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Identification of a Monoclonal Antibody-Defined Breast Carcinoma Antigen in Body Fluids

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Abstract—*The monoclonal antibody NCRC-11 defines antigens associated with secretory glandular epithelia as well as most epithelial malignancies. These components have been identified in, and isolated from, normal body fluids including urine and skim milk. The immunoadsorbent purified antigens from urine and milk were very similar to those purified from breast and ovarian carcinomas; by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and immunoblotting, NCRC-11 antibody-binding antigens from all sources were of high apparent molecular weight (> 400 kD) with the major component(s) present as a single band or a doublet. Also, by analysing epitope profiles, all purified antigen preparations were shown to react in a characteristic manner with a panel of monoclonal antibodies which were originally produced against human milk products or materials from tumours.*

Since it was shown that NCRC-11 antigens were released from tissues in a soluble form, the possibility that these antigens might represent a diagnostic marker for breast cancer was evaluated. The findings obtained indicated that NCRC-11 antigens were elevated in the serum of advanced breast cancer patients in comparison to healthy control females, so that access to the circulation was available to these products released from the tumour but not to those released from normal epithelia.

INTRODUCTION

THE ANTI-BREAST carcinoma monoclonal antibody, NCRC-11, reacts with most malignancies of epithelial origin and also with the luminal surfaces of specialized epithelia [1]. The target antigen for this antibody has been isolated from breast and ovarian carcinomas and it has been shown to be a high molecular weight glycoprotein [2, 3]. These molecules also bear epitopes for a number of other monoclonal antibodies some of which were originally raised against products derived from human milk and others against human tumour cells. Clearly, these breast and ovarian carcinoma antigens are immunologically complex [3].

In the present study, the relationship between NCRC-11 antigens produced by tumours and those found in normal tissues and fluids has been investigated. It was determined that NCRC-11 antigens were shed or exfoliated from normal epithelia into secretions (milk) and excretions (urine). This suggested that the antigen might also be released from developing tumour into the local environment of the tumour, and thence into the circulation of the cancer patient. In this way, the possibility that circulating NCRC-11 antigen might be a diagnostic marker for active disease has been evaluated.

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MATERIALS AND METHODS

Monoclonal antibodies

NCRC-11 (IgM) was originally prepared using spleen cells from a BALB/c mouse immunized against dissociated breast carcinoma cells [1]. The following anti-human milk fat globule membrane antibodies were employed: HMFG-1 (IgG1) and HMFG-2 (IgG1) [4]; an anti-human epithelial membrane antigen monoclonal antibody termed EMA (IgG2a) also known as E29 [5] from Dakopatts a/s (High Wycombe, Bucks.); LICR-LON-M8 (IgG1), LICR-LON-M18 (IgM) and LICR-

LON-M24 (IgM) abbreviated to M8, M18 and M24 [6]; 115D8 (IgG1), 115F5 (IgG2) and 115G2 (IgG2) [7]. The antibody Ca1 (IgM) was prepared against wheat germ agglutinin-binding glycoproteins from cultured human laryngeal carcinoma H.Ep2 cells [8], and Ca2 (IgG1) and Ca3 (IgG1) were both prepared by immunization with the purified Ca1-defined antigen [9].

NCRC-11 antibody was purified from ascitic fluid by its binding to and elution from a Sepharose-lentil lectin affinity column (Pharmacia, Uppsala, Sweden), its protein concentration being determined assuming $E^{1\%}_{280\,nm} = 11.9$.

Antigen preparations

Fresh human milk was divided into cream and skim milk by centrifugation for 30 min at 1200 ***g*** [10]. The skim milk fraction was centrifuged at 100,000 ***g*** for 60 min and stored at −20°C. The cream fraction was washed 5 times with phosphate-buffered saline, pH 7.3 (PBS) (30 min at 1200 ***g*** each time) to remove whey proteins and then quickly frozen. The cream was slowly thawed, rupturing membrane globules and vigorously shaken in PBS containing 1 mM $MgCl_2$. When butter formation was observed, the suspension was centrifuged at 100,000 ***g*** for 60 min and the membranes were resuspended in PBS and stored at −20°C [11].

