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Lack of β-Casein Production by Human Breast Tumours Revealed by Monoclonal Antibodies

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Abstract—*An immunohistochemical study with four monoclonal antibodies to human β-casein was carried out to examine the expression of this milk protein in a wide range of normal tissues, in 127 breast tumours and in a heterogeneous panel of 42 malignancies of other histogenesis. The only normal tissue stained positively by the antibodies was the mammary gland in late pregnancy, during lactation and in the post-lactional regression period. None of the tumours of non-mammary origin showed any staining. Furthermore, only two of 40 benign breast lesions and one anaplastic primary carcinoma with its metastasis (among 87 breast carcinomas) showed any reactivity. The immunohistochemical results were supported by immunoblotting data and suggested β-casein expression has no role to play as a marker in the diagnosis or monitoring of human breast cancer.*

INTRODUCTION

HUMAN milk contains numerous products produced by the fully differentiated mammary gland the most characteristic of which are the caseins. The attractive idea of looking for casein in tumour tissues [1, 2] and body fluids [3, 4] in an attempt to define its potential value as a marker in characterizing breast cancer from a functional point of view has been pursued by several investigators (see also Refs [5, 6] for further references). Such studies have, however, produced conflicting results. Immunohistochemical detection of casein in human mammary carcinomas using polyclonal antisera suggested that casein was produced by most, if not all, breast tumours [1, 2, 7, 8]. It has become evident, however, from the work of other investigators, that the production of conventional polyclonal antisera specific for casein appears to be difficult due to the weak immunogenicity of casein itself and to the contamination of casein preparations by significant amounts of immunodominant milk components ([5, 9], Simickova, personal communication). Thus, the comparative immunohistochemical and biochemical study by Ormerod *et al.* [10] demonstrated that a small amount of the immunogenic mucin molecules (termed by the authors EMA or epithelial membrane antigen) in the acid-precipitated casein used for immunization resulted in a predominantly anti-EMA antiserum which revealed a false 'casein' positive in normal human tissues and tumours.

In order to resolve these inconsistencies and to be able to study the process of differentiation and its relation to malignancy in the human breast, we have developed four monoclonal antibodies to human casein and established their specificity of reaction [6].

We have now examined in detail the reaction of a broad spectrum of normal human tissues as well as of a large number of benign and malignant breast tumours with the four anti-casein monoclonal antibodies. The results of this immunohistochemical study, supported by immunoblotting data, suggest that the production of β-casein by human breast tumours is an extremely rare event, thus making the potential usefulness of this milk protein as a marker of breast cancer highly unlikely.

MATERIALS AND METHODS

Monoclonal antibodies

Four mouse monoclonal antibodies designated F20.2 (IgG2a), F20.4, F20.10 and F20.14 (all three of the IgG1 subclass) to human β-casein were described in detail previously [6]. One of them, F20.10, also recognizes an epitope present on human α-lactalbumin [6]. Control reagents included monoclonal antibodies BA16 and BA17 specific for human 40 kDa keratin [11] which were

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Table 1. Staining intensity of anti-casein monoclonal antibodies on tissue sections processed in different ways

Antibody	Ig subclass	Specificity	Staining intensity* on: Frozen sections Unfixed	M/A†	Paraffin sections MC‡	FKFM§	F‖
F20.2	IgG2a	β-Casein	++	++	++	+	−
F20.4	IgG1	β-Casein	++	++	+/++	+	−
F20.10	IgG1	β-Casein and α-lactalbumin	++	++	+/++	+	−
F20.14	IgG1	β-Casein	++	++	+/++	±	−

*Staining intensity in an indirect immunoperoxidase technique is expressed as: (++) strong positivity, (+) clear but less intensive staining, (±) weak and variable positivity, (−) no detectable staining.
†M/A = fixed in cold methanol/acetone(1:1).
‡MC = fixed in methacard (a mixture of methanol, chloroform and acetic acid, 6:3:1).
§FKFM= fixed in acidic formol saline with methanol (methanol, 40% formaldehyde, acetic acid and distilled water, 8:2:1:9, with 8.5 g NaCl per litre).
‖F = fixed in routine formol saline.

used to verify the epithelial origin of the lesions under study, and the antibody TF-1 against pig transferrin [12] used as a negative control. In both immunohistochemistry and staining of immunoblots, undiluted hybridoma tissue culture supernatants were used.

