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Acknowledgements—This work was supported by Public Health Service grant 5R01CA14054 from NIH. S.I. was a recipient of a Cancer Research Institute/Concern Foundation for Cancer Research grant. We thank S. Gordon for F 4/80 antibody. The technical assistance of M.-L. Solberg, M. Hagelin, K. Benedek, M. Hedenskog and A. Karlsson is acknowledged.

Pergamon

European Journal of Cancer Vol. 30A, No. 7, pp. 1002–1007, 1994
Elsevier Science Ltd
Printed in Great Britain
0959-8049/94 $7.00+0.00

0959-8049(94)E0177-6

Expression of P-Glycoprotein and *In vitro* or *In vivo* Resistance to Doxorubicin and Cisplatin In Breast and Ovarian Cancers

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The expression of P-glycoprotein (P-gp) was studied by immunocytohistochemistry, using the C219 monoclonal antibody, in 39 locally advanced breast cancers and 20 ovarian cancers from previously untreated patients. P-gp was expressed in 46 and 35% of breast and ovarian tumours, respectively. A significant association was observed in both tumour types between P-gp expression and *in vitro* resistance to doxorubicin. We also observed a higher clinical response rate to doxorubicin ± vincristine in patients with breast cancers not expressing P-gp. Conversely, no correlation was found between P-gp expression and *in vitro* resistance to cisplatin or *in vivo* response to cisplatin ± cyclophosphamide treatment in ovarian cancers. Our results support the relevance of P-gp expression as a specific indicator of resistance to certain drugs, such as doxorubicin and vincrinstine, involved in the phenomenon of multidrug resistance in breast and ovarian cancer cells.

Key words: *P*-glycoprotein, multidrug resistance, doxorubicin, breast cancer, ovarian cancer, immunocytohistochemistry, chemosensitivity assays
Eur J Cancer, Vol. 30A, No. 7, pp. 1002–1007, 1994

INTRODUCTION

INTRINSIC OR acquired resistance to various chemotherapeutic agents is a major problem in cancer treatment. Among the different markers, P-glycoprotein (P-gp) is the most consistently described as an indicator of multidrug resistance (MDR) in a wide variety of animal and human tumour cell lines [1–9]. In fact, an increased P-gp level has been reported to be associated with MDR amplification or overexpression in experimental systems [1]. More recently, significantly raised P-gp levels have been detected in untreated and previously treated human

tumours such as sarcomas, ovarian, colon, hepatic and breast carcinomas, and non-lymphoblastoid leukaemias [10–20]. Hence P-gp represents the stable product of the MDR1 gene, and consequently its overexpression can be considered an indication of the presence of MDR cells and used to predict failure to respond to drug treatment. Several available antibodies against different domains of P-gp allow direct screening on individual human tumours for the presence and the degree of the MDR phenomenon [21–24]. However, results mainly obtained with cell suspensions may be biased owing to the poor representativeness of the original cell population for some solid tumour types.

We evaluated P-gp expression by immunocytohistochemical assay with the C219 monoclonal antibody, which recognises a cytoplasmic domain of P-gp, on sections and cytospin preparations from 39 breast and 20 ovarian human cancers. P-gp expression was analysed in relation to *in vitro* resistance to doxorubicin and cisplatin by primary cultures of breast and ovarian cancers. Furthermore, the expression of P-gp was analysed in relation to clinical response to doxorubicin ± vincristine in breast cancer, and to cisplatin ± cyclophosphamide in ovarian cancer.

PATIENTS AND METHODS

Patient population

The case series comprised 39 patients with locally advanced (T3b-T4) primary breast cancer and 20 patients with advanced ovarian cancer who underwent surgery at the Istituto Nazionale Tumori of Milan. All the patients were previously untreated. Breast cancer patients were subsequently treated with three or four cycles of pre-operative doxorubicin, alone or in association with vincristine, according to regimens adopted at the Istituto Nazionale Tumori of Milan [25]. Patients with ovarian cancers were treated with cisplatin, alone or in association with cyclophosphamide.

Human tumour samples

Immediately after surgery, specimens from 39 primary breast cancers, 17 primary ovarian cancers and three metastatic lesions were placed in Hanks' balanced salt solution, trimmed of adipose tissue or areas of necrosis, and cut into small fragments. *In vitro* chemosensitivity of breast cancer was assessed directly on tumour fragments by using a previously described metabolic assay [25], which has been found to be highly feasible and reliable on this tumour type. Conversely, for ovarian cancer (which grows in semi-solid medium) a proliferative assay was performed on cell suspensions enzymatically obtained from solid fragments by using 0.01% DNase and 0.14% collagenase type I, as described previously [26].

