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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

P.D. Rye and R.A. Walker

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

potentially useful markers by non-immunogenic methods. In a study analysing the glycoproteins released by non-malignant and malignant human breast, we identified a considerably higher incidence of glycoproteins in the molecular weight range M_r 210000 to M_r 280000 from carcinomas than benign samples [17]. In particular, a fucosylated glycoprotein of M_r 230000 was identified from 65% of carcinomas but no benign tissues. This clearly has potential as a tumour-associated marker, which could provide prognostic information. In the present study, we have raised a monoclonal antibody by *in vitro* immunisation against the fucosylated glycoprotein of M_r 230000 identified from breast carcinomas. The reactivity of this antibody has been assessed in a range of breast tumours and the relationship between antibody reactivity and clinico-pathological features has been determined.

MATERIALS AND METHODS

Antibody generation

The murine monoclonal antibody used in this study, LU-BCRU-G7, is a second generation antibody derived from a polyclonal antiserum P5252 [18], raised against a fucosylated glycoprotein identified in medium from primary cultures of human breast carcinomas [17].

The polyclonal antiserum P5252 was coupled to sepharose 6B (Pharmacia-LKB, Milton Keynes, U.K.) in borate buffer pH 11. Culture medium from the human breast cell line Hs578T was applied to the column in Tris buffer pH 8.6 and bound glycoprotein was eluted with acetate buffer pH 3.6. This preparation was identified as a broad band of M_r 230000 on SDS-PAGE and was used as the immunogen for raising the monoclonal antibody LU-BCRU-G7.

The second generation antibody was raised using an *in vitro* immunisation method. For each immunisation a spleen cell suspension was prepared from a 3-week-old BALB/c mouse and resuspended in 30 ml of IVIM (*in vitro* immunisation medium); DMEM (Dulbecco's modified eagles medium) supplemented with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin, 10% thymoma cell medium (Sigma, Poole, U.K.), 1% MEM non-essential amino acids (Sigma) and 15% fetal calf serum containing 5×10^{-5} mol/l 2-mercaptoethanol. The P5252 affinity isolate was added to the cell suspension at a concentration of 1 μg/ml, and the spleen cell suspension seeded in a 75-cm^2 flask at 10^7 cells/ml and incubated for 5 days undisturbed at 37°C in an atmosphere of 5% CO_2, 95% air. After incubation, the flask contents were agitated to dislodge the loosely adherent blast cells and the resulting cell suspension centrifuged at 500 *g* for 5 min. Adherent cells were scraped off, washed in serum-free DMEM and seeded into the fusion plates. After washing the non-adherent blast cell pellet once in serum-free DMEM, the cells were fused with $5–8 \times 10^5$ cells of the myeloma line SP2-0-Ag14 in the presence of 50% polyethylene glycol (1500 Da). After diluting with 40 ml SDMEM (supplemented DMEM); DMEM containing 10% fetal calf serum (FCS), 2 mmol/l L-glutamine and 1% MEM non-essential amino acids, aliquots of the cell suspension were dispensed into 96 16-mm wells, and incubated at 37°C in an atmosphere of 5% CO_2, 95% air. After 24 h, 0.5 ml of SDMEM, containing the additional supplements at double strength, hypoxanthine-aminopterin-thymidine (HAT) medium, 20% thymoma cell medium (Sigma) and 20% hybridoma supplement (Sigma) were added to each well. Half-medium changes were performed every 3 days using SDMEM with single strength supplements.

Antibody assay

Supernatant from each well was assayed after day 12, using a cell-based ELISA method using the human breast cancer cell line Hs578T, known to express the glycoprotein of M_r 230000. Positive wells were identified, cloned by serial dilution and retested for antibody production and specificity using immunohistochemistry on known positive breast cancer tissues [17]. Antibody typing was determined by a murine isotyping kit (Amersham International, Aylesbury, U.K.).

