

ARTICULOS COMPLETOS/ FULL PAPERS

TWO MONOCLONAL ANTIBODIES IDENTIFYING HUMAN BREAST CARCINOMA ASSOCIATED ANTIGENS

Mauro Alfonso¹, Lourdes Bouzó¹, Ignacio Ansotegui², Mahmood Jeddi- Tehranio²,
Alvaro Velandia¹, Enrique Rengifo¹, Rubén Ferhandler³, Ana M. Vázquez¹, Anders
Rosén²

¹Center of Molecular Immunology, P.O. Box 16040, Havana 11600, Cuba. ²Karolinska Institute; Box 60400, S-10401 Stockholm, Sweden. ³National Institute of Oncology and Radiobiology, 29 y E, Vedado, Havana, Cuba

Recibido en diciembre de 1992. Aprobado en diciembre de 1993

Key words: Breast carcinoma antigens, monoclonal antibodies

SUMMARY

Two monoclonal antibodies (Mabs), ior-cama1 and ior-cama2, were generated by hybridoma technology, after immunization of Balb/c mice with the breast cancer cell line MCF-7. Both Mabs identify antigens abundantly expressed in membranes of breast cancer tumors, normal breast and other epithelial tissues. Immunoprecipitation of radiolabeled membrane extracts of different cell lines showed that ior-cama1 Mab recognized a large protein antigen of 165/236 kDa in SDS-PAGE under reducing conditions, whereas ior-cama2 reacted with a complex antigen composed of a wide band around 35 kDa and a weaker one of 41 kDa molecular weight. These Mabs recognized antigens released into the body fluids of breast cancer patients where ior-cama1 reacts with an epitope similar or structurally related to CA 15-3 and ior-cama2 seems to be recognizing a high molecular weight secreted antigen.

RESUMEN

Dos anticuerpos monoclonales (AcMs), ior-cama1 e ior-cama2, fueron generados por la tecnología de hibridomas a partir de la inmunización de ratones Balb/c con la línea celular de cáncer mamario, MCF-7. Ambos AcMs identifican antígenos expresados abundantemente en membranas celulares de los tumores mamarios, tejido normal de la mama y otros tumores de origen epitelial. La inmunoprecipitación de extractos de membranas radiomarcados provenientes de diferentes líneas celulares demostró que el AcM ior-cama1 reconoce un antígeno proteico de 165/236 kDa, mientras que ior-cama2 reaccionó con un antígeno complejo que aparece como una banda ancha alrededor de 35 kDa y otra más débil de 41 kDa de peso molecular. Estos AcMs reconocieron antígenos liberados a los fluidos corporales de pacientes con carcinomas mamarios, en los cuales el ior-cama1 reacciona con un epitope similar o estructuralmente relacionado con el CA 15-3 y el ior-cama2 parece reconocer un antígeno secretado de alto peso molecular.

INTRODUCTION

Breast carcinomas continue being the leading malignant tumors in women in most countries (Henney and De Vita, 1988). Thus, reliable markers for breast cancer are of great clinical interest for diagnostic and therapeutic purposes (Thor *et al.*, 1988; Oettgen, 1990). Particular efforts have been made to produce monoclonal antibodies with high tumor specificity and although it has been impossible to find tumor specific Mabs or even organ-specific Mabs, some have shown to be useful for histological, serological and in vitro diagnostic applications when used within a tumor marker panel (Zenklusen *et al.*, 1988).

Numerous markers applicable in the prognosis and diagnosis of breast cancer have been reported to date: Ki67 (Locker *et al.*, 1992), carcinoembryonic antigen (CEA), tissue polipeptide antigen, high molecular weight epithelial mucins, such as CA 15-3, MCA (Miserez *et al.*, 1991), CA M26, CA M29 (Bieglmayer *et al.*, 1991), tumor associated glycoprotein 72 (Soomro and Shousha, 1992) and CA-549 (Shurbaji *et al.*, 1989) are some of the most representative members of this family.

Several tumor associated antigens are released into the circulation of patients. The demonstration of antigens shed by tumors cells into the circulation offers the possibility of assessing the clinical course of disease in cancer patients by monitoring changes in serum levels of such tumor associated antigens (Yedema *et al.*, 1991).

We report here the generation and characterization of two Mabs, ior-cama1 and ior-cama2, that recognize antigens expressed in tumoral cells and that are also released into body fluids of breast cancer patients.

