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Immunocytochemical reactivity of breast cancer tissue with antibodies to neuron-specific enolase and an adenocarcinoma-associated glycolipid antigen

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Summary. The immunocytochemical reactivity of breast cancers to antibodies raised against neuron-specific enolase (NSE), carcinoembryonic antigen (CEA) and an adenocarcinoma-associated glycolipid antigen (IR-14) was studied in relation to the long-term outcome of the neoplastic disease.

The patients whose tumours exhibited reactivity with the IR-14 and anti-NSE antibodies had a considerably better 5-year and long-term survival than those without such reactivity. Assessment of DNA-ploidy of the tumour cells was also relevant for long-term prognosis, immunohistochemistry giving additional prognostic information among aneuploid tumours. Reactivity with polyclonal CEA antibodies was of no prognostic value.

It is suggested that tumors carrying the IR-14 reactive epitope, which was originally isolated from circulating antigen-antibody complexes, might evoke a favourable immune response and thus improve the survival of the patient.

Key words: Breast cancer – tumor markers – Immunohistochemistry – Glycolipid antigen – Neuron-specific enolase

Introduction

The pathological assessment of breast carcinomas is based on multiple factors such as the cytological appearance, histological subtype, grade of differentiation and pattern of infiltration (Bloom and Richardson 1957; Wallgren et al. 1976). Chemical findings such as the presence of different hormone receptors are also important (Roberts et al. 1978; Westerberg et al. 1978). All these factors may contribute to the choice of therapy and to the prognostic evaluation. The survival of an individual patient is still very difficult to predict on the basis of these criteria, however, and the search for further prognostic factors is therefore of considerable importance.

In recent years a number of studies have been undertaken to evaluate the prognostic significance of the nuclear DNA content. Retrospective studies on formalin-fixed paraffin-embedded archival material has indicated that DNA analysis may provide prognostic information of importance for the clinical management of individual patients (Erhardt et al. 1986).

Immunocytochemistry offers another approach for assessing breast tumours. The technique has thus been used to establish the site of origin of the primary tumour and to explore the possibilities of specific therapeutic regimens (Roberts et al. 1978; Westerberg et al. 1978; Gatter and Mason 1982; Gatter et al. 1982; Colcher et al. 1984).

In the study of breast cancer a number of epitopes have been examined. Different antibodies have been raised against delipidated human milk fat globule membranes and "epithelial membrane antigen" (EMA), (Heyderman et al. 1979; Arklie et al. 1981; Taylor-Papadimitriou et al. 1982). These antibodies may be of special interest, since it has also been shown that the fusion of murine myeloma cells with lymphocytes from patients with metastatic mammary carcinoma can yield clones producing antibodies with reactivity to the same antigen, that is to say, the human host organism is capable of producing antibodies against this tumour-related structure. When these antibodies were administered as immunotherapy, a certain response was noted (Burnett and Hayden 1985) but the reactivity did not appear to be related to the prognosis, measured as 5-year survival.

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Many of the tumour markers presently used have been obtained after immunization experiments with use of rather crude preparations of the antigen, most often membrane enriched cell homogenates of tumour tissue. The probability of finding immunoglobulins with reactivity to the tumour is high, but this technique does not select epitopes according to their ability to elict an antitumour response in the human.

An alternative approach to the isolation of epitopes has been proposed by Singhal et al. (1986). They isolated antigens as circulating antibody-antigen complexes from patients with carcinoma of the breast; all these antigens had thus initiated the production of antibodies in the patient. One of the monoclonal antibodies, named IR-14, raised against the antigen fraction of this preparation was shown to react with human breast, colonic and liver adenocarcinomas. Since this epitope obviously can be recognized as foreign by the host, it is possible that in patients with tumours exhibiting reactivity with the IR-14 antibody, the related antigen may also elict an anti-tumour response, perhaps implying a better prognosis in these patients.

The aim of the present investigation was therefore to study the reactivity of breast cancer tissue with the IR-14 antibody and with antibodies against NSE. Since both epitopes in question are virtually unaffected by formalin fixation and dehydration, an attempt was also made to elucidate the possible prognostic importance of such reactivity in a more than 20-year-old archival material. For comparison, we included studies of the reactivity with polyclonal CEA antibodies, since the reactivity of these has been reported to be unrelated to the biological behavior of the tumour. The DNA ploidy pattern, which is known to be of prognostic importance, was also studied in order to investigate its possible correlation with the IR-14 reactivity.