Normal urine was dialysed against PBS + 0.02% NaN_3 and centrifuged at 100,000 ***g*** for 60 min before storage at −20°C.

Concentrations of protein solutions were determined from their absorbance at 280 nm (assuming $E^{1\%}_{280\,nm} = 6.7$, as for bovine serum albumin, BSA) and protein concentrations for milk fat globule membranes were determined by the method of Lowry *et al.* [12] using BSA as standard.

NCRC-11-defined antigen preparations were isolated from detergent (Nonidet P-40) solubilized subcellular membranes from breast and ovarian mucinous carcinomas by immunoadsorbent chromatography using Sepharose-linked NCRC-11 antibodies as previously described [3]. Samples of skim milk and normal urine were also employed as the starting material for NCRC-11 antigen isolation although detergent was not included in the initial sample solution or washing buffers. In all cases, NCRC-11 antigens were dialysed overnight against PBS, centrifuged at 100,000 ***g*** for 60 min and stored at − 20°C [3].

Radioisotopic antiglobulin assay

Milk fat globule membranes, skim milk (at 50 μg/ml in PBS) and urine (at 500 μg/ml) were adsorbed to Terasaki microtest plates as previously described for purified NCRC-11 antigen preparations [3]. Briefly, the radioisotopic antiglobulin assay was performed as follows: after antigen adsorption, the wells were washed 4 times with a washing buffer of PBS + 0.1% BSA + 0.1% rabbit serum (RbS) + 0.02% NaN_3. During the final wash cycle, the wells were incubated for 30 min with washing buffer to complete the blocking of non-specific adsorption binding sites.

Monoclonal antibodies or washing buffer were added at 10 μl/well. All monoclonal antibodies were added at concentrations or dilutions predetermined to be at saturation (i.e. neat hybridoma supernatants, ascitic fluids at 1/1000 and purified IgG or IgM antibodies at 1 or 5 μg/ml, respectively). After incubation for 1–2 h at room temperature, the wells were aspirated and washed 4 times with washing buffer. ^{125}I-Labelled affinity purified $F(ab')_2$ fragments of rabbit anti-mouse Ig were added at 10^5 c.p.m./10 μl/well (radioiodination of this reagent was performed using the chloramine T procedure of Jensenius and Williams [13] using 18 MBq ^{125}I per 25 μg protein). Incubation was continued for 1–2 h at room temperature. The wells were then aspirated, washed 6 times, after which the radioactivity in each well was determined. The non-specific binding of antibodies to 'PBS-coated' and 'BSA/RbS-blocked' wells was determined and the values obtained were subtracted from those determined with antigen-coated, BSA/RbS-blocked and antibody-treated wells.

Double determinant or 'sandwich' radioimmunoassays

NCRC-11 ascitic fluid (1/1000 in PBS + 0.02% NaN_3) was adsorbed on to the wells of Terasaki microtest plates. After incubation at 5°C for 18 h, the wells were aspirated and washed 4 times with washing buffer. On the 4th wash cycle, the plates were incubated with a washing buffer for 1 h in order to block any remaining non-specific binding sites. Aliquots (10 μl) of normal or breast cancer patients' sera (at a dilution of 1/5 or 1/10) or washing buffer alone were added to the wells. Patients' sera was obtained from advanced breast cancer patients with progressive disease at the time of sampling. After incubation for 1 h at room temperature, the wells were aspirated and washed 4 times. [^{125}I]NCRC-11 antibody (radiolabelled according to Fraker and Speck [14]) was added at 10^5 c.p.m./10 μl/well and incubated for 1 h at room temperature. The wells were then aspirated, washed 6 times after which the radioactivity in each well was determined.