MATERIALS AND METHODS

Generation of Mabs

Hybridomas were obtained by fusing splenocytes from Balb/c mice immunized with membrane extracts of MCF-7 breast cancer cell line (Engel and Young, 1978) and P3-X63-Ag8-653 non-secreting myeloma cells, as previously described (Iacobelli et al., 1985). The screening of hybridoma supernatants was performed by ultra-microELISA (UME) (see below), and the selected hybrids were subcloned twice by limiting dilution. Isotypes were determined by immunodiffusion (Ouchterlony and Nilsson, 1978) on culture supernatants, using class and subclass specific antibodies (Sigma, St. Louis, USA). Monoclonal antibodies were purified from mouse ascites fluid by Protein A-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography. The IgGs were then labeled with alkaline phosphatase (AP) (Grade VII, Sigma, St. Louis, USA) according to the method of Voller et al. (1976).

Table 1
Description of different human cell lines used in this study

Cell line	Origin	Reference
MCF-7	Breast adenocarcinoma	ATCC HTB22
MDA-MB-231	Breast adenocarcinoma	ATCC HTB29
MDA-MB-134	Breast, ductal carcinoma	ATCC HTB23
MDA-MB-157	Breast, medular carcinoma	ATCC HTB24
A-431	Epidermoid carcinoma	ATCC CRL 1554
U-1752	Squamous lung cell carcinoma	Bregh et al., Anticancer Res. 1, 317, 1981
SW-1116	Colorectal carcinoma	ATCC CCL 233
A-375	Malignant melanoma	ATCC CRL 1619
Fetal Fib.	Fetal lung fibroblasts	Hybridoma group Karolinska Inst., Stockholm, Sweden
Raji	B cell Burkitt's lymphoma	ATCC CCL 86
CCRF-CEM	T cell acute lymphoblastic leukemia	ATCC CCL 119
Jutkat	T cell acute lymphoblastic leukemia	ATCC CRL 8179

ATCC=American Tissue-type Culture Collection

Cell lines

Hybridomas and cell lines (table 1) were cultured in an Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 µg/mL) and 2 mM L-glutamine. Insulin (0.6 µg/mL) was added only to breast cancer cell lines.

Preparation of the membrane antigens

Pieces of tissue from normal (breast) or tumor origin (melanoma, lung, colon, breast, and ovarian) were homogenized on ice in 10 mM tris-HCl buffer, pH 7.4, 2 mM Phenylmethylsulfonyl-fluoride (PMSF) using a Polytron homogenizer. The extract was then centrifuged at 100000 xg for 10 min at 4°C. Supernatant was centrifuged again for 1h at 100000 xg at 4°C. Finally the pellet was resuspended in PBS and stored at -70°C (Skoog et al., 1986). Also a mixture of membranes and cytosols from MCF-7 cell line was obtained after homogenization and centrifugation at 10000 xg for 10 min (Bray et al., 1987).

Indirect and sandwich ELISA

The UME was performed indirectly or as a sandwich technique, essentially as described previously (Vázquez et al., 1992). Wells of UME plates (Immunoassay Center, Havana) were coated with 10 µL of the membrane extracts from tumoral tissues of different histological types in carbonate-bicarbonate buffer pH 9.6 over night at 4°C. After washing with distilled water with 0.05% Tween 20, 10 µL of ior-cama1 or ior-cama2 culture supernatants or purified antibody were added. After incubation for 2 h at 37°C and washing, 10 µL of alkaline phosphatase-conjugated sheep anti-mouse immunoglobulins (Sigma, St. Louis, USA) were added for 1 h at 37°C. After washing, the wells were incubated at room temperature with 10 µL of substrate buffer (0.13 mg/mL of 4-methylumbelliferyl phosphate in diethanolamine buffer pH 9.8) and fluorescence at 420-529 nm was measured on a SUMA 101 multifluorimeter. Results were expressed as relative fluorescence units (RFU), each unit correspond to 17 micromoles of hydrolyzed substrate. In sandwich UME, the wells were coated with 20 µg/mL of purified ior-cama2 Mab, then incubated with human ascites fluid from breast cancer patients, at different dilutions, for 2 h at 37°C. Plates were washed and AP-conjugated ior-cama2 was added.

