Virchows Arch. A Path. Anat. and Histol. 365, 15—21 (1975)

Immunofluorescence Detection of Casein in Human Mammary Dysplastic and Neoplastic Tissues

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Received July 26, 1974

Summary. To detect and localize casein in human mammary dysplastic and neoplastic tissues, an indirect immunofluorescence method has been devised. Anti-casein antibodies have been obtained from rabbits immunized with casein isolated from human milk. Cryostat sections post-fixed in alcohol and paraffin sections from routinely formalin-fixed tissues proved suitable.

The immuno-fluorescence method revealed the presence of casein in epithelial cells in mammary dysplasia and in some, but not all, cases of carcinoma of the breast. Well differentiated (Grade I) carcinomas were shown to contain casein, mainly localized at the inner border of the epithelial cells; the milk protein was present also in cells metastatic in lymph nodes.

In Paget's disease of the nipple, casein could be detected in neoplastic cells in the ducts and also in cells identifiable with the typical intra-epidermal Paget cells.

This finding is evidence of a functional differentiation of Paget cells along lines proper to the mammary epithelium.

Introduction

The detection and cellular localization of milk proteins has been investigated in the rat (Turkington, 1969; Young and Nelstrop, 1970) and cow (Kihm, 1973) lactating mammary glands by means of immunofluorescence techniques: casein as well as other milk proteins could be detected. The former, presenting a marked antigenic resistance, could be localized also in formalin-fixed, paraffin embedded tissues.

Turkington (1970) furthermore applied immunofluorescence techniques to study the presence and localization of casein in experimental rat mammary tumours, to obtain information on the production of this specific endogenous protein, and therefore on the functional properties and homogeneity of tumour cell lines.

In the present work an immunofluorescence method for the localization of human casein has been devised and applied to the study of cases of mammary dysplasia and carcinoma. The detection of casein, a protein specific to the mammary epithelial cells, could in fact provide information on the secretory activity and the degree of differentiation of epithelial cells in human dysplastic and neoplastic lesions of the breast. In addition, since milk protein secretion is known to be under hormonal control, the presence of such proteins might possibly be an index of the status of hormonal balance and control in the tumours.

Material and Methods

Casein was separated from milk, kindly supplied by lactating women, either by acidification at pH 4.6 (McKenzie, 1967) or by centrifugation at 35000 × *g* for 30 min., according to the method extensively reported by Young and Nelstrop (1970). The protein content of the final solution of casein in distilled water was brought to 1 mg/ml [measured by spectrofluorometry against a known solution of bovine B casein (Nestlé)]. Gel electrophoresis confirmed the presence of protein bands corresponding (Groves and Kiddy, 1968) to casein in different states of aggregation.

Anti-casein antibodies were obtained from rabbits immunized by several subcutaneous injections of casein, at intervals of 2 weeks. The first three injections of antigen plus Freund's complete adjuvant (Difco) were followed by five booster injections of antigen alone, 2 mg of casein being given on each occasion. The animals were bled 7 days after the last injection.

The presence of anti-casein antibodies was confirmed by a positive "ring test" and by the presence of a single precipitation line in Ouchterlony plates.

Mammary gland tissues were collected from surgical biopsies. Cryostat sections from fresh-frozen tissues were postfixed in 95% ethyl alcohol for 20 min. at room temperature. Paraffin sections from formalin-fixed, routinely processed tissue blocks were de-paraffinized. All the sections were finally brought to phosphate-buffered saline for immunofluorescence tests. Serial sections were stained with haematoxylin and eosin, and the following diagnosis made: lactating (2 cases), "normal", i.e. without evidence of disease (2 cases), pre-puberal (1 case), atrophic (2 cases) mammary gland, benign mammary dysplasia (16 cases) and carcinoma (25 cases). Of the latter, 1 case was a medullary carcinoma, 2 were mucous carcinomas, 1 was a papillary carcinoma, 3 were intraductal carcinomas with occasional cribriform patterns, and 3 were Paget's disease of the breast. Of the left 15 cases of infiltrating carcinoma, 7 showed a high (Grade I), 3 an intermediate (Grade II) and 5 a low (Grade III) degree of histological differentiation. In 2 cases of Grade I carcinoma, lymphnode metastases were also investigated.

