive, the more positive cells of normal appearance were encountered, particularly in the presence of lobular hyperplasia (Fig. 1).

Breast benign lesion tissue (*Table* 2). In fibroadenomas all the epithelial cells were positive in a homogeneous pattern which differentiated them from the negative peripheral connective tissue (Fig. 2). In the fibrocystic diseases there was marked heterogeneity from one area to another. Lobular epithelial hyperplasia were all positive and generally showed a homogeneous pattern (10 out of 15), whilst 21 of 30 with duct hyperplasia showed heterogeneous staining (Fig. 3). The secretory polarity of positive cells always appeared to be preserved in the benign areas, in contrast to carcinomas *in situ*, where the secretory polarity disappeared. The epithelium in cysts showing apocrine metaplasia were frequently positive, whilst when the epithelium was flat the cells were negative.

Malignant tumors

As shown in Table 1 31 of the 124 breast tumors contained no alphalactalbumin immunoreactivity. Duct, tubular, medullary and intra-epithelial duct cancers contained varying proportions of alphalactalbumin. The cytoplasmic distribution was homogeneous in lobular carcinomas, in which all cells were positive (Fig. 4). In the other histological types, such as invasive duct cancers (Fig. 5), distribution of positive cells was heterogeneous, and in the positive cells the cytoplasmic distribution was perinuclear (Fig. 6). In undifferentiated cancers, such as lobular cancers, the presence of alphalactalbumin in the cell allowed its recognition in the dense collagen from fibroblasts. The cells of the comedo carcinomas were frequently negative (0%). All of the cells of the hemangiosarcoma, which is a connective tissue tumor, were negative (0%).

Menopausal status did not influence the presence of alphalactalbumin in breast cancer cells.

Lactalbumin and estrogen and progesterone receptors

Forty-five out of 50 (90%) tumors containing both estrogen and progesterone receptors showed alphalactalbumin immunoreactivity. When

Table 2. *Benign breast lesions and alphalactalbumin staining*

Histological type	Total No.	Alphalactalbumin		
		Heterogeneous	Homogeneous	Negative
Fibroadenoma	15	0	15	0
Intraductal papilloma	3	3	0	0
Tubular ademona	2	2	0	0
Fibrocystic disease:	30			
cyst with flat epithelium	28	0	0	28
cyst with apocrine metaplasia	30	20	10	0
ductal epithelial hyperplasia	30	21	0	9
lobular epithelial hyperplasia	15	5	10	0
sclerosing adenosis	10	2	0	8

receptors were undetectable, 27 out of 41 (65%) tumors still showed alphalactalbumin positivity (Table 3).

Lactalbumin and histological grade

No correlation was found between the presence of lactalbumin in a tumor and its grading according to Scarff and Bloom: 16 out of 20 (80%) grade 1, 42 out of 50 (84%) grade 2 and 32 out of 48 (66%) grade 3 tumors showed alphalactalbumin positivity. No significant difference could be found when tissue differentiation, nuclear polymorphism and mitosis were studied separately (Table 4).

DISCUSSION

The localization of a protein using the immunoperoxidase technique principally depends on the quality and specificity of the antiserum used.

The validity of the immunoperoxidase technique used here was established using adequate controls; thus non-immune rabbit serum gave no reaction and there was no interference from endogenous peroxidases. Further, the reaction of the antiserum with the cellular antigen was completely inhibited by extraction of the specific anti-human anti-alphalactalbumin antibody from the antiserum by passage over an alphalactalbumin affinity column. Specific anti-alphalactalbumin immunoglobulins purified similarly showed the same type of cell binding as the total specific antiserum. There were therefore antigenic similarities between the cellular material binding the anti-alphalactalbumin antiserum and human alphalactalbumin; this allows us to regard the material as immunoreactive lactalbumin-like material (ILLM).

Fixation of sections in formalin and their embedding in paraffin did not lead to extraction of the immunoreactive material and did not destroy the antigenic determinants when the technique for removing paraffin of De Lellis *et al.* [12] was used as described by Walker [13], Clayton *et al.* [14] and Bailey *et al.* [15]. Retrospective studies are therefore possible on sections prepared in this way. Absent or weak alphalactalbumin immunoreactivity in the normal breast glandular tissue surrounding tumors confirmed the observations of Walker [13], Clayton *et al.* [14] and Bailey *et al.* [15]. Our results showed marked positivity in cases of lobular hyperplasia, which appears to increase the risk of cancer development [16].