Immunoblotting

Urine samples (concentrated approx. 15-fold), skim milk (at 2 mg/ml) or NCRC-11 antigen preparations (in the range 10–100 μg/ml) were diluted 1 : 1 in SDS PAGE reducing sample buffer and then applied to 5–15% gradient polyacrylamide gels, with 3% stacking gels (which had been pre-run

Table 1. *Reactivity of a panel of monoclonal antibodies with various antigen preparations*

Monoclonal antibody	Mean cpm ± S.D. (−background) bound to:				
	NCRC-11 antigen from				
	Breast carcinoma	Ovarian carcinoma	Milk fat globule membranes	Skim milk	Normal urine
NCRC-11	7705 ± 96	7274 ± 116	3252 ± 275	1428 ± 54	2206 ± 211
HMFG-1	6207 ± 464	9380 ± 257	13,182 ± 2099	11,393 ± 814	1213 ± 147
HMFG-2	7551 ± 107	7485 ± 449	6712 ± 213	4811 ± 359	3087 ± 328
EMA	8954 ± 166	7786 ± 214	10,772 ± 1158	5615 ± 341	2588 ± 230
115D8	15,330 ± 471	13,238 ± 305	13,674 ± 617	12,627 ± 495	3697 ± 206
115F5	6963 ± 299	6343 ± 467	14,003 ± 453	7780 ± 441	1807 ± 264
115G2	598 ± 150	2843 ± 105	6407 ± 559	7622 ± 286	192 ± 78
M8	7089 ± 48	7176 ± 241	9991 ± 294	4221 ± 198	2807 ± 247
M18	291 ± 31	1980 ± 235	6884 ± 224	7309 ± 349	572 ± 58
M24	646 ± 161	117 ± 61	5577 ± 161	6142 ± 378	277 ± 102
Ca1	6107 ± 40	3867 ± 232	5799 ± 625	2343 ± 81	415 ± 56
Ca2	6818 ± 219	6954 ± 206	2882 ± 297	1037 ± 126	2984 ± 312
Ca3	7072 ± 246	5946 ± 38	3760 ± 146	2199 ± 73	2120 ± 259

for 15 h at 15 V). Electrophoresis was continued at 50 V for a further 15 h at 50 V using the discontinuous buffer system of Laemmli [15].

Electroblotting onto nitrocellulose membranes was performed essentially as described by Towbin *et al.* [16] using the Biorad Transblot Apparatus for 20 h at 50 V and 200 mA in 25 mM Tris, 192 nM glycine buffer, pH 8.3, containing 20% methanol. The membranes were blocked for 1 h at room temperature using 1% BSA in PBS, and washed 6 times over 30 min. Incubation with NCRC-11 hybridoma tissue culture supernatant was carried out for 3 h at room temperature and thereafter the membranes were washed as before. Horse radish peroxidase conjugated to $F(ab')_2$ fragments of sheep anti-mouse Ig (Amersham International, Amersham, Bucks., U.K.) at a dilution of 1/1000 in 1% BSA in PBS was applied to the nitrocellulose membranes and incubated for 1 h. After washing 9 times with PBS over 45 min, peroxidase activity was detected by addition of 25 mg of 3,3'-diaminobenzidine hydrochloride (B.D.H., Poole,et), in 50 ml 10 mM Tris-HCl, pH 7.4 with 50 μl 30% H_2O_2.

RESULTS

NCRC-11 antigens were isolated and purified from breast and ovarian carcinomas. These showed a characteristic profile of reactivity with a variety of monoclonal antibodies against products derived from human milk and the antibodies Ca1, Ca2 and Ca3 originally prepared against glycoproteins human laryngeal carcinoma cells [8]. The findings presented in Table 1 are in accord with previous results using the same panel of antibodies [3]. This panel of antibodies was also tested against milk fat globule membranes, skim milk and normal urine. All 13 of the antibodies examined reacted with epitopes in products from human milk, with antibodies HMFG-1 and 115D8 being the most reactive. The antibodies M18 and M24 which were of minimal reactivity with the purified antigens, bound strongly to milk fat globule membranes and skim milk (Table 1). The reactivity profile of the panel of antibodies with normal urine was similar to that obtained with the antigens isolated from breast or ovarian tumours.