In vitro *chemosensitivity testing*

Drugs. Doxorubicin (Farmitalia-Carlo Erba, Milan, Italy) was used at a single concentration of 3.5 μg/ml in the metabolic assay [25], and at different concentrations (0.01–1.0 μg/ml) in the proliferative assay. Cisplatin (Bristol-Myers, Evansville, Illinois, U.S.A.) was used in the proliferative assay at concentrations ranging from 0.1 to 10 μg/ml. Both the drugs were initially dissolved in 0.9% sodium chloride and then diluted with fresh medium immediately before each experiment.

Metabolic assay. Breast cancer fragments of about 1 mm^3 sampled from different areas of the tumour were incubated *in vitro* for 3 h at 37° C [25]. Doxorubicin-treated and control samples were run in triplicate. At the end of the second hour of incubation, 5 μCi (specific activity 25 Ci/mmol) of [^{3}H]uridine was added to the culture medium. Nucleic acids were extracted from trichloroacetic acid (TCA)-precipitable material, and precursor incorporation was evaluated by liquid scintillation counting and expressed as fractional incorporation [25]. Drug effect was expressed as the ratio between incorporation values in drug-treated versus control samples. A tumour was considered sensitive when the inhibition of [^{3}H]uridine incorporation induced by doxorubicin exceeded the median coefficient of variation observed in control samples (20%), according to previously-defined sensitivity criteria [25].

Proliferative assay. Ovarian cancer cells were cultured according to the method of Tanigawa *et al.* [27], modified as needed [26]. Layers of 1 ml 0.5% agarose in supplemented medium were prepared in 35-mm diameter plastic dishes. Cells were suspended in 1 ml of 0.4% agarose in supplemented medium and poured over these underlayers. A final concentration of 0.333×10^3 viable cells/ml was plated in each culture. Triplicate cultures were set up for each experimental point. The drug or diluent used for reconstitution was added to the tumour cell suspension immediately before plating, thereby providing continuous exposure to drugs for the course of the assay. In each experiment, positive control wells were plated following the addition of 100 μl of mercuric chloride to a final concentration of 10 μg/ml. The cells were incubated at 37°C in 5% CO_2:95% air. After 72 h, each well was labelled with 5 μCi of [^{3}H]thymidine ([^{3}H]dT, specific activity 5 Ci/mmol) and incubated for an additional 24 h. Cellular DNA was precipitated with 10% TCA and solubilised with potassium hydroxide for liquid scintillation counting. Experiments were considered evaluable when the radioactivity in control samples was more than 1000 cpm, the coefficient of variation < 40% and inhibition of [^{3}H]dT incorporation by mercuric chloride > 90%. A tumour was considered sensitive to the tested drugs when the concentrations of drugs able to inhibit [^{3}H]dT incorporation by 50% (IC_{50}) were lower than 0.1 for doxorubicin and 1.0 μg/ml for cisplatin.

P-gp determination

The expression of P-gp was detected by using the C219 monoclonal antibody (Cis Diagnostici, Trezzano Vercellese, Vercelli, Italy). Bouin-fixed sections (4 μm thickness) for breast cancers and 3.7% formalin-fixed cytospin preparations from ovarian cancers were incubated at 4°C for 2 h with 10 μg/ml of C219 monoclonal antibody in a humid chamber. Samples were washed twice in phosphate-buffered saline (PBS) and treated with a goat anti-mouse immunoglobulin IgG2a (Amersham International, U.K.) diluted 1/40 for 30 min. The specimens were then incubated with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories, U.S.A.) for 30 min at room temperature. Two cell lines resistant to doxorubicin—a human breast cancer line (MCF7 Dx, resistance index 300) and a colon cancer line (LoVo Dx, resistance index 14), both markedly expressing P-gp—were used as positive controls. The doxorubicin-sensitive parent MCF7 and LoVo cell lines were used as negative controls.

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Revised 7 Feb. 1994; accepted 1 Mar. 1994.

The stained preparations were evaluated independently by two observers, unaware of *in vitro* or clinical sensitivity to drugs. The positivity was classified according to the intensity of the plasma membrane and Golgi region staining using a four-point scale (absent, weak, intermediate and strong).