Materials and methods

The study comprised 83 patients with breast cancer operated on consecutively in 1962. These patients represents all cases of operable breast cancers detected within a geographically defined area, representing approximately one fifth of the Stockholm county. In nine of these patients information on the long-term survival was not available. These patients were excluded from the study, leaving 74 cases to be analyzed. The mean age of the 74 patients was 61 years (range 36–85, standard deviation 12 years). Each patient had undergone mastectomy with or without removal of the axillary lymph nodes. The histochemical and immunological reactions were performed on parallell sections using one paraffin block from each case, the block being selected to contain adequate amounts of the primary lesion. The tumours were reclassified according to the WHO classification (Azzopardi et al. 1981).

The IR-14 antibody was obtained as a gift from Imré Corp. (Seattle, WA, USA) through Medscand AB (Malmö, Sweden). Antibodies to NSE (rabbit) and CEA (rabbit) were obtained from Dakopatts, Denmark (cat nos. A 589 and A 115 respectively). Biotin-avidin reagents with peroxidase label were purchased as a Vectastain (R) kit (PK-4002; Vector laboratories, Burlingame, CA, USA). Nonspecific rabbit and swine sera, swine-antirabbit bridging antibodies, and rabbit-PAP complexes were all obtained from Dakopatts (cat. nos. X 902, X 901, Z 196 and Z 113 respectively).

All immunocytochemical analyses were performed on formalin-fixed, paraffin-embedded sections which were dewaxed in xylene and immersed in ethanol. Prior to the immunohistochemical reactions, non-specific peroxidase reactivity was blocked in methanol containing 0.3% H_2O_2 for 30 min at 20° C. Reactivity with the IR-14 was monitored after the avidin-biotin-peroxidase procedure (Vectastain kit, Vector laboratories Inc.). The specific antibody was diluted 1:400 and the incubation was performed at 4° C overnight. Nonreactive mouse serum (1:20) was used as a negative control. Diaminobenzidine (DAB; 0.05% in a Tris buffered saline at pH 7.6, and with 0.03% H_2O_2 added immediately prior to use) was used to demonstrate enzyme label and the stain was developed for 5 min at room temperature.

The anti-NSE antibodies were used at a dilution of 1:400. The presence of an antigen-antibody reaction was detected with the PAP technique as described by Sternberger (1986), using swine-anti-rabbit bridging antibodies (Dakopatts). Here again DAB was used to demonstrate the enzyme label. To visualize possible endocrine activity, parallel sections were stained according to the Grimelius method (1968). The CEA antibodies were used at a dilution of 1:200. Bound antibodies were detected with the PAP method, using the DAB reaction as above. All slides were counterstained with Harris haematoxylin for 1 min. Nonreactive rabbit serum (1:20) was used as a negative control.

Sections intended for DNA analysis were dewaxed in xylene, immersed in ethanol and postfixed in 10% formalin (Caspersson et al. 1983) and subsequently stained according to the Feulgen method. The nuclear DNA content was then measured on the histological sections, using a high-resolution integrating microspectrophotometer (UMSP), with preselection of measuring fields.

Parallel selections from each block of tumour tissue were

Table 1. Immunohistochemical reactivity to IR-14, NSE, and CEA in 74 mammary carcinoma

	IR-14 pos[a]	IR-14 neg[a]	Total
NSE pos[a]	12	0	12
CEA pos[a]	5	0	5
CEA neg[a]	7	0	7
NSE neg[a]	47	15	62
CEA pos[a]	21	6	27
CEA neg[a]	26	9	35
Total	59	15	74
	26	6	32
	33	9	42

[a] A positive immunocytological reaction was defined arbitrarily as one one in which a distinct stain could be seen in more than 20% of the tumour cells