Electrophoresis and immunoblotting

Conditioned culture medium from the breast cancer cell line Hs578T was concentrated by centrifugation (Macrosep. Filtron Technology Corporation, Massachusetts, U.S.A) and subjected to electrophoresis in 7.5% polyacrylamide gels under reducing conditions according to the method of Laemmli [19]. After electrophoresis, the gels were blotted onto polyvinylidene difluoride membrane (Immobilon P, Millipore, Watford, U.K.) essentially as described by Towbin *et al.* [20], in the absence of methanol. Molecular weight standards run on the gel were stained on the membrane with 0.1% Coomassie blue in methanol/acetic acid/water. Blots were immunostained either with the polyclonal antiserum P5252, or the murine monoclonal antibodies LU-BCRU-G7, and HMFG 2 (Unipath Ltd., Bedford, U.K.). Primary antibody was detected using a biotin-labelled secondary antibody and the streptavidin-biotin peroxidase complex (ABC) detection system (Dako Ltd., High Wycombe, U.K.). The enzyme was developed with diaminobenzidine (0.5 mg/ml in phosphate-buffered saline (containing 0.015% H_2O_2).

Patients

Primary breast carcinomas excised from 190 patients between 1981 and 1989 were studied. Node status was known for 168 cases, with 98 having evidence of node metastasis. 34 cases were of advanced stage. Primary treatment was either mastectomy or lumpectomy and radiotherapy. All patients with axillary lymph node metastasis received radiotherapy. A small number of patients were given adjuvant chemotherapy. Clinical follow-up was from 7 to 134 months with a median follow-up period of 35 months. Selection of cases was based on availability of tissue and data. In addition to the 190 invasive carcinomas, four examples of normal breast tissue, four cases of fibrocystic change with regular ductal hyperplasia, two fibroadenomas, one example of atypical ductal hyperplasia, two cases of lobular carcinomas *in situ* and 10 cases of ductal carcinoma *in situ* were examined. These tissues had been fixed and processed in a similar manner.

Tissues and histology

All cases were received immediately after resection. A standard block was fixed in 4% formaldehyde in saline for 18–36 h and processed through to paraffin wax. Samples were also frozen and stored in liquid nitrogen. Haematoxylin and eosin-stained sections were examined for classification using WHO criteria and graded for differentiation using a modification of the Bloom and Richardson system [21].

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Revised 23 Feb. 1994; accepted 21 Mar. 1994.

Immunohistochemistry

Before applying the primary antibody all sections were blocked with non-immune rabbit serum. Endogenous peroxidase activity in tissue sections was inhibited by incubation with 0.3% hydrogen peroxide in ultra pure water for 30 min. The mouse monoclonal LU-BCRU-G7 was used at 1/200 dilution for 2 h at room temperature. The primary antibody was detected using the ABC complex detection system. Controls involved the omission of the primary reagent and inclusion of a known positive case in each staining batch. The proportion of reactive cells was determined as a percentage value and categorised into six groups: negative, < 10%, 10–25%, 25–50%, 50–75% and > 75%. This was performed by one observer without knowledge of patient outcome. Intra-observer variation was checked by repeat evaluations of 25 cases 2 months after the initial assessment, without knowledge of the results of the original assessment.

Selected cases were pretreated with β-galactosidase (EC 3.2.1.23) 1.0 U/ml, sialidase (EC 3.2.1.18) 1.0 U/ml, or β-*N*-acetylhexosaminodase (EC 3.2.1.30) 10 U/ml, (Oxford GlycoSystems, U.K.), at 37°C for 18 h before blocking with non-immune rabbit serum.

Oestrogen receptor (ER) and progesterone receptor (PgR) data was available for 130 cases. This had been determined using frozen sections stained with the ERICA kit (Abbott, U.K.) and the monoclonal antibody NCL-PgR (Novocastra, U.K.) with an ABC complex detection system.

Statistics

χ^2 tests were used to examine correlations between antibody reactivity and clinico-pathological parameters. Life table analysis was performed on survival data.