Clinical response

In patients with locally advanced breast cancer, clinical response was evaluated through clinical and physical examinations following three or four cycles of doxorubicin [60 mg/m^2 intravenously (i.v.)], alone or in association with vincristine (1.2 mg/m^2 i.v.) every 21 days and before starting local-regional treatment. Clinical response was defined according to the standard clinical criterion of tumour volume reduction and was evaluated as the product of the two largest tumour diameters. Tumours were defined as sensitive when a volume reduction of at least 50% was achieved after primary chemotherapy. Progression was defined as the occurrence of new disease manifestations starting from the date of local-regional treatment (surgery or radiotherapy).

In patients with ovarian cancer, clinical response was assessed through physical, gynaecological and surgical evaluations following three cycles of cisplatin (100 mg/m^2 i.v., every 28 days) or seven cycles of cisplatin (1 mg/kg weekly) plus cyclophosphamide (800 mg/m^2 on weeks 1, 4 and 7). Patients were defined as sensitive when complete response (i.e. the histological disappearance of disease) was documented at second-look laparotomy.

Statistical analysis

The Wilcoxon test was used to assess differences in ranking of P-gp expression as a function of *in vitro* data. The association between P-gp expression and *in vitro* or *in vivo* resistance to drugs was tested by means of the kappa test [28].

RESULTS

Immunostaining was always localised in the plasma membrane and/or in the Golgi region of cells, and staining intensity varied widely. No reactivity to C219 was observed in 54% of breast cancers and in 65% of ovarian carcinomas. Weak, intermediate and strong intensity of staining were similarly distributed in the two tumour types. Examples of P-gp-negative (P-gp$^-$) and P-gp-positive (P-gp$^+$) human tumours are shown in Figure 1a and b.

When the intensity score was related to *in vitro* sensitivity to doxorubicin (Figure 2), an inverse quantitative relation was observed. In fact, in breast cancer, the median percentage of inhibition induced by doxorubicin on RNA precursor incorporation was 35% in P-gp$^-$ tumours and only 6% in tumours expressing P-gp ($P < 0.01$). Similarly, in ovarian cancer, a remarkably lower median IC_{50} value for doxorubicin was observed for P-gp$^-$ than for P-gp$^+$ tumours (0.047 versus 0.2 μg/ml, $P = 0.02$). However, no correlation was observed, among P-gp$^+$ tumours, between the degree of immunoreactivity (weak, intermediate or strong) and the level of *in vitro* doxorubicin resistance, in terms of IC_{50} or percentage inhibition of [^{3}H]uridine incorporation. Qualitative analysis of the relation between P-gp positivity and *in vitro* resistance to doxorubicin showed a significant association for both tumour types (Table 1). In particular, more than 85% of P-gp$^+$ breast and ovarian cancers were resistant *in vitro* to doxorubicin. Conversely, about 85% of P-gp$^-$ breast and ovarian cancers were sensitive *in vitro* to doxorubicin. The degree of association between *in vitro* resistance to doxorubicin and P-gp immunoreactivity was similar for the two tumour types.