Positive staining of the epithelial cells of all fibroadenomas was in agreement with the observations of Clayton *et al.* [14] and was greater than the degree of positivity observed by Bahu *et al.* [17] (50%) and Bailey *et al.* [15] (9%). The cells surrounding cysts were positive only in the presence of idrosadenoid metaplasia. In the fibrocystic dysplasias, ILLM was found mainly in cases of lobular epithelial hyperplasia. This condition is known to increase the risk of breast cancer four-fold [16].

Our study confirms the presence of human alphalactalbumin in the epithelial cells of breast cancers, as previously shown in culture where *de novo* synthesis has been established [18, 19], and in breast cancer cytosols [20]. It is in agreement with the immunocytochemical studies of Bahu *et*

Table 3. Demonstration of alphalactalbumin as a function of the presence or absence of oestrogen and progesterone receptors

Oestrogen receptors + >10 fm/mg protein	Progesterone receptors	Total No.	Alphalactalbumin immunoperoxidase-positive	Alphalactalbumin immunoperoxidase-negative
+	+	50	45 (90%)	5
-	-	41	27 (65%)	14
+	-	32	21 (65%)	11
-	+	1	-	1
Total No.		124	93 (73%)	31

Table 4. Demonstration of alphalactalbumin as a function of the histopathological grading of Bloom

Grade	Total No.	Percentage of lactalbumin-positive cells 0%	1-25%	26-70%	71-100%
I	20	4	3	10	3
II	50	8	8	25	9
III	48	16	7	22	3

al. [17], Clayton *et al.* [14] and Walker [13]. In contrast, our results do not agree with the studies of Bailey *et al.* [15], who were unable to detect ILLM in 44 breast cancers whilst they were able to show this milk protein to be present in breast cells of pregnant and lactating women. The employment of different antisera and the particular treatment of the antiserum used by Bailey *et al.* [15] could explain the difference in results.

In our series all lobular carcinomas were positive and the labelling of cells by the anti-alphalactalbumin antiserum was homogeneous, as all cells of these cancers showed ILLM. The degree of positivity of these lobular cancers was greater than that seen by Clayton *et al.* [14] (42%), Bahu *et al.* [17] (8%) and Walker *et al.* [13] (16%), but were similar to the degree of positivity observed for casein [21]. These lobular cancers show the highest frequency of estrogen receptors as compared with other breast neoplasms [22].

Furthermore, this study confirmed the raised frequency (75%) of positive cells in duct cancers found by Clayton *et al.* [14] (77%), Bahu *et al.* [17] (63%) and was higher than that observed by Walker [13] (49%). The distribution of the positive cells in contrast to that in lobular cancer was heterogeneous; within the cells, the material was perinuclear.

No correlation could be established between the presence of ILLM in a tumor and the histopathological grading of Bloom nor any one of its three components. Similar observations were made by Clayton *et al.* [14], Walker [13] and Bahu *et al.* [17].

No correlation could be established between the presence or absence of oestrogen receptors and alphalactalbumin immunoreactivity as previously shown by Bahu *et al.* [17]. Nevertheless, when both progesterone and estrogen receptors were present together, 90% of tumors contained lactalbumin. This observation seems to indicate the preservation of the sequence of receptor interaction and synthesis of milk proteins as seen under physiological conditions. The presence of alphalactalbumin immunoreactivity (65%) in the cells or tumors containing neither estrogen nor progesterone receptors could be due to several causes. Immunocytochemistry demonstrates immunoreactivity within the cell, whereas the receptors are studied in a total tumor extract. The techniques used could therefore detect heterogeneous labeling of cells with anti-alphalactalbumin, whereas this technique does not allow receptor detection. An error of omission could therefore appear in the receptor assays if only a small proportion of the cells contained receptors. Furthermore, lactalbumin synthesis primarily depends on prolactin [19], the receptors of which we have not studied for.

A functional classification of tumors could therefore complement the histopathological classification with which there was no correlation. Alphalactalbumin synthesis by a tumor is in fact direct evidence of adequate hormonal synergism acting on a breast cell still showing integrity of the receptor/protein synthesis sequence. The presence of alphalactalbumin could therefore be considered as a marker of hormone dependency as it is present particularly in the most hormone-dependent cancers such as lobular tumors and is particularly associated with the simultaneous presence of estrogen and progesterone receptors. Further, it could establish the proportion of hormone-dependent cells within the same tumor which cannot be established by overall determination of estrogen and progesterone receptors.

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