The nature of the NCRC-11 defined antigens in urine samples was examined by SDS PAGE, followed by electrophoretic transfer onto nitrocellulose membranes which were 'stained' using the NCRC-11 antibody. As shown in Fig. 1 the NCRC-11 antigens were identified in normal urine samples either as a single strong band of high apparent molecular weight (> 400 kD) or as a doublet (with an indication of minor high molecular weight bands) and the mobility of these varied slightly between individuals. These variations and banding patterns of components reacting with the NCRC-11 antibody resemble the genetic polymorphism in the expression of peanut lectin-binding urinary mucins (i.e. products of the 'PUM' locus [17]), and recent studies have established that this system may be defined using the peanut lectin and tumour-binding monoclonal antibodies [18] including NCRC-11 (Swallow and Price, unpublished findings).

NCRC-11-defined antigens were purified from both skim milk and normal urine by their binding to and elution from an immunoadsorbent of Sepharose linked NCRC-11 antibodies. The purified antigens (which were isolated from specimens obtained from single individuals, rather than from pooled samples)

Table 2. Reactivity of a panel of monoclonal antibodies with NCRC-11 defined antigen preparations isolated from skim milk and normal urine

Monoclonal antibody	Mean cpm ± S.D. (− background) bound to NCRC-11 defined antigen preparations isolated from:	
	Skim milk	Normal urine
NCRC-11	2514 ± 372	9715 ± 488
HMFG-1	6519 ± 208	12,158 ± 295
HMFG-2	3701 ± 60	9523 ± 542
EMA	4362 ± 81	10,823 ± 126
115D8	9363 ± 365	15,145 ± 391
115F5	3798 ± 206	9492 ± 141
115G2	5370 ± 230	2730 ± 278
M8	4934 ± 304	10,125 ± 638
M18	5107 ± 249	2395 ± 64
M24	4235 ± 200	243 ± 159
Ca1	1098 ± 152	3951 ± 134
Ca2	2534 ± 177	15,871 ± 339
Ca3	2844 ± 217	9589 ± 229

were examined by SDS PAGE and immunoblotting using the NCRC-11 antibody. As shown in Fig. 2, the various antigens tested (ovarian mucinous carcinoma antigens—track 1; skim milk antigen—track 2; normal urine antigen—track 3) exhibited either one or two major bands of high apparent molecular weight (> 400 kD) reactive with the NCRC-11 antibody. These banding patterns were reproducible on repeated testing and there was no evidence for a trivial explanation (e.g. sample deterioration) for the presence of doublets in purified antigens or unfractionated samples.

The purified antigens from skim milk and urine were examined for their reactivity with the panel of antibodies which had been employed to probe epitopes on purified antigens from breast and ovarian carcinomas. As shown in Table 2, the profile of reactivity of antibodies with these antigens from healthy individuals was similar to that obtained using antigens from tumours (Table 1). Notably the antibody 115D8 consistently displayed high binding with all antigens tested. NCRC-11 showed comparatively lower binding with purified skim milk antigen in relation to its reaction with the other antigens (Tables 1 and 2). The antibody M18, which reacts with Galβ1 → 4GlcNAcβ1 → 6– sequences [19] was reactive with all NCRC-11-defined antigens with the exception of that from breast carcinoma (Tables 1 and 2) although previous studies have shown that this sequence is masked by sialic acid in breast carcinoma antigens and may be exposed by neuraminidase treatment [3]. The determinant defined by the M24 antibody was apparently only found on the purified antigen isolated from skim milk and was not present on any of the other antigen preparations.

Clearly, NCRC-11-defined antigens obtained from detergent-solubilized tumour membrane preparations were similar to NCRC-11-defined antigens isolated directly from skim milk or normal urine, neither of which had been treated with detergent to render the antigens soluble. Therefore, the antigen may be secreted or shed in a soluble form from tissues. SDS PAGE analysis and Western blotting of serum samples from normal individuals and advanced breast cancer patients indicated that patients' serum contained detectable high molecular weight components reactive with the NCRC-11 antibody whereas similar components in normal sera were not identified or only barely demonstrable, with at best, only extremely faint staining of the nitrocellulose membranes. Serum samples were analysed using a 'sandwich' radioimmunoassay with NCRC-11 antibodies bound to the wells of microtest plates; these were treated with diluted serum samples and the capture of NCRC-11 antigens from the sample was then detected by the binding of ^{125}I-labelled NCRC-11 antibodies. Figure 3 summarizes the results of 3 tests. Firstly, in panel a serum samples were tested at a dilution of 1/10 and in panel b samples were tested at a dilution of 1/5. With both approaches, the specificity of the assays was excellent with only one of the normal serum samples above the upper limit of normal reactivity, this being defined as the mean value for normal control samples plus two standard deviations. The sensitivity of these assays was, however, such that 41% of samples in panel a and 51% of samples in panel b displayed values above the upper limit established using the normal serum samples.