Immunocytochemical studies

Cell suspension (1 x 10⁶ cells/mL) of the different culture cell lines was prepared in tris buffered saline (TBS) containing 1% BSA. One hundred microliters of the cell suspension was added onto slides and dried for 18 h at 24°C. The cells were fixed in acetone:methanol (1:1) for 90 sec and washed in TBS for 5 min. The binding of Mabs to different cell lines was evaluated by the APAAP method (Cordell et al., 1984)

Immunoprecipitation of radiolabeled surface proteins

The surface proteins of 100 x 10⁶ cells of different cell lines were radiolabeled with ¹²⁵I-Na using the lactoperoxidase method (David and Reisfeld, 1974) and immunoprecipitations with ior-cama1 and ior-cama2 Mabs were performed as previously

described (Ansotegui et al., 1991). The immunoprecipitated proteins were then analyzed using 8% homogeneous SDS-PAGE. Amersham radioactive high molecular weight markers were used as standards. Gels were autoradiographed on Kodak XAR film at -70°C.

Enzymatic and chemical treatment

MCF-7 membrane extracts were incubated in 0.1 N NaOH during 5 h at 37°C and then coated on UME well plates. Also, wells previously coated with MCF-7 membrane extracts were treated with 5 mM sodium periodate in 50 mM sodium acetate buffer pH 4.5, pronase E (Sigma, St. Louis, USA) at 0.6 mg/mL in PBS, neuraminidase from *Vibrio cholera* (Type II, Sigma, St. Louis, USA) at 0.006 U/well in 0.05 M sodium acetate buffer pH 5.5; according to methods described before (Vázquez et al., 1992). Methanol lipid extraction was performed for 30 min at 4°C. After treatment, the binding of Mabs was detected by indirect ELISA.

Blocking assays

Blocking of Mabs activity was performed by mixing one volume of a pre-tested concentration of Mab with an equal volume of antigen samples, e.g. breast carcinoma patient ascites fluid at various dilutions. The mixture was incubated for 3 h at 37°C and the reactivity of monoclonal antibodies was measured by an indirect UME in plates coated with MCF-7 membrane extracts described above. PBS or culture medium were used as negative control to avoid a background reactivity. In parallel experiments, samples of ascites fluid of a breast cancer patient were incubated with different dilutions of Mab concentrated supernatants during 16 h at 4°C and after, the presence of CA 15-3 antigen was detected by the immunoradiometric assay ELSA-CA 15-3 (CIS, France).

Fractionation of ascites fluid from a breast cancer patient

Ascites was centrifuged at 37000 rpm for 15 min and filtered through a 0.22 µm nitrocellulose steril filter. One hundred microliters of the supernatant fluid was applied to a Superose 6B

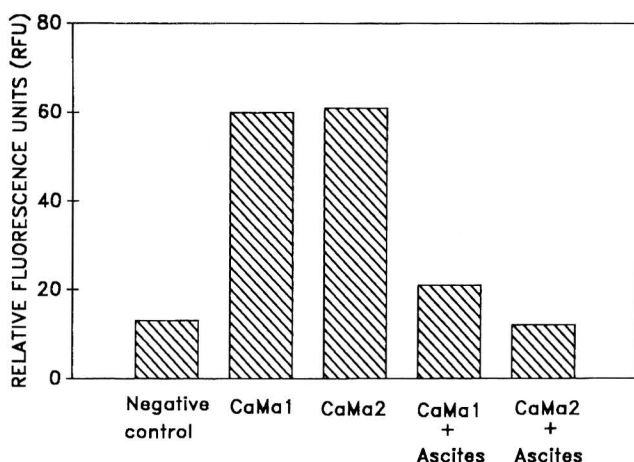


Fig. 1. Recognition of different membranes extracts by ior-cama1 and ior-cama2 Mabs. (NB) normal breast, (CRC) colorectal carcinoma, (MEL) melanoma, (MED) mediastine, (BAC) breast adenocarcinoma, (ECL) lung epidermoid carcinoma, (BDIC) breast ductal invasive carcinoma, (OC) ovarian carcinoma.

TABLE 2

Immunocytological reactivities of ior-cama1 and ior-cama2 Mabs with different cell lines.

Cell line	% Positive Cells		Intensity	
	cama1	cama2	cama1	cama2
Breast cancer MCF-7	100	100	+++	+++
MDA-MB-231	100	100	+++	+++
MDA-MB-134	100	100	+++	+++
MDA-MB-157	100	100	+++	+++
Epidermoid carcinoma A-43	25	50	+	+
Lung cancer U-1752	70	80	+++	+
Colorectal cancer SW-1116	30	30	+	+
T cell leukemia CEM	0	0	-	-
B lymphoma Raji	0	0	-	-

column HR 10/30 (Pharmacia Fine Chemicals) equilibrated in PBS pH 7.4, containing 0.1% sodium azide. The elution was performed at 0.5 mL/min flow rate. Fractions (0.25 mL) were collected and assayed by sandwich UME, using ior-cama2 Mab.