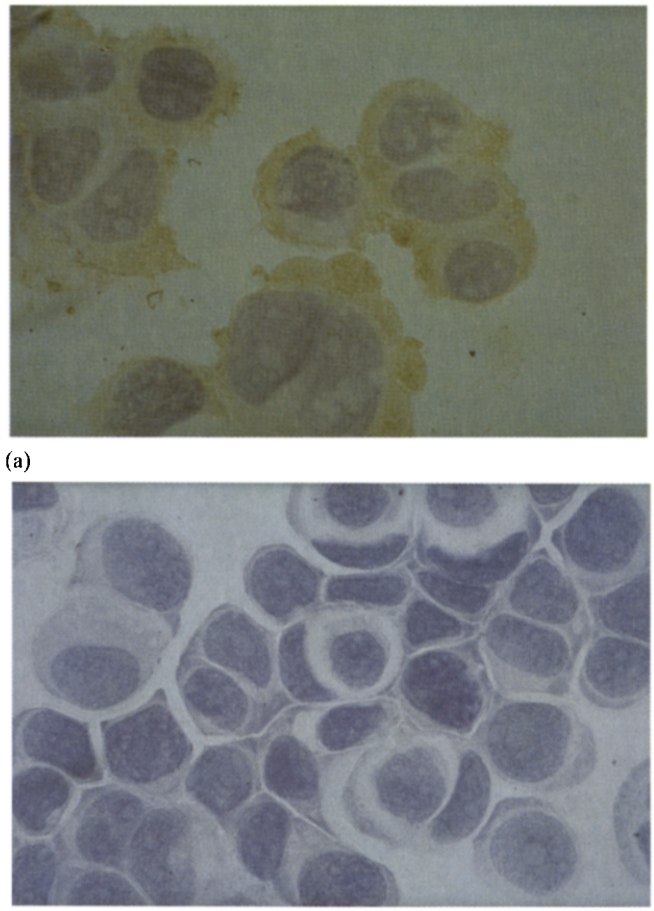

Figure 1. P-glycoprotein reactivity with monoclonal antibody C219 in MCF7 cell lines (peroxidase detection method: ×100). (a) positive sample (MCF7 Dx cell line); (b) negative sample (parent MCF7 cell line).

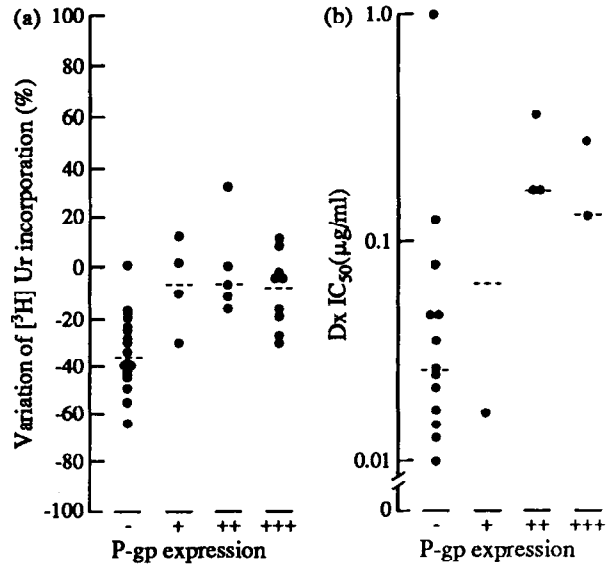

Figure 2. Relation between P-gp expression and *in vitro* sensitivity to doxorubicin. (a) Breast cancer; (b) ovarian cancer. Broken lines indicate the median value of the percentage variation in (a) [^{3}H]uridine incorporation or (b) the median IC_{50}.

Table 1. P-gp staining and in vitro *doxorubicin sensitivity in breast and ovarian cancers*

	No. of cases	Sensitive	Resistant
P-gp$^-$			
Breast	21	18	3
Ovarian	13	11	2
P-gp$^+$			
Breast	18	2	16
Ovarian	7	1	6

Association between *in vitro* results: $P = 0.0005$ for breast and $P = 0.002$ for ovarian cancers.

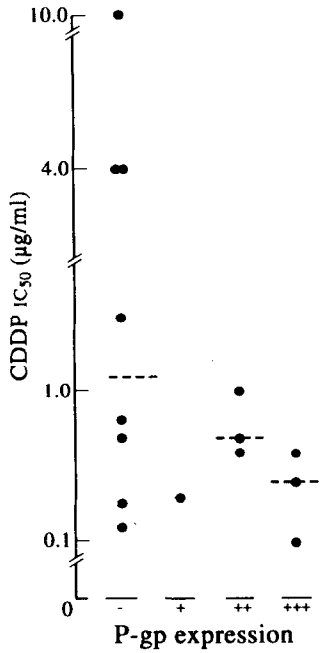

Figure 3. Relationship between P-gp expression and *in vitro* sensitivity to cisplatin in ovarian cancer. Broken lines indicate the median IC_{50}.

P-gp expression in ovarian cancers was also analysed in relation to *in vitro* sensitivity to cisplatin, a drug not involved in MDR phenotype (Figure 3). There was no correlation between P-gp positivity and resistance to the alkylating agent, but only a trend of a lower median IC_{50} value in P-gp$^+$ than in P-gp$^-$ tumours.