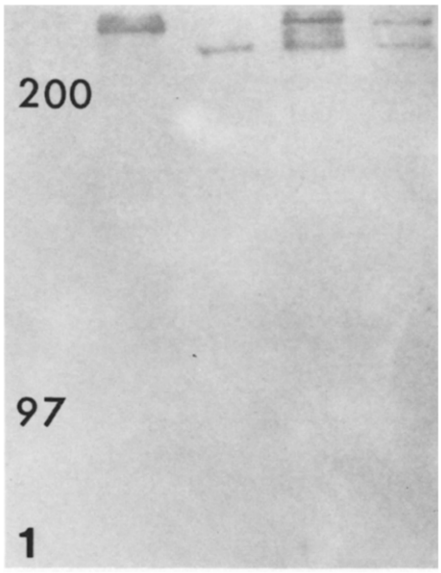

Fig. 1. Immunoblotting of concentrated urine samples from four different individuals. Samples were separated by SDS PAGE, electrophoretically transferred onto nitrocellulose paper and 'stained' with the NCRC-11 antibody. Staining was visualized by incubation with peroxidase conjugated anti-mouse Ig followed by diaminobenzidine. Positions of two molecular weight marker proteins (molecular weight $\times 10^{-3}$ *k) are shown to the left.*

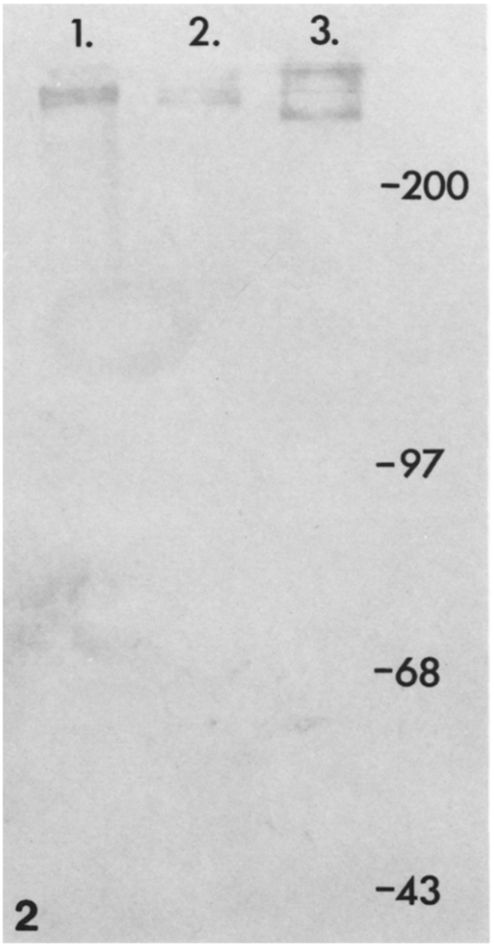

Fig. 2. Immunoblotting of three preparations of immunoadsorbent-purified NCRC-11 antigens. NCRC-11 antigens were isolated from: ovarian mucinous carcinoma (track 1); skim milk (track 2); normal urine (track 3). Molecular weight marker proteins (molecular weight $\times 10^{-3}$ *k) are shown to the right.*