The histological typing of breast tumours as proposed by the World Health Organization (Scarff and Torloni, 1968) has been adopted.

Two cases of adeno-carcinomas of the rectum were also examined, to test the specificity of the results. Some of the above cases were examined both on cryostat and paraffin sections, others on paraffin sections only.

An *immuno-fluorescence* indirect "sandwich" method has been employed. The sections were first treated with the anti-casein immune serum diluted 1:10 in saline, washed, and subsequently treated with fluorescein- or rhodamine-conjugated goat anti-rabbit globulin serum (from Behringwerke, Marburg, or Biochemical Check-up, Milan). Occasionally, treatment with normal goat serum preceded the immunofluorescence staining sequence.

Controls were made by omitting the first step or substituting the anti-casein serum with normal rabbit serum undiluted or diluted 1:10. Immune serum absorbed with casein solution (1 mg/ml) to get a final dilution 1:10 was also employed.

The immuno-fluorescence preparations were examined with a Leitz Ortholux fluorescence microscope equipped with a Ploem illumination system, or with a Leitz Dialux microscope equipped with an iodine lamp, for fluorescence microscopy. The films used were Ilford Pan F and Kodak High Speed Ektachrome daylight type.

Results

The indirect immunofluorescence method for the detection of casein was tested on sections of human lactating mammary glands. When anti-casein immune serum was employed, fluorescent material was observed lining the surface or filling the cavities of dilated alveoli. Casein could also be detected in intra-epithelial position, mainly along the acinar border. Formalin-fixed, paraffin embedded tissues proved suitable for immuno-fluorescence; controls were negative.

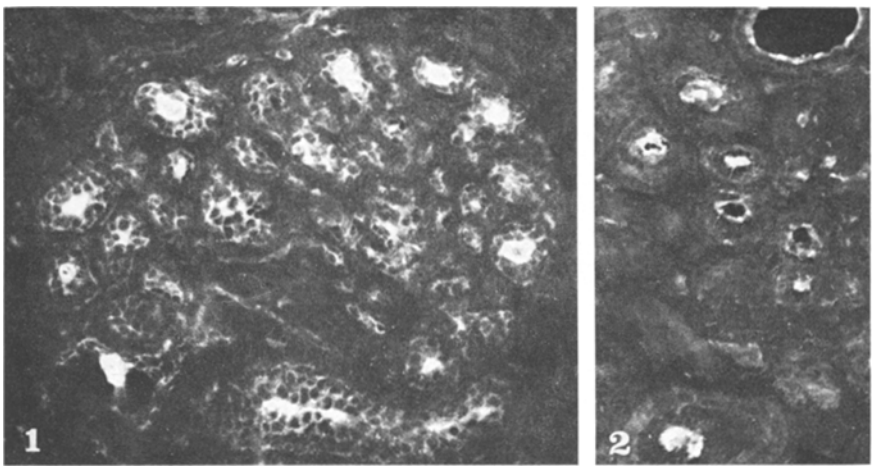
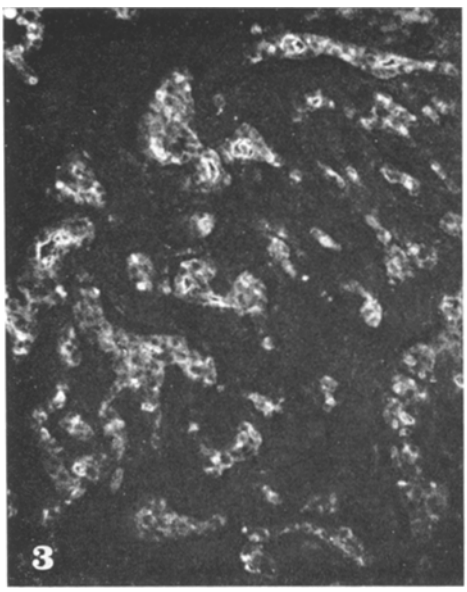