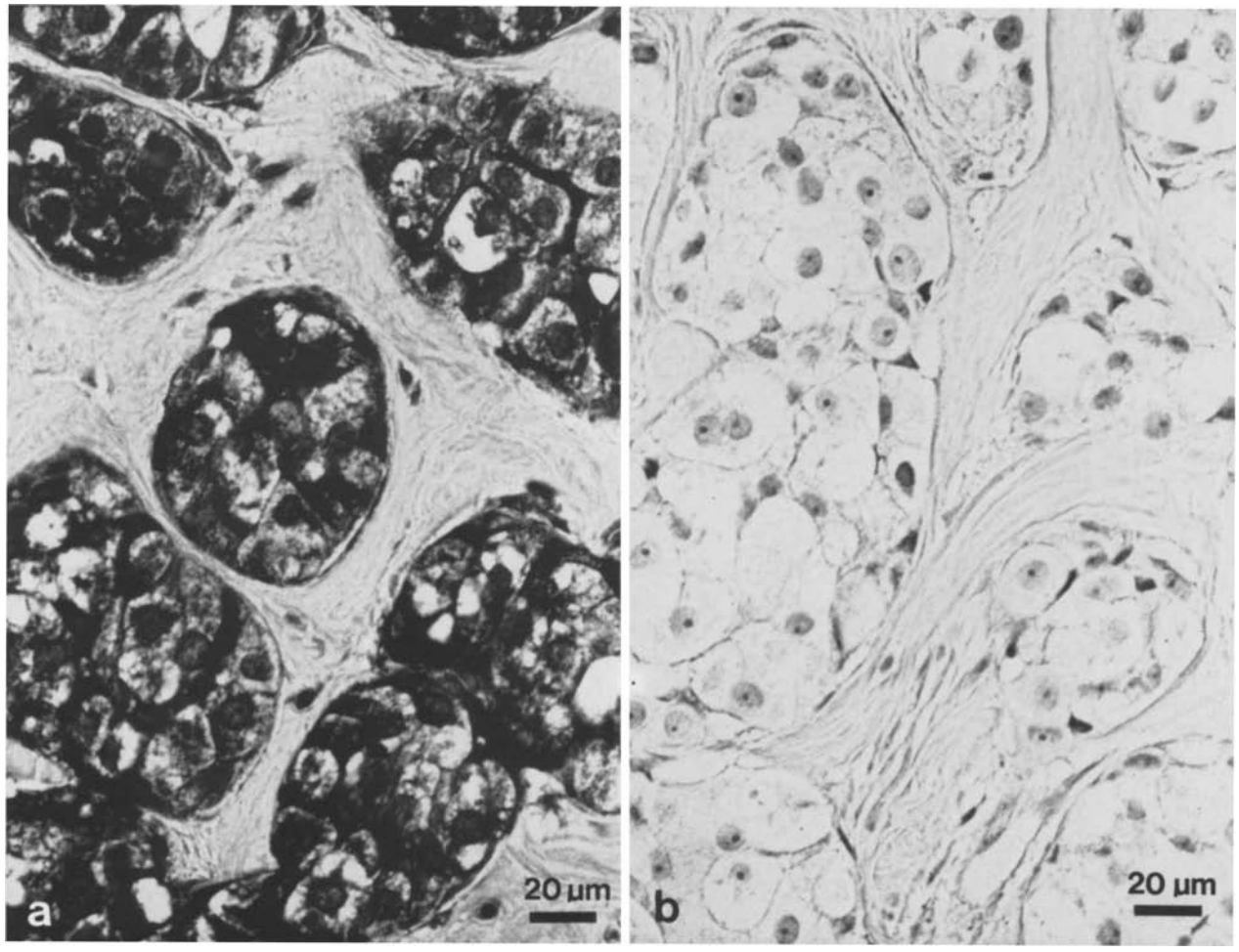

Fig. 1. Immunocytochemical demonstration of the IR-14 antibody (**a**), with intense cytoplasmic reactivity. In the parallel section used as a negative control (**b**), the corresponding area exhibits the counter-stain only. (Immunoperoxidase and Harris' haematoxylin stain; blue interference filter 350–480 nm; ×460)

evaluated with respect to the histological subtype of the tumour (Azzopardi et al. 1981), the proportion of the tumour that presented the antigenic determinants and the DNA ploidy profile (Auer et al. 1980; Caspersson et al. 1983). The DNA histogram patterns were thus classified into diploid and/or tetraploid (types I–II) and aneuploid (types III–IV) subgroups (Auer et al. 1980). After the morphological and immunological evaluation, information was obtained regarding the survival of the individual patients. No attempt was made to distinguish between death from cancer and death from intercurrent diseases.

The possible statistical dependence of the group on any variable was tested by means of two-by-two contingency table tests (chi-square). In all cases the hypothesis tested was that the two characteristics were independent. The tests were made at a significance level of 0.05. Yates' correction for continuity was used. To eliminate the possible effect of differences in age distribution, the 5-year-survival rates were compared also after age-matching using the standard population method.

Results

Parallel sections from a total of 74 tumours were studied immunocytochemically with the three antibodies described (Table 1), and also by Feulgen-DNA analysis and with the argyrophilic stain. A positive immunological reaction (Fig. 1) was defined arbitrarily as one in which a distinct stain could be seen in more than 20% of the tumour cells. The staining intensity or distribution was not graded in further detail. Such reactivity with the IR-14 antibodies was detected in 80% of all the tumours, while NSE reactivity was seen in only 16%, and CEA in 43%. The argyrophilic reaction was negative in all cases, and indications of true neuro-endocrine differentiation was not obtained in any case. The nine tumours excluded from the study because of a lack of clinical information did not differ from the remaining material concerning the immunological or histochemical reactivity. All tumours binding the anti-NSE antibodies also reacted with the IR-14 ones, while CEA was unrelated to the other reactions.