RESULTS

Antibody production

After cloning suitable fusion products, 13 hybridomas were identified as having reactivity with a breast tumour cell line known to express the high molecular weight glycoprotein (M_r 230 000). Further study in breast tissue sections revealed one of these clones, G7, which showed a promising staining pattern and was the subject of this study. This antibody, like all those produced by this method, were of the IgM class. The G7 clone was identified as an IgM with kappa light chain and has been given the notation LU-BCRU-G7.

Immunoblotting

The LU-BCRU-G7 monoclonal identified a single band on 7.5% gels corresponding to the glycoprotein of M_r 230 000 (Figure 1). A higher molecular weight component as recognised by both the HMFG 2 monoclonal antibody and the P5252 antiserum, which migrated as a broad band on 7.5% gels with a mobility corresponding to an M_r 400 000. Two faint bands with a mobility corresponding to approximately M_r 150 000 and M_r 160 000 were also identified by the P5252 antiserum.

Glycosidase treatment

A decrease in reactivity of the LU-BCRU-G7 monoclonal antibody was observed after pretreatment of sections with β-galactosidase but not sialidase or β-*N*-acetylhexosaminodase.

Immunohistochemistry

There was no staining of the normal or benign breast, either within the selected cases or adjacent to breast carcinomas, nor of the atypical ductal hyperplasia or lobular carcinoma *in situ*. Staining of 6 cases of ductal carcinoma *in situ* was seen with 3 having 10–25% of positive cells, 2 with 50–75% cells staining and 1 with greater than 75% positive cells.

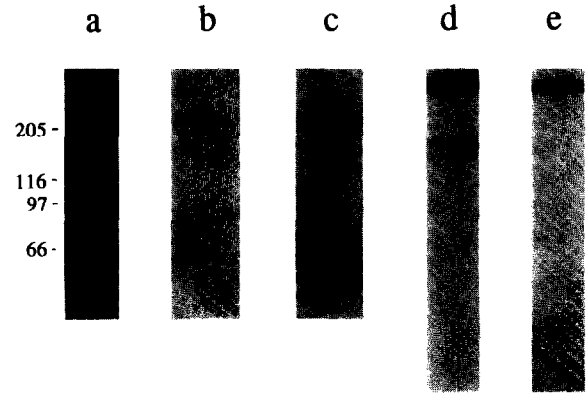

Figure 1. Western blotting of Hs578T-cultured medium, immunostained with (b) clone B8, (c) clone G7, (d) polyclonal antiserum P5252, and (e) HMFG 2. A portion of the blot (a) containing molecular weight markers was stained for protein.

The reactivity of invasive carcinomas is shown in Table 1. Staining was always cytoplasmic (Figure 2). In all analyses, 10% was taken as the cut-off point between positive and negative; 28% of carcinomas were, therefore, negative. Further analyses were undertaken, including the 10–25% category with the negative and < 10% group, since this was closer to the mean.

Relationship to tumour characteristics

The majority of the carcinomas were infiltrating ductal type. There were 19 infiltrating lobular carcinomas and 14 specialised types (mucinous, papillary, tubular, medullary). Half of the specialised types had > 10% of cells staining, with only three having a staining of more than 25% of the cells, in comparison to the overall figure of 54%. Seventeen of the infiltrating lobular carcinomas had more than 10% of positive cells with 10 cases greater than 75%.

There was no relationship between staining with LU-BCRU-G7 and node status or stage (Table 1).

With the cut-off point at 10% there was no relationship between staining and grade [χ^2 2.81, 2 degrees of freedom (df), $0.25 > P > 0.2$]. If the 10–25% group was included with the negative and < 10% categories there was an association (χ^2 7.83, 2 df, $P = 0.02$). This was due to the higher numbers of well-differentiated carcinomas with lower antibody reactivity.

There was no relationship between reactivity and ER and PR status with both cut-off levels.