Tissues and tumours

Normal human tissues came from the tissue banks of both the Research Institute of Clinical and Experimental Oncology in Brno and the Imperial Cancer Research Fund Laboratories in London as well as from the Departments of Forensic Medicine of the J.E. Purkyne University in Brno. Tissues from primary and secondary tumours were obtained from the Departments of Surgery and Gynaecology, RICEO in Brno and from Dr. Rosemary Millis at the ICRF Clinical Oncology Unit at New Cross Hospital in London. Frozen sections were either air-dried only or fixed in a cold mixture of methanol and acetone (1:1) for 10 min before staining, while pieces of tissue to be embedded in paraffin were fixed in one of the fixatives of the precise composition of which is given in Table 1.

Immunoperoxidase staining of tissue sections

The indirect immunoperoxidase technique used in this study was performed as previously described [11] using peroxidase conjugated rabbit anti-mouse immunoglobulin antiserum (Dako, Copenhagen, Denmark) as the second antibody, diaminobenzidine (Sigma London U.K.) as chromogen and haematoxylin to counterstain nuclei.

Detection of antigens in Western blots

The details of human casein purification and the electrophoretic separation of milk components were published previously [6]. The whole-tissue lysates from several 30 nm frozen sections of both benign and malignant breast tumours were made directly in sample buffer as described by Taylor-Papadimitriou *et al.* [13]. The proteins were separated by SDS-PAGE on 10% or 12.5% polyacrylamide gels with a 5% stacking gel. Electrophoretic transfer of the separated proteins onto nitrocellulose membrane and immunoenzymatic staining were performed essentially as described [11, 14] using 0.1% Tween 20 to prevent non-specific binding of the reactants and biotinylated second antibody followed by preformed complexes of streptavidin with biotinylated peroxidase (Amersham, U.K.).

RESULTS

Immunohistochemical studies with the four antibodies against casein showed that none of the epitopes were detectable in normal resting breast while all antibodies revealed heterogeneous positivity of the luminal cells and of the luminal contents in alveoli of late-pregnant as well as post-lactational regressing breast (Fig. 1) and almost homogeneous expression of epitopes in lactating human breast tissue. The availability of breast tissue samples fixed in different fixatives enable us to compare the effects of various treatments on the target epitopes studied. On the basis of such comparisons, the results of which are summarized in Table 1, either frozen sections fixed in methanol/acetone or methacarn-fixed paraffin-embedded samples were selected for the subsequent immunohistochemical study of human β-casein expression in other normal human tissues, in breast tumours and in tumours of other histogenesis.

The immunoperoxidase staining of a wide range of normal human tissues (including brain, cerebellum, striated and smooth muscle, cartilage, spleen, thymus, lymph nodes, myokard, epidermis and its appendages, tongue, oesophagus, intestine, salivary glands, liver, pancreas, gall bladder, adrenal gland, thyroid, respiratory tract, and male and female genito-urinary systems) gave negative results with