Binding of IR-14 antibodies was observed in

Table 2. Immunohistochemical reactivity to IR-14, NSE and CEA related to histological type

	Antibody		
	IR-14	NSE	CEA
Histological tumour type			
Ductal (57)	46 (81%)	11 (19%)	30 (53%)
Lobular (9)	7 (78%)	0	1 (11%)
Colloid (4)	2 (50%)	0	0
Commedo (4)	4 (100%)	1 (25%)	1 (25%)
Total (83)	59 (80%)	12 (16%)	32 (43%)

Number of tumours with positive reaction and its percentage within each histological group

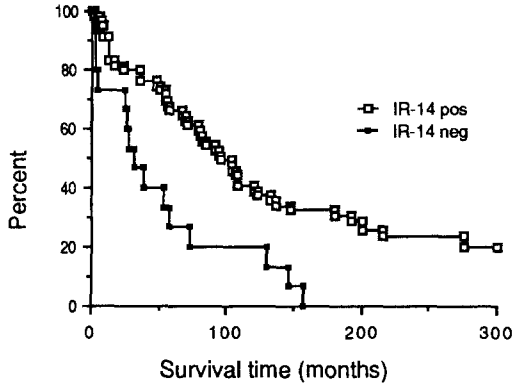

Fig. 2. Long-term survival of 54 mastectomized breast cancer patients in whom the tumour tissue was found to react with the IR-14 antibody, compared with that of 20 patients in whom this reaction was not observed

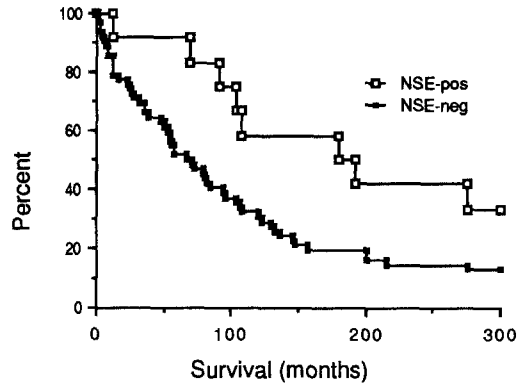

Fig. 3. Long-term survival of 12 mastectomized breast cancer patients in whom the tumour showed reactivity with anti-NSE antibodies, and that of 62 patients in whom this reactivity was not seen

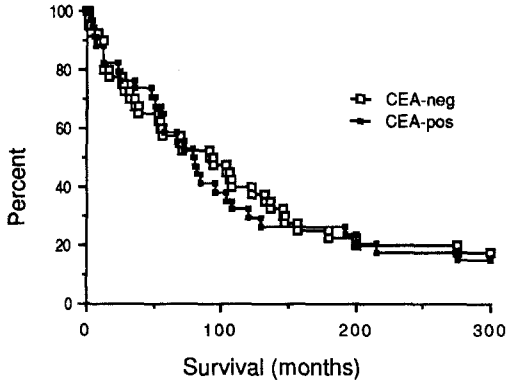

Fig. 4. Long-term survival of 32 mastectomized breast cancer patients in whom the tumour showed CEA reactivity, and that of 42 patients in whom this reactivity was not observed

all histological groups (Table 2). In 32 of the cases metastatic involvment of lymph nodes or distant organs was known or detected at the time of operation. Of these, 15 patients survived for more than 5 years, all of whom showed a positive IR-14 reaction. Among patients with shorter survival only 10/17 were reactive; the difference being statistically significant at a 5% rejection level. Similar, but smaller differences were observed in patients without known metastases at the time of surgery. The size of this sample is, however, too small to allow a further stratification according to the TNM system or the histological type.

When the binding of the three studied antibodies was considered in relation to patient survival, a lower mortality rate was found among the patients with tumours binding the IR-14 antibody than among those with nonreactive tumours (Fig. 2). This difference was slightly greater among patients with tumours that also displayed NSE reactivity (Fig. 3). These effects on the prognosis are not evident, however, until about 5 years after the operation. The prognostic differences measured as 5-year-survival rates were most pronounced among patients over the age of 75. Correction for discrepancies in age distribution, using the standard population method, resulted in an even greater difference in prognosis. The occurrence of a detectable reaction against CEA (Fig. 4) did not influence the prognosis in this material.