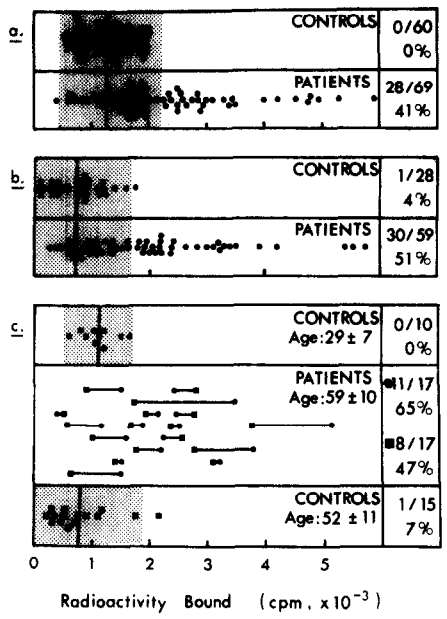

Fig. 3. Double determinant immunoassay (sandwich immunoassay) for NCRC-11 antigen in the serum of advanced breast cancer patients. Serum samples were tested at a dilution of 1/10 *in panels a and c, and at a dilution of* 1/5 *in panel b.*

The data in panel c describe the results of 2 tests separated in time by 6 months and performed with different batches of reagents. The patient samples gave similar results in the 2 tests and the control groups (laboratory controls in the first test and age matched controls in the second test—panel c), although small, also generated comparable ranges for normal values.

DISCUSSION

The conclusions of this investigation are as follows: NCRC-11-defined antigens are present in a soluble form in the milk and urine of normal healthy individuals (the presence of antigen in the urine is not sex-linked). NCRC-11 antigens from normal sources were identified as high molecular weight components which migrated in SDS PAGE gels as a major single band or doublet. This is equivalent to the behaviour of NCRC-11 antigens isolated from breast and ovarian carcinoma subcellular membranes following their solubilization with the non-ionic detergent, N-40. In addition, the profiles of reactivity of a panel of monoclonal antibodies with antigens purified from normal or malignant sources were essentially the same (Tables 1 and 2). The determination of monoclonal antibody-defined epitope profiles of different antigens is a sensitive procedure for the comparison of antigen preparations from different sources.

The results of the immunoblotting tests indicate that the NCRC-11-defined antigens from tumour, skim milk or urine display variations in their mobility in SDS PAGE gels and that the banding patterns are characteristic for samples from different individuals. These findings are entirely consistent with the view that the antibody, NCRC-11, defines the same family of urinary mucins, originally identified by their binding to peanut lectin, and which exhibit a genetic polymorphism [17]. Recent collaborative studies have confirmed this proposal, although at present it remains unknown as to whether, or to what extent, this genetic polymorphism may be involved in any aspect of tumour development or susceptibility to malignant disease.

NCRC-11 antigens from skim milk or urine did not require the action of detergent to effect their solubilization. It was this finding that prompted further testing to determine whether these products gain access to the circulation when they are released from a developing and invasive tumour which is disrupting normal breast tissue architecture. The results in Fig. 3 would indicate that this is indeed the case, and at the present stage of development, the immunoassay for NCRC-11 antigen in breast cancer patients' sera displays excellent specificity for tumours but the sensitivity of the assay requires improvement. Comparable sandwich immunoassays for the detection of serum antigens have been reported using antibodies against human milk fat globule membranes. Antigens reactive with both HMFG-1 and HMFG-2 antibodies have been assayed in patients' sera and 30% of sera from advanced breast cancer patients contained elevated levels of the HMFG-1 antigen as compared with 6% of sera from healthy control women, whereas 53% of patients showed elevated levels of HMFG-2 antigen compared with 17% of controls [20].

Sandwich immunoassays, based upon the use of the 115D8 antibody, have also been employed to detect antigens in the sera of breast cancer patients [21, 22]. Elevated levels of 115D8 antigen were found with increasing frequency according to the staging of the disease so that up to 79% of sera from patients with advanced disease (Stage IV) were positive in the assay. The potential of this serum marker was further illustrated by showing that increasing or decreasing 115D8 antigen levels correlated with breast cancer progression or regression, indicating that the assay can be used to monitor the course of the disease during therapy [22].

It is evident from the present investigation that NCRC-11-defined antigens belong to a descrete family of glycoproteins which are currently being evaluated for their diagnostic potential in a number of laboratories using several different antibodies directly against separate epitopes on what must be a series of immunologically complex molecules.

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