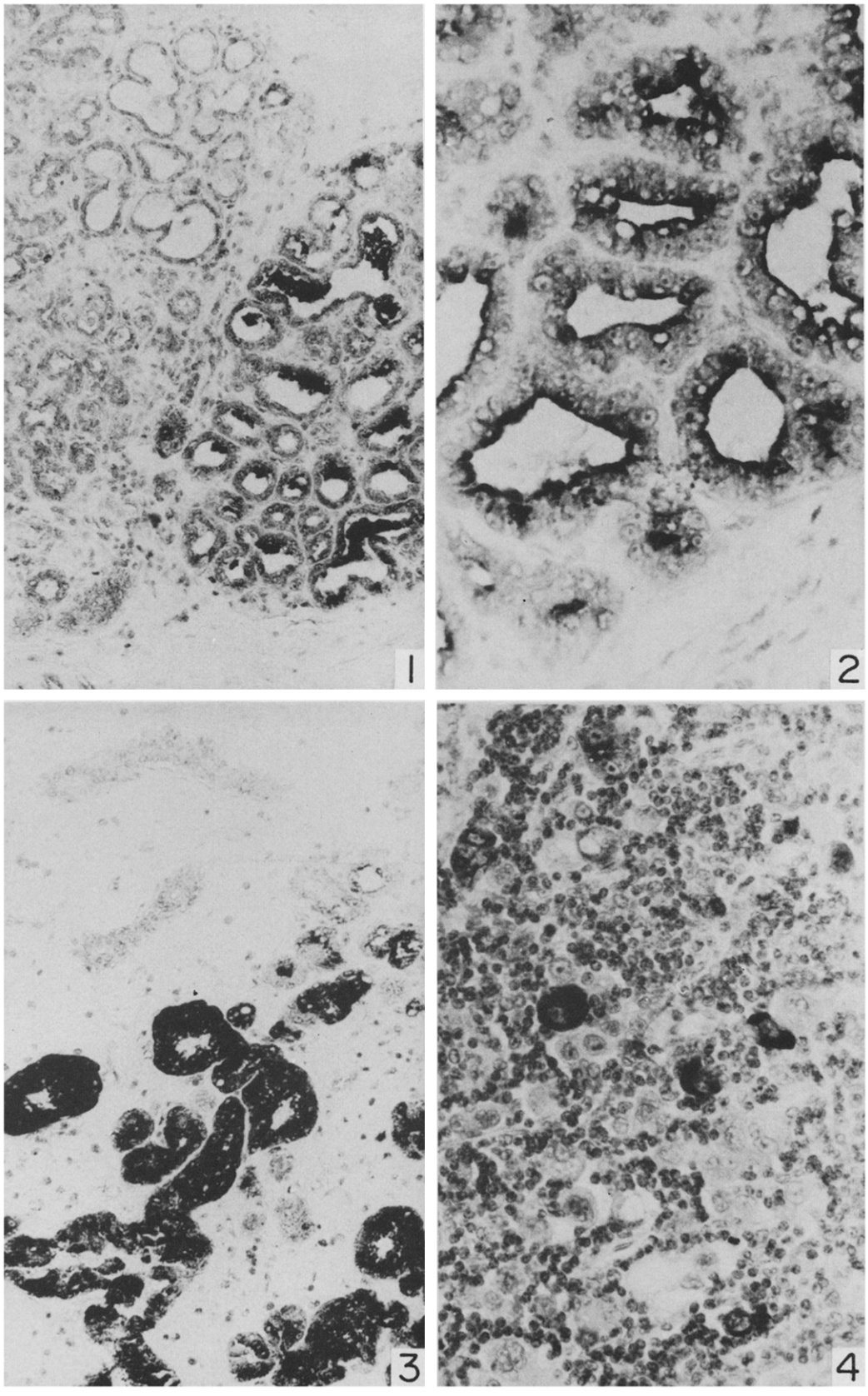

Fig. 1. Immunoperoxidase staining with the antibody F20.2 showing β-casein secretion by one out of several terminal ductal lobular units in postlactationally regressing human mammary gland. × 160

Fig. 2. Almost homogeneously positive staining of a lactating adenoma by the antibody F20.10. × 250.

Fig. 3. Heterogeneous expression of β-casein as revealed by immunoperoxidase staining with the antibody F20.2 in a fibroadenoma. × 160.

Fig. 4. Production of β-casein by some cells of the secondary anaplastic breast carcinoma in a lymph node, demonstrated by the antibody F20.14. × 250.