Relationship to recurrence and survival

This was only considered for stage I and stage II carcinomas. 45 patients had developed recurrent disease during the time period and 29 had died. The relationship of node status and grade with recurrence and survival was examined. There was an association between node status and the development of recurrence (χ^2 6.92, $0.01 > P > 0.005$) and survival (χ^2 6.23, $0.02 > P > 0.01$), with a weaker association for histological differentiation (recurrence, χ^2 7.26, 2 df, $0.05 > P > 0.025$; survival, χ^2 7.37, 2 df, $P = 0.025$). The relationships between reactivity with LU-BCRU-G7 and recurrence and survival are shown in Figures 3 and 4. There was a significant relationship

Table 1. Reactivity of breast carcinomas with LU-BCRU-G7 in relation to tumour characteristics

	Extent of staining					
	Negative	< 10%	10–25%	25–50%	50–75%	> 75%
Total	31(16.3%)	23(12.1%)	33(17.4%)	23(12.1%)	39(20.5%)	41(21.6%)
Node status						
Positive	13	10	19	11	26	19
Negative	14	11	9	6	10	20
Stage						
Early	23	17	26	11	29	31
Advanced	4	4	3	7	8	8
Grade						
I	6	5	9	3	2	3
II	4	13	17	11	26	28
III	11	5	7	9	11	10
Oestrogen receptor						
Positive	14	14	17	12	16	21
Negative	5	4	5	6	10	6
Progesterone receptor						
Positive	11	13	12	12	11	14
Negative	8	5	10	6	15	13

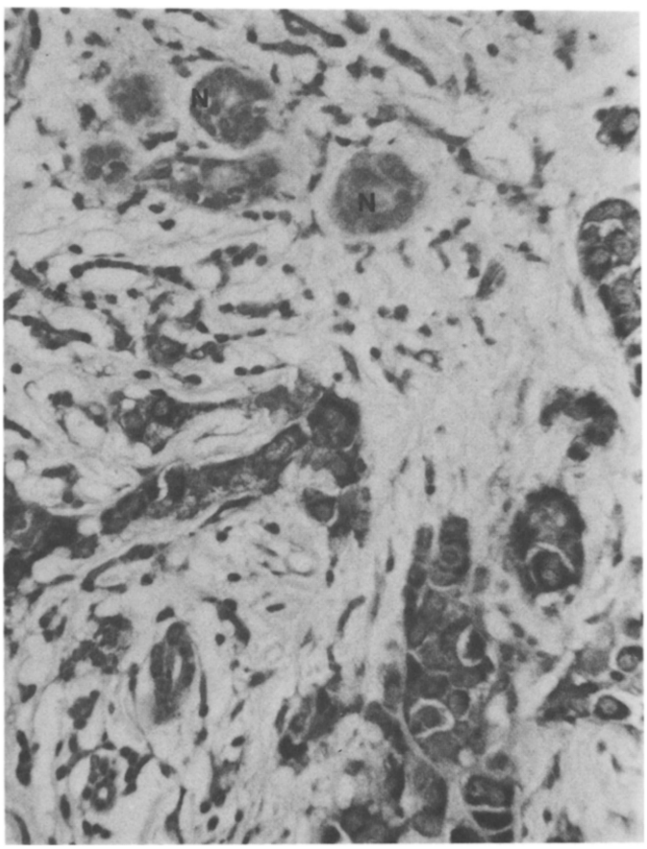

Figure 2. Infiltrating ductal carcinoma showing reactivity of LU-BCRU-G7, in tumour cells but not in normal cells (N). Scale: (9mm) = 20 μm.

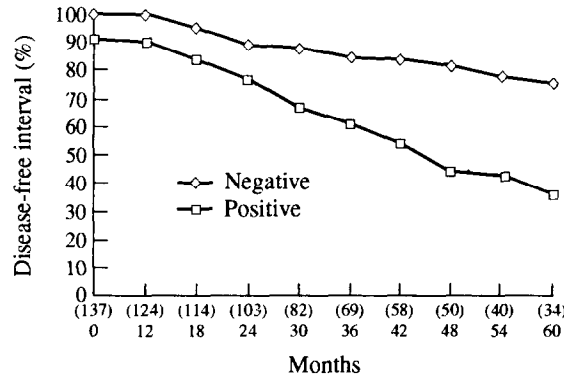

Figure 3. Disease-free interval for patients presenting stage I and II breast carcinomas with reactivity of the monoclonal antibody LU-BCRU-G7 (negative = less than 10% cells staining; positive = greater than 10% cells staining). Figures in parentheses on the abscissa show the total number of patients free from disease at the considered interval.