Fig. 1. Benign mammary displasia. Cryostat section post-fixed in alcohol. Immuno-fluorescence method for human casein: the protein is present in the lumen and in the epithelial cells in alveoli and ductules of a lobule. (× 150)

Fig. 2. Mammary dysplasia. Cryostat section; post-fixed. Casein is revealed along the inner border of some ductules and one small cyst (top). (× 100)

The pre-puberal and the atrophic mammary gland gave negative results, while in "normal" breast occasional presence of fluorescent material could be seen along the inner border of epithelial cells of some alveoli and ductules. The presence of casein could be detected in all cases of mammary dysplasia: minute amounts of fluorescent material could be seen in most ductules and acini (Figs. 1 and 2). Both cryostat and paraffin sections proved suitable, but the results were more definite and the localization more precise in the latter. Casein was localized mainly at the acinar border of the epithelial cells; homogeneous material occasionally seen filling the lumen of ductules was also positive. Fluorescent material could in addition be observed lining the inner surface of some small cysts and ducts and also of some cysts with apocrine metaplasia of the epithelium. Controls confirmed the specificity of the results.

In some, but not all, mammary carcinomas the presence of casein could be detected and localized by immunofluorescence: this was correlated with the specific histopathological diagnosis in each case. Most neoplastic cells in medullary and Grade III carcinomas were negative; in some areas in these tumours and especially in Grade II carcinomas (Fig. 3), casein-containing cells could be detected, displaying a thin rim of specific fluorescence at the periphery of the cytoplasm. A similar type of cytoplasmic fluorescence could also be observed in some areas in intra-ductal, in papillary and in mucous carcinomas. In intra-ductal carcinomas with cribriform patterns casein was seen lining and sometimes also filling intercellular spaces. In Grade I, well differentiated, carcinomas of the breast casein was present, and mainly localized at the inner border of gland-like structures (Fig. 4); a diffuse cytoplasmic fluorescence could also occasionally be observed. In the 2 cases examined, cells metastatic in the lymph nodes were positive, arranged in small clusters in the peripheral sinus (Fig. 5) or scattered in the lymphatic tissue.



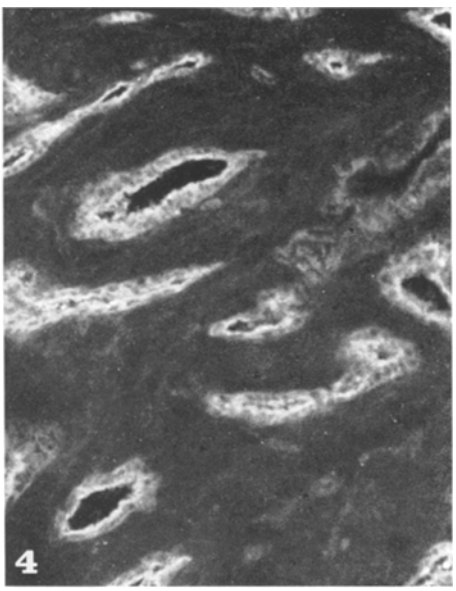


Fig. 3. Grade II carcinoma of the breast. Paraffin section from formalin-fixed tissues. Only some neoplastic cells contain casein, diffuse in the cytoplasm or along the inner border of cells circumscribing small inter-cellular spaces. (× 150)

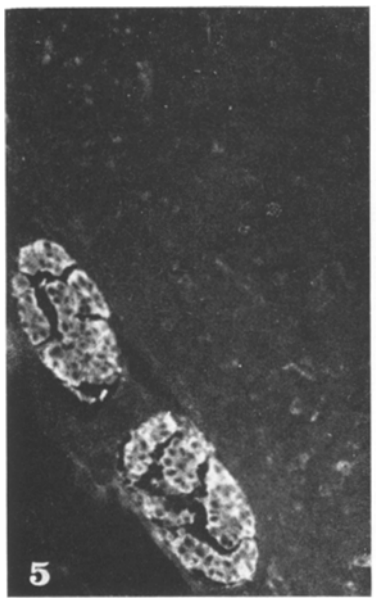
Fig. 4. Grade I carcinoma. Paraffin section. Immunofluorescence method for casein: the protein is present in neoplastic cells, mainly along the inner border of gland-like structures. (× 150)