RESULTS

The fusion with splenocytes of mice immunized with membrane extracts of MCF-7 cell line, two clones (ior-cama1 and ior-cama2) producing IgG1 Mabs were selected on the basis of the UME reactivity to MCF-7 membrane extracts. Further characterization of the reactivity of these Mabs with different membrane extracts showed that they not only recognize membrane extracts of mammary tumors but also a wide pattern of reactivity with membrane

TABLE 3

Effect of chemical and enzymatic treatment of MCF-7 membrane extracts on the binding of ior-cama1 and ior-cama2 Mabs.

Treatment	% of binding reduction	
	ior-cama1	ior-cama2
NaOH	0	9
Pronase	60	70
Neuraminidase	3	8
Sodium periodate	2	0
Methanol	0	6

extracts from normal or tumoral tissues of different histological types was found, including lung carcinoma, colorectal carcinoma, ovarian carcinoma, normal breast, etc (figure 1).

Immunocytochemical studies showed that both Mabs were positive in stainings of several breast cancer cell lines and others of epithelial origin, whereas no reaction was observed in lymphoid cell lines, e.g. Raji and CEM (table 2). These results correlate with the reactivity of both Mabs in immunohistochemical stainings on frozen or paraffin-embedded sections of human tissues which have shown that the recognition pattern of these Mabs is not restricted to breast tumors (data not shown).

To determine the biochemical nature of the epitope recognized by these Mabs, enzymatic and chemical treatments were tested on binding to breast carcinoma

membrane extracts. In both Mabs the reactivity against these membrane extracts was inhibited approximately in 60 and 70% respectively after protease digestion using pronase E (table 3), whereas no significant binding reduction, neuraminidase and alkali treatments, which suggests that the epitopes recognized by these Mabs are located on the protein backbone of the antigenic molecule. Because carbohydrate epitopes may be attached to different carrier molecules and the carbohydrate composition of glycoproteins may vary between individuals (Stahli *et al.*, 1988), generally less variable protein epitopes such as those recognized by ior-cama1 and ior-cama2 Mabs, may be preferable in assays for the quantification of highly glycosylated circulating antigens.

Analysis of radiolabeled membrane immunoprecipitates (IP) in SDS-PAGE showed that