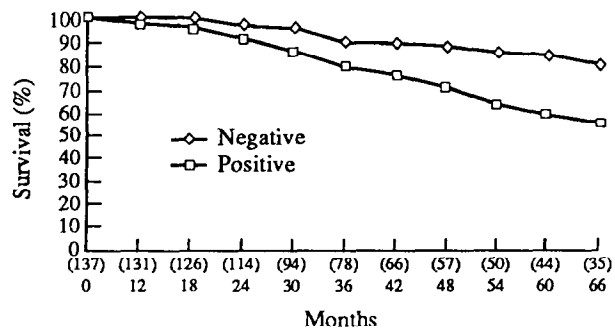

Figure 4. Survival curve for patients presenting stage I and II breast carcinomas with reactivity of the monoclonal antibody LU-BCRU-G7 (negative = less than 10% cells staining; positive = greater than 10% cells staining). Figures in parentheses on the abscissa show the total number of patients alive at the considered interval.

between freedom from recurrence and a lower level of reactivity (< 10% cells staining, χ^2 12.8, $P < 0.001$; < 25% cells staining, χ^2 10.62, $0.005 > P > 0.001$). There was a significant relationship with survival which was greater for those cases having < 25% cells staining (χ^2 7.89, $0.01 > P > 0.005$) than for those with < 10% positive cells (χ^2 6.14, $0.02 > P > 0.01$).

The relationship of staining to survival of cases subdivided on the basis of node status was considered (Figure 5). There was a significant difference in survival for the node-negative cases with less than or greater than 10% cells staining (χ^2 4.29, $0.05 > P > 0.025$): those node-negative cases with < 10% cells staining doing much better than those showing > 10% cells staining. This effect was not so significant in the node-positive group (χ^2 2.90, $0.1 > P > 0.05$).

DISCUSSION

Several monoclonal antibodies have been generated with the aim of detecting tumour-associated antigens [4–12]. However, the majority detect high molecular weight mucins which although expressed in a high proportion of carcinomas are also detectable in normal breast. The use of a fucosylated glycoprotein of M_r 230000 which we have previously demonstrated to be tumour-associated [17], has resulted in the generation of a monoclonal antibody which is different from others in two aspects; there is no staining of normal or benign breast tissue and the cellular localisation in carcinomas is always cytoplasmic. This contrasts with the staining seen with antibodies against high molecular mucins which show a membrane localisation in better differentiated areas and is similar to that seen in normal breast [22]. Two other antibodies have been described which detect glycoproteins of similar size to those reported by us. The one generated by Frankel *et al.* [23] reacted with a higher percentage of carcinomas but also showed reactivity with normal breast. This was not available for comparison studies. The breast carcinoma-associated glycoprotein recognised by the monoclonal antibody CU18 is of a similar molecular weight to that detected by LU-BCRU-G7 but has features typical of a high molecular weight mucins [24]. LU-BCRU-G7, therefore, appears to detect a novel tumour-associated glycoprotein.

Staining was observed in 72% of carcinomas which corresponds to our previous biochemical studies [17] where two-thirds of carcinomas secreted the fucosylated glycoprotein of M_r 230000. The lack of reactivity in the benign tissues, including the premalignant atypical ductal hyperplasia, but staining with ductal carcinoma *in situ*, is also consistent with our observations

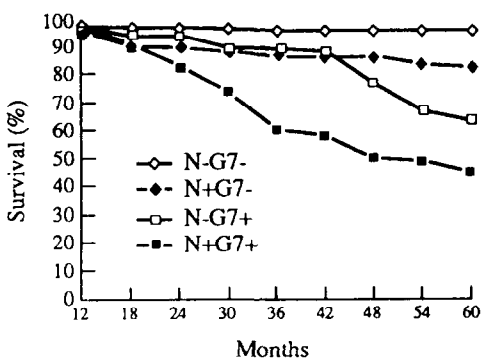

Figure 5. Survival curve showing the relationship of lymph node status with LU-BCRU-G7 reactivity (node status negative = N−; node status positive = N+; G7- = less than 10% cells staining with antibody; G7+ = greater than 10% cells staining with antibody).