P-gp expression was also analysed in relation to clinical response to doxorubicin ± vincristine in breast cancer patients (Table 2). Objective clinical response was observed in 67% of patients with P-gp$^-$ tumours and in 39% of patients with tumours expressing P-gp. An overall correlation between P-gp immunoreactivity and objective clinical response was observed in 25/39 cases (64%, $P = 0.037$), with an accuracy of P-gp expression in predicting clinical resistance or sensitivity of 61 and 67%, respectively. The results were no different when clinical response was analysed in relation to the modulation of intensity of P-gp expression. Progression rates within 2 years of local-regional treatment were similar for patients with P-gp$^+$ (61%) and Pgp$^-$ (57%) tumours. Moreover, in the subset of patients with P-gp$^+$ tumours, the rate of progression was not influenced by the achievement of clinical response. Conversely, in ovarian cancers, clinical response to cisplatin ± cyclophosphamide, i.e. to drugs not involved in MDR, was unrelated to P-gp expression (data not shown).

Table 2. P-gp expression and clinical response in breast cancer

	No. of cases	Responders	Non-responders
P-gp$^-$	21	14	7
P-gp$^+$	18	7	11

$P = 0.037$.

DISCUSSION

In the present study we evaluated the expression of P-gp by immunocytohistochemistry with the C219 monoclonal antibody in previously untreated advanced human breast and ovarian cancers. P-gp was expressed in about half of the breast tumours. This incidence is within the range of the frequencies previously reported for this tumour type [15] using immunoblotting to detect P-gp expression.

In previously untreated ovarian cancers, P-gp expression was observed in about one-third of the cases. Results from immunocytohistochemical studies on this tumour type are discordant. In fact, P-gp expression was detected only occasionally by Rubin *et al.* [12] or in most untreated cases or those previously treated with alkylating agents by Van der Zee *et al.* [29]. MDR1 gene expression was recently detected using a highly sensitive polymerase chain reaction in about 80% of untreated ovarian cancers [31], i.e. in a fraction of tumours higher than the frequency of ovarian cancers clinically resistant to drugs involved in MDR.

In our study, we found a significant relationship between P-gp expression and *in vitro* doxorubicin resistance, evaluated by metabolic or proliferative assays, in breast and ovarian cancers. In particular, in ovarian cancers immunocytochemical detection of P-gp expression was associated with *in vitro* resistance to doxorubicin in more than 90% of cases. Conversely, as expected, we did not find any correlation between P-gp positivity and *in vitro* resistance to cisplatin, a drug not included in the MDR phenotype. Results on breast cancer are in keeping with those previously reported by Salmon *et al.* [17] who showed in a preliminary series of tumours a significant relationship between P-gp expression, as immunocytochemically detected by JSB1 and C219 monoclonal antibodies, and *in vitro* resistance to doxorubicin, evaluated with the clonogenic assay. A correlation betwen P-gp expression, assessed by immunoblotting, and poor *in vitro* cytotoxicity of doxorubicin and vincristine has also been reported by our group on another series of breast cancers [15].

In the present study we also demonstrated a significant correlation between P-gp expression and objective clinical response to doxorubicin–vincristine treatment in locally advanced breast cancer patients. These data confirm a previous finding of Verrelle *et al.* [16], who reported a correlation between a strong P-gp positive staining (> 75% of cells) and lack of initial response to chemotherapy, but also showed a shortened time to progression in a series of 20 locally advanced breast cancers.

However, in our experience the association between P-gp expression and objective clinical response to doxorubicin-includ-

ing regimens, although better than that purely expected by chance, was observed in only two-thirds of the cases and was not improved by considering the modulation of staining intensity. The modest specificity and sensitivity of the assay in predicting clinical response are in keeping with published reports and could be ascribed to different reasons. The limited specificity (67%) suggests the existence of drug-resistance mechanisms unrelated to P-gp, as already described in experimental and clinical models [31–35]. However, other biological reasons cannot be excluded. For example, the subset of P-gp$^-$ tumours which failed to respond to clinical treatment was characterised by a marked biological aggressiveness, as indicated by the highest median [^{3}H]thymidine labelling index (10%) as compared to the other subgroups, whose median proliferative rates ranged from 3.6 to 3.9%. Considering sensitivity, the success of doxorubicin treatment observed in about 40% of P-gp$^+$ tumours might be ascribed to criteria used to define the positivity threshold of P-gp expression or to the accuracy of clinical response assessment. With regard to the first point, in this case series positivity criteria seemed to play a marginal role, since six of the seven tumours clinically responsive were strongly positive and only one weakly positive. As regards clinical response, tumour reduction (> 50%) is the standard criterion used to assess short-term clinical response in advanced tumours. However, it is only a partial indicator of long-term clinical outcome since it does not strictly reflect tumour biological eradication. In fact, in this case series, the progression rate at 2 years was similar for patients with P-gp$^+$ tumours, regardless of whether or not objective clinical response was achieved.