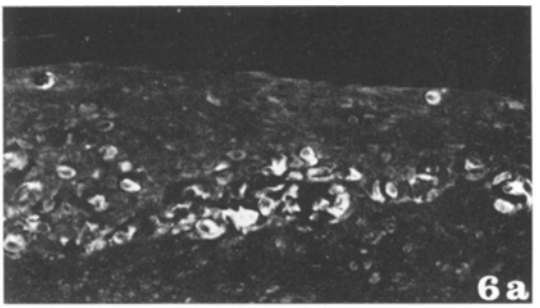
In three cases of Paget's disease of the breast, the immunofluorescence method allowed the detection of casein in neoplastic cells in the ducts; in the nipple epidermis many fluorescent cells could be observed when anti-casein serum was employed: such epithelial cells were isolated or grouped in small clusters, arranged towards the base or a few also at the upper third of the epidermis (Figs. 6a and 7a). The number of these casein-containing cells was in some areas quite prominent, and at least most of them appeared to correspond to the typical Paget cells seen in routine histology (Fig. 7b). Controls carried out in serial sections invariably gave negative results (Fig. 6b).

The 2 cases of adeno-carcinomas of the rectum gave negative results.

Discussion

The results we have obtained in the human mammary gland confirm previous results of others in the rat (Turkington, 1969; Young and Nelstrop, 1970) and cow (Kihm, 1973) lactating mammary glands: casein can be detected and localized by an immuno-fluorescence method; such protein is fixation-resistant and retains its antigenic properties also in routine formalin-fixed paraffin-embedded tissues. The specificity of the immuno-fluorescence reaction was demonstrated by negative controls, also when the immune serum was absorbed with casein; the presence of anti-casein antibodies was confirmed by positive ring and Ouchterlony tests. The





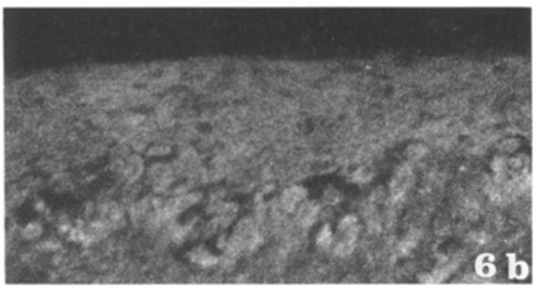
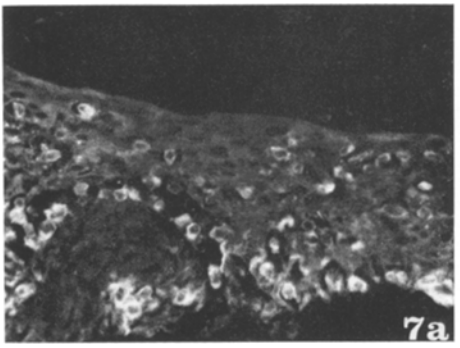


Fig. 5. Axillary lymph node with tumoral cells metastatic from a breast carcinoma. Paraffin section from tissues fixed in Serra fluid (formol-alcohol-acetic acid). Casein-containing neoplastic cells are present in the peripheral sinus; the lymphatic tissue is negative. (× 150)

Fig. 6a and b. Paget's disease of the nipple. Paraffin section. (a) The immunofluorescence method for casein reveals numerous epithelial cells, mainly along the base of the epidermis. (b) Serial section of the same area. In the control, no fluorescent cells can be seen (film overexposed). (× 250)