that LU-BCRU-G7 detects a tumour-associated antigen. There was no correlation between antibody reactivity and node status or grade. The lack of association with node status is similar to the findings for high molecular weight mucin antibodies [15], with the exception of CaMBr8 [25]. A correlation between staining with NCRC 11 and histological differentiation has been found in several studies [12, 14, 15].

The main findings of this study have been the association between absence of staining for the glycoprotein and improved disease-free interval and, to a lesser extent, survival in the early stage carcinomas. The value of LU-BCRU-G7 as a prognostic marker appears to be greater after 4–5 years of follow-up. This is suggested by the differences in survival in the node-negative group which do not appear until after 42 months. The behaviour of breast cancer is such that it is important to identify markers which will be of value as prognostic indicators for short, medium and long periods of follow-up. Of additional interest has been the finding that the LU-BCRU-G7 antibody can identify differences in behaviour within the node-negative group. This could be of value in the selection of patients within this group who would benefit from further therapy. Follow-up of the patients in this study will be continued to establish the value of LU-BCRU-G7 as a prognostic marker over a longer duration.

Several other antibodies have been investigated for their value as prognostic markers. Wilkinson *et al.* [26] found complete absence of staining with HMFG1 to be associated with an extremely poor prognosis and extracellular staining with this antibody to be associated with a favourable prognosis. HMFG1 is considered to detect a differentiation-associated antigen since high staining tumours are more likely to respond to endocrine therapy and contain steroid receptors [27]. Other studies have failed to find an association between HMFG1 reactivity and prognosis [15, 28] and are all agreed on the lack of prognostic value of HMFG1 [15, 26, 28]. The glycoprotein detected by LU-BCRU-G7, unlike HMFG1, shows no features associated with differentiation in that it is not detected in normal breast and has no relationship with steroid receptors. More prominent staining with NCRC 11 in early stage carcinomas is associated with a better prognosis [12, 14, 15] but it is not an independent indicator due to its close association with histological grade. Studies of the Mam series of antibodies [6] have shown Mam 3b to give prognostic information but to be no better than standard methods [29] and the Mam 6 group of antibodies provides no prognostic information [15]. Expression of the antigen CaMBr8 is associated with nodal metastasis, high mitotic activity and c-*erb*B-2 amplification. However, in a long-term follow-up study this was shown to be associated with a poorer prognosis in only a small subset of breast cancers [25]. Other approaches used in the search for markers of prognosis have utilised the carbohydrate-binding lectins. A survey of many different lectins showed none to be of value in determining short-term prognosis [15]. There has been recent interest in the *N*-acetyl galactosamine-binding lectin, *Helix pomatia*, which has been reported to be of value as a prognostic marker [30, 31]. However, those studies that have found a relationship of *Helix pomatia* with prognosis appear to agree that it is due to a correlation with node status [32]. It is, therefore, different from the findings with LU-BCRU-G7.

Aberrant glycosylation is a common feature of breast carcinomas [16]. It is, therefore, likely that the glycoprotein detected by LU-BCRU-G7 contains glycan structures not normally expressed in non-malignant breast but found in a proportion of carcinomas. Glycosidase treatments have shown that non-

reducing terminal β1-3 or β1-4 galactose is involved in the LU-BCRU-G7 antibody binding. Structural analysis of both the glycan and protein core is required to determine its possible function and relationship to normal glycosylated components.

In conclusion, the antibody LU-BCRU-G7 generated against a tumour-associated glycoprotein of M_r 230 000 appears to be a useful prognostic marker in early stage breast carcinoma, particularly in the node-negative group.

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Acknowledgement—This work was supported by the Cancer Research Campaign.