In ovarian cancers, we found no correlation between P-gp expression and clinical response in patients treated with cisplatin ± cyclophosphamide. This finding is consistent with the data reported by Holzmayer *et al.* [30], who demonstrated that the presence of MDR1 mRNA, even at low levels, was correlated with lack of a response to regimens, including P-gp-transported drugs whereas it failed to be indicative of the outcome in patients treated with drugs not involved in the MDR phenomenon.

Our results, together with those of other authors [35], support the relevance of P-gp expression as an indicator of specific resistance to drugs involved in the MDR phenotype rather than of general chemoresistance, as demonstrated by the lack of relationship between P-gp positivity and *in vitro* or clinical response to cisplatin in ovarian cancers. However, our data also showed that P-gp is strongly indicative of sensitivity or resistance to doxorubicin, as evaluated by short-term *in vitro* tests, but it is largely unable to predict clinical response to treatment. The lack of predictivity of short-term clinical outcome could be partially ascribed to the imprecision of tumour shrinkage evaluation and/or to the prevalence of biologically-aggressive, drug-resistant cell subpopulations.

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Acknowledgements—Supported in part by grants from the Special Project PF ACRO (n. 92.02369.PF39) of the Italian Research Council (CNR) and from the Associazione Italiana Ricerca Cancro (AIRC). The authors thank Ms R. Vio and Ms B. Johnston for editing the manuscript.

Pergamon

European Journal of Cancer Vol. 30A, No. 7, pp. 1007–1012, 1994
Elsevier Science Ltd
Printed in Great Britain
0959–8049/94 $7.00+0.00

0959-8049(94)E0179-8

Prognostic Value of a Breast Cancer-associated Glycoprotein Detected by Monoclonal Antibody LU-BCRU-G7

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The immunohistochemical reactivity of a second generation murine monoclonal antibody (LU-BCRU-G7), raised against a novel fucosylated glycoprotein of M_r 230 000, has shown a significant association with prognosis of early stage carcinomas. Staining was observed in 72% of the 190 breast carcinomas tested. No relationship with steroid receptor status, stage or node status was found. An association with grade was observed (χ^2 7.83, 2 degrees of freedom, $P = 0.02$) only when the negative cut-off level was raised from < 10% cells staining to < 25%. Antibody reactivity was always cytoplasmic. Immunoblotting shows the antibody is reactive with a component of M_r 230 000 not detected by HMFG 2. A significant association was found between lack of reactivity and improved disease-free interval ($0.005 > P > 0.001$) and survival ($0.02 > P > 0.01$). Subdivision of cases on the basis of node status showed that staining could refine survival data. A decreased reactivity of LU-BCRU-G7 was observed after pretreatment with β-galactosidase but not a sialidase or β-*N*-acetylhexosaminodase indicating that non-reducing terminal galactose residues in β1-3 or β1-4 linkages may be involved in the antibody binding site. This approach has identified a useful and novel prognostic marker in early stage human breast carcinoma.

Key words: glycoprotein, breast cancer, prognosis, *in vitro* immunisation, murine monoclonal antibody
Eur J Cancer, Vol. 30A, No. 7, pp. 1007–1012, 1994

INTRODUCTION

GLYCOSYLATION CHANGES in malignancy have become a well-recognised phenomenon [1–3]. The role of glycoconjugates in normal cell interactions suggests that specific alterations in glycoconjugate expression associated with malignancy could be significant in determining tumour cell behaviour. This has resulted in the search for tumour-associated glycoconjugates of biological and clinical interest, which has been facilitated by the introduction of monoclonal antibody technology. For breast cancer, a variety of different immunogens have been used, including milk fat globule membranes [4–6], breast cancer cell lines [7–9], and membrane-enriched extracts of human metastatic mammary carcinomas [10–12]. Although a large number of antibodies have been generated, they nearly all detect similar high molecular weight mucin molecules [13] which, although well expressed in carcinomas, are present to some extent in normal and benign breast epithelium. Only one antibody, NCRC11, appears to provide prognostic information about the behaviour of early breast cancer [14], but this is due to its close link with tumour differentiation [15].

A significant disadvantage of the immunological approaches used thus far has been their selectivity for the more immunogenic components of the polymorphic epithelial mucins or PEMs [16]. In spite of this problem few workers have attempted to identify