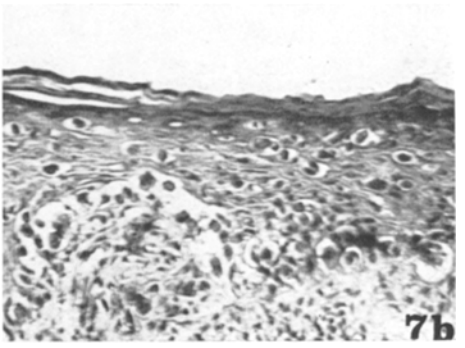


Fig. 7a and b. Paget's disease of the nipple. Paraffin section from formalin-fixed tissues. (a) Casein-containing cells in intra-epidermal position. (b) After staining of the section with H and E. Most of the immunofluorescent cells correspond to typical Paget cells. (× 250)

site of casein production in the human lactating mammary gland was shown to be similar to that observed in the rat and cow, i.e. within the acini and along the acinar border of the secretory cells. Our observation of the presence of casein in epithelial structures in mammary dysplasia gives evidence of a specific secretory function of the epithelial cells in such disease, and is in agreement with previous

electronmicroscopical investigations (Wellings and Roberts, 1963; Ozzello, 1971) reporting the presence of small intracytoplasmic "secretory" granules in epithelial cells, also with apocrine metaplasia, in mammary dysplasia and sclerosing adenosis. Similar cytoplasmic granules have also been described in neoplastic cells in human breast carcinomas (Bush and Merker, 1968; Ozzello, 1971). Milk protein production by mammary carcinomas has been extensively investigated in experimental animals by means of biochemical (Hilf, 1967; Turkington and Riddle, 1970; Hohmann, Bern and Cole, 1972) and immunofluorescence (Turkington, 1970) techniques. These studies have shown casein production to be a specific feature of some experimental rat tumours, and to be enhanced by estrogen administration "in vivo" (Hilf, 1967) and by "in vitro" culture in the presence of insulin, cortisol and prolactin (Turkington and Riddle, 1970; Hohmann *et al.*, 1972). Similar studies have not been reported, to the best of our knowledge, in human breast carcinomas. The numerous immunofluorescence investigations on such tumours were in fact mainly directed to the detection, in the sera of patients, of antibodies against antigens of fetal (Edinak, Lardis and Vrana, 1971) or viral (Taylor and Odili, 1970; Edinak, Hirshaut, Bernhard and Trempe, 1972; Hoshino and Dmochowski, 1973) origin, or of auto-antibodies directed against mammary neoplastic cells (Loisillier, Buffe, Tan, Burtin and Grabar, 1965; Priori, Seman, Dmochowski, Gallager and Anderson, 1971). Some of these previous studies, especially those reported by Loisillier and collaborators (1965) might possibly fit with our observations on the presence of casein in some carcinomas of the breast: the "auto-antibodies" were in fact removed by prior absorption with milk proteins.

Our immunofluorescence investigations indicate that casein production is a functional characteristic of only some mammary carcinomas: when immunofluorescence was correlated with the histological pattern, most neoplastic cells in poorly differentiated carcinomas were seen to be negative, while Grade I carcinomas were positive. The rather small number of cases investigated does not allow to draw general conclusions; it seems however not unreasonable to interpret these findings as evidence of a specific functional as well as morphological differentiation of Grade I carcinomas of the breast. Casein production appeared to be maintained also in neoplastic cells metastatic in lymph nodes.

The immunofluorescence demonstration of the presence of casein in intra-epidermal "Paget" cells and in neoplastic cells in the underlying ducts in Paget's disease of the breast is evidence of a functional differentiation of the intra-epidermal cells along lines proper to the mammary epithelium: this finding seems to fit with theories (Jacobaeus, 1904; Muir, 1931; Muir and Aitkenhead, 1934) on the mammary origin of Paget cells.

One of us (G.B.) is supported by grants from the Italian C.N.R. We thank Dr. P. Arese, Dr. P. Comoglio and Prof. A. Carbonara for help in isolating casein, in gel electrophoresis and in immuno-diffusion, respectively.

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