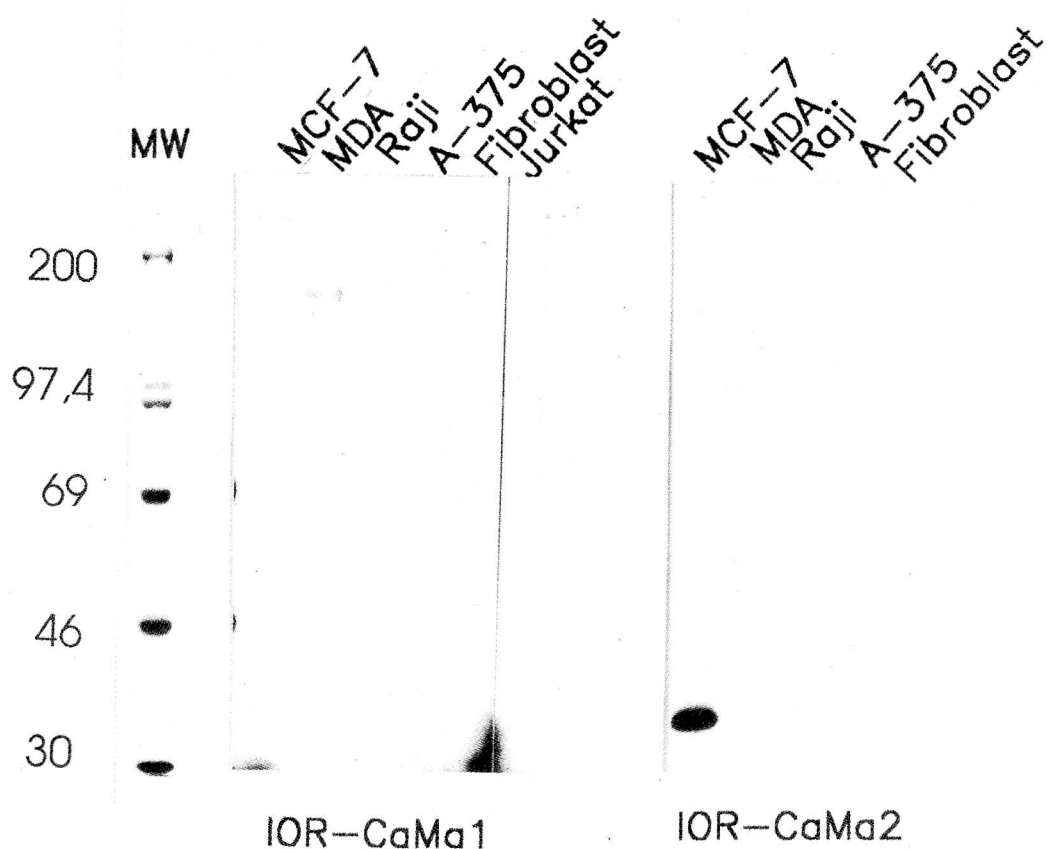


Fig. 2. SDS-PAGE analysis of iodinated antigens precipitated from breast cancer cell lines by ior-cama1 and ior-cama2 Mabs. Other cell lines were included in this study. Vertical scale: molecular weight markers expressed in kDa

ior-cama1 precipitated a high molecular weight protein in two breast cancer cell lines: 236 kDa in MCF-7 and 165 kDa in MDA cell line; whereas ior-cama2 recognized a lower molecular weight protein complex composed of a broad protein band around 35 kDa and a weaker one of 41 kDa in MCF-7 cell line (figure 2). Unexpectedly, an appreciable band of lower molecular weight than those corresponding to the breast cancer cell lines was immunoprecipitated using radiolabeled proteins from Raji cell line with ior-cama1 Mab. This result contradicts previous findings on immunohistochemical analysis which have

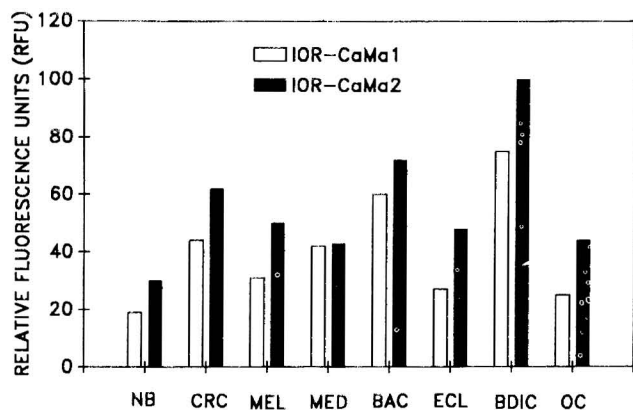


Fig. 3. Inhibition of Mab reactivity by ascites fluid from a breast cancer patient.

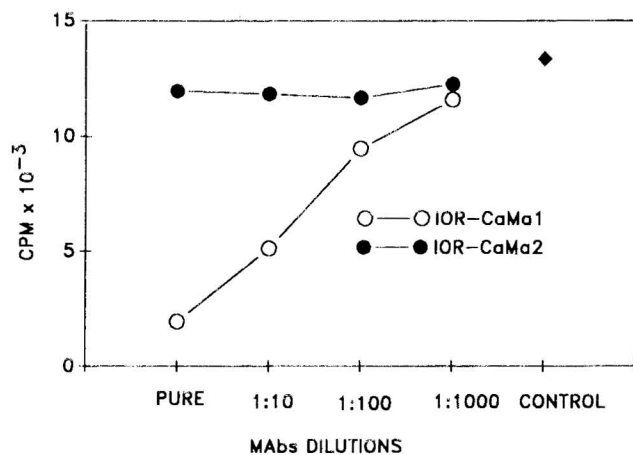


Fig. 4. Blocking of CA 15-3 antigen binding after incubation of malignant ascites fluid with ior-cama1 and ior-cama2 Mabs.

confirmed the absence of reactivity between ior-cama1 and lymphoid cells. Several cell lines of other linages were negative in the immunoprecipitation experiments and also no staining was produced by an isotype-matched antibody of irrelevant specificity (data not shown).