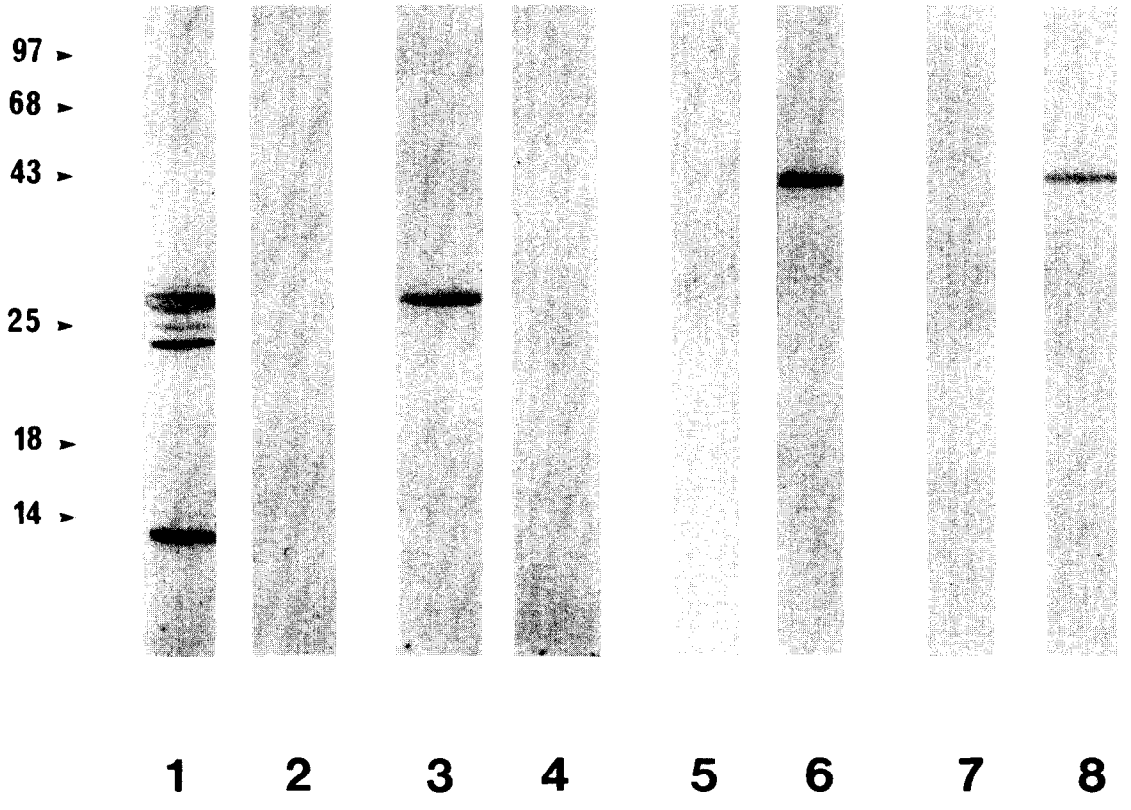

Fig. 5. *Immunoblots of gel-separated protein mixtures and tumour extracts stained with the antibody F*20.10 (*tracks* 1,2,3,4,5,7) *and with the antibody BA*17 (*tracks* 6,8). *Micelle preparation from human milk* (*track* 1), *human milk fat globule membrane preparation* (*track* 2), *human* β*-casein* (*track* 3), *normal human serum* (*track* 4), *fibroadenoma* (*tracks* 5,6), *infiltrating ductal carcinoma of the breast* (*tracks* 7,8). *The molecular weight of the markers is given in* kDa × 10^{-3}.

Table 2. Staining patterns of benign and malignant breast tumours with monoclonal antibodies to human β-casein

Tumour type	Total number of tumours examined	Staining pattern: Negative	Focally positive	Homogeneously positive
Benign lesions				
Fibroadenoma	12	11	1	0
Fibrocystic disease	20	20	0	0
Lactating adenoma	1	0	0	1
Benign phyllodes tumour	7	7	0	0
Pure in situ *carcinoma*	4	4	0	0
Malignant lesions				
Infiltrating ductal carcinoma	43	43	0	0
Infiltrating lobular carcinoma	10	10	0	0
Special types of carcinoma	17	16	1	0
Metastases	13	12	1	0

all four antibodies.

In order to determine whether human breast tumours produce any β-casein, 127 benign and malignant lesions were examined. The staining results obtained with our four anti-casein antibodies are summarized in Table 2. Only two out of 40 benign lesions were found positive, one of them being a lactating adenoma which showed nearly homogeneous positivity with all four antibodies (Fig. 2), the other one was a fibroadenoma with only some areas (mainly those with morphological features of lactational foci) stained with the antibodies to β-casein (Fig. 3). No obvious difference could be observed between the staining patterns showed by the F20.10 antibody cross-reacting with human α-lactalbumin and the patterns revealed by the three antibodies specific for β-casein only. Seventy primary infiltrating breast carcinomas and four pure *in situ* carcinomas were examined (see Table 2). One lesion, an anaplastic carcinoma, contained occasional foci positive with all four antibodies on parallel sections. Interestingly, the lymph node metastasis of this particular carcinoma also showed a mosaic pattern of positive (about 5–15%) and negative malignant cells (Fig. 4). No reactivity with any anti-casein antibody was found in the metastases from a further 12 patients. The metastatic carcinoma cells were clearly identified on the basis of their strong positive staining with the antibody BA17 specific for human keratin 19 [11, 15].