In accordance with previous studies (Auer et al. 1980; Erhardt et al. 1986) the prognosis was found to be related to the DNA ploidy pattern as determined by microspectrophotometry (Fig. 5). In the aneuploid group a majority of the cases (49/54) also reacted with the IR-14 antibody. The improved prognosis associated with the IR-14 reactivity was also noted in this group. Thus among the patients in whom the tumours were aneuploid, and at the same time IR-14 reactive, the 5-year

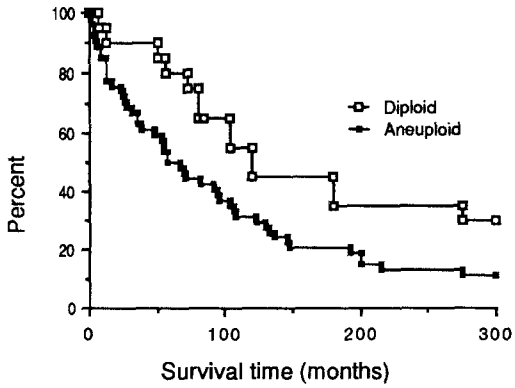

Fig. 5. Long-term survival of 54 mastectomized breast cancer patients in whom tumour showed a diploid/tetraploid DNA pattern, and that of 20 patients in whom the tumours were aneuploid

survival rate was 60%, compared to the 21% found among those with nonreactive aneuploid tumors. Most of the diploid/tetraploid tumours displayed IR-14 reactivity, but the number of cases in this group was too low to allow further analysis. Similarly, NSE reactivity seemed to indicate a better prognosis irrespective of the DNA ploidy pattern and the occurrence of IR-14-related epitopes. However, also these figures were too small for a more detailed statistical analysis.

Discussion

At present two laboratory tests, namely the microspectrophotometric analysis of tumour cell ploidy and the determination of hormone receptors, measured either chemically or immunocytochemically, seem to provide useful data for the assessment of the prognosis in breast cancer. For these tests, however, it is necessary to have access to sophisticated equipment, and in the case of hormone receptor analysis it is important to use fresh tumour material. In contrast both the NSE and the IR-14 reactive epitopes are virtually unaffected by fixation and embedding procedures, which simplifies the immunocytochemical analysis of these structures. In addition the tests are relatively inexpensive and do not necessitate any special tissue process or equipment.

In the present material reactivity with both these antibodies was associated with a considerably better long-term survival, while no such effect was observed with CEA. Similar differences in survival rates were noted when diploid and polyploid breast cancers were compared with one another (Erhardt et al. 1986).

The prognostic value of the IR-14 antibody may be explained in terms of the nature of the antigen used for its production. As discussed above, this glycolipid was originally isolated as a circulating antigen-antibody complex showing that the antigen had already evoked an immunological response in the patient (Singhal et al. 1986). The reactivity was not detected in benign mammary tissue, and it may therefore be speculated that the antibody detects a tumour-related structure, to which the human host tissue can react immunologically, and as a consequence of this an antitumour response is induced. The results obtained thus support the idea that the immune system is of significance in the tumour-host interaction and that immunological reactions in the patient may be of importance for her survival.

It is more difficult to propose an explanation for the correlation between NSE reactivity and prognosis. Thus although one could speculate that the NSE-positive tumors have neuro-endocrine features and that their better prognosis reflects their "carcinoid-like" nature (Cubilla and Woodruff 1977). However, none of these preparations showed argyrophilic reactivity and the mere existence of such a tumour entity in the breast is still a matter of debate (Taxy et al. 1981; Nesland et al. 1986; Bussolati et al. 1987). In our experience from tumours at other sites, the polyclonal anti-NSE antibody will react with quite a number of adenocarcinomas even when these tumours show no other signs of neuro-endocrine differentiation.

A finding of reactivity with IR-14 and anti-NSE antibodies in an individual patient may thus constitute important information on the biological potential of her tumour, while its possible implications on the therapeutic attitude remains to be elucidated. The information given by this reactivity is additional to that obtained by examination of metastatic involvment or determination of the ploidy pattern, especially when the tumours are aneuploid. We have not yet investigated whether or not this immunocytochemical information is additional to that obtained from hormone receptor analysis. It seems as if the assessment of the prognosis in patients with breast cancer would be improved if all these determinations were performed and weighted together. Studies on considerably larger series are required, however, to determine the algorithms necessary for the estimation of prognosis based on these different analysis.

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