To see if any of the common human cancers produce β-casein ectopically, a heterogeneous group of 42 tumours of non-mammary histogenesis was also included in the immunohistochemical study. None of the tumours including melanomas, lymphomas, sarcomas, colon carcinomas, urinary bladder carcinomas, lung carcinomas and testicular germ-cell tumours showed detectable staining with any of the anti-casein antibodies.

The immunohistochemical results mentioned above were confirmed on a selected panel of several normal human tissues and benign as well as malignant mammary tumours using the immunoblotting technique after separation of the proteins from the whole-tissue lysates by SDS-PAGE. An example of this kind of analysis is demonstrated in Fig. 5 with the antibody F20.10. Only purified human β-casein (track 3) and the casein fractions and α-lactalbumin of human milk (track 1) reacted with the F20.10 antibody while neither lysates of breast tumours (tracks 5, 7) nor complex protein mixtures like preparation of the human milk fat globule membranes (track 2) and normal human serum (track 4) showed any positive reaction with the antibody. That the tumour lysates contained enough protein material of epithelial origin can be judged from the strong reaction of the anti-keratin monoclonal antibody BA17 used on the strips of the same blots (tracks 6, 8). Similar negative results on tumour lysates were obtained with the remaining three anti-casein antibodies (data not shown).

DISCUSSION

In the present study we have demonstrated the lack of β-casein production by normal human tissues, with the exception of late-pregnant, lactating and regressing mammary gland. Moreover, we have

also shown that β-casein secretion by human breast tumours is extremely rare. Our immunohistochemical results with the four monoclonal antibodies differ markedly from those published by other authors using conventional polyclonal antisera to human casein [1, 2, 7, 8, 16]. The specificity of our monoclonal antibodies is supported by the results of an extensive characterization in ELISA and immunoblotting of both one- and two-dimensional gel electrophoreses, using a broad spectrum of purified human proteins as well as complex antigenic mixtures including human milk, serum, tissue, and cell line homogenates ([6], this paper, and our unpublished data). The fact that F20 antibodies recognize at least two (the F20.10 epitope being shared by α-lactalbumin) and probably three different epitopes of the human β-casein molecule ([6], our unpublished data) further substantiates the interpretation of the staining patterns observed.

The contamination of polyclonal anti-casein antisera by antibodies to the immunogenic milk antigens other than casein was clearly demonstrated by Ormerod *et al.* [10] and Simickova (personal communication) and the reactivity of such antisera with various human epithelial tissues [16, 17] also suggests the cross-reaction of the polyclonal reagents with molecules different from casein. The conflicting results obtained with polyclonal antisera almost certainly reflect the lack of their specificity. A similar discrepancy has also been noted with another milk protein, α-lactalbumin, where positive staining of breast cancers with polyclonal antiserum was observed even though mRNA transcripts could not be detected in these tumours [18].

Monoclonal antibodies of the F20 series have proved to be useful reagents for monitoring the differentiation of human mammary epithelial cells *in vitro* [19] as well as *in situ* [20]. We believe this is the first report of the evaluation of β-casein production by human breast tumours using a panel of monoclonal antibodies. Two monoclonal antibodies to human casein, LICR-LON-14.1 and LICR-LON-32.2, have recently been published by Early and McIlhenney but data concerning their reactivity with human breast tumours were not presented [21]. The antibody LICR-LON-14.1 is specific for human K-casein while LICR-LON-32.2 recognizes an epitope of human β-casein shared by caseins of some other species including bovine β-casein [20]. Our four monoclonal antibodies of the F20 series cross-react neither with human α-casein nor with bovine β-casein (unpublished data) and, therefore, they appear to bind casein epitopes different from those recognized by either of the two antibodies mentioned above. The staining results of both LICR-LON-14.1 and LICR-LON-32.2 on normal human tissues are identical with those of the F20 series of antibodies reported here and it would be of interest to see whether there is any significant reactivity of the two LICR-LON antibodies with breast carcinomas.

The lack of β-casein expression in a large number of benign as well as malignant mammary lesions observed in this study suggests this major milk protein cannot be used as a marker in the diagnosis or clinical monitoring of breast tumours.

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