Inhibition assays demonstrated that binding of the Mabs to the breast cancer membrane antigens from MFC-7 cell line were drastically blocked by pre-incubation with ascites fluid derived from a breast cancer patient (figure 3), indicating that antigens recognized by these Mabs were released into the ascites fluid. The immunoradiometric

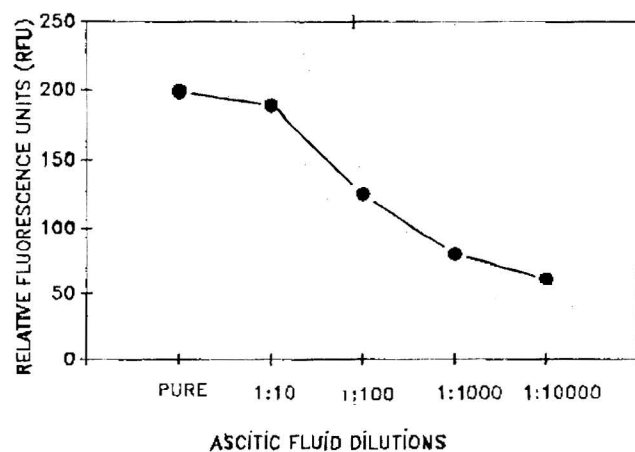


Fig. 5. Recognition of the antigen in malignant fluid ascites by ior-cama2 Mab using a sandwich UME.

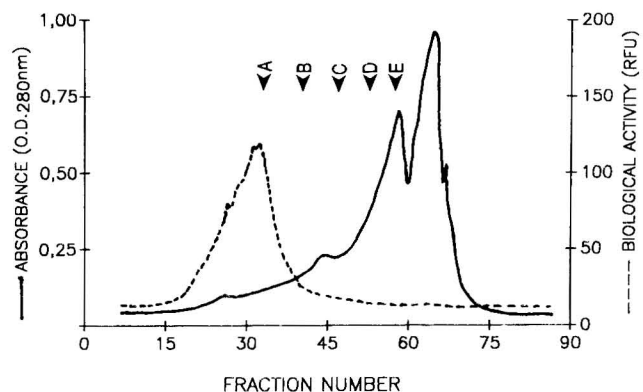


Fig. 6. Gel filtration chromatography of human ascites fluid from a breast cancer patient using Superose 6B. Fractions were monitored for protein by absorbance at 280 nm (continuous line) and for the antigen (discontinuous line) by sandwich UME using ior-cama2 Mab. The column was calibrated with Blue dextran (A), Thyroglobulin (B), Ferritin (C), Catalase (D) and BSA (E) molecular weight markers.

assay (IRMA) for the determination of the well-established tumor marker CA 15-3 showed positive values of this antigen for the human ascites fluid tested. We observed that ior-cama1 Mab blocked the reactivity of the ascites sample in the ELISA CA 15-3 system in a concentration dependent manner, whereas ior-cama2 did not block the binding in any of the concentrations tested (figure 4). Thus, ior-cama1 seems to recognize an epitope similar or structurally related to the CA 15-3 epitopes while ior-cama2 detects a different breast cancer associated secreted antigen.

As the epitope recognized by ior-cama2 Mab seems to be repeatedly expressed on the antigenic molecule, an homogeneous sandwich UME was developed using ior-cama2 both as capture antibody (20 µg/mL) and as probe. A titration curve was obtained using different dilutions of samples containing the breast cancer antigen (figure 5).

Fractionation of breast carcinoma ascites fluid on a Superose 6B gel filtration column showed that the secreted antigen recognized by ior-cama2 eluted in the void volume, indicating a molecular weight equal to or greater than 1.5×10^6 kDa (figure 6).

The presence of the antigen in the different fractions collected from the gel filtration chromatography was detected using the sandwich UME previously described.

DISCUSSION

In view of the high incidence of breast cancer in women, many attempts have been made to arise unique clones producing antibodies by hybridoma technology, which may have potential diagnostic value (Ceriani *et al.*, 1990; Stein *et al.*, 1991).

The reported lack of specific breast cancer Mabs, is not a serious problem because the binding ratio tumor/normal cell has been high enough for practical purpose required for operational specificity (Perrone *et al.*, 1990).

In the present paper we have studied two new Mabs recognizing tumor associated antigens. We used the strategy of immunization with whole cell membrane extracts followed by screening of Mabs in ELISA against various target membrane antigens of breast and non-breast lineages. The two Mabs studied in detail were selected due to their stronger reactivity with breast carcinoma compared to normal breast and other epitheloid cell lines. Pan-epithelial epitopes of

this type have been reported previously (Skilton *et al.*, 1990; Pancino *et al.*, 1991). However, all the described breast tumor associated antigens have shown to be not truly specific for tumors since they also react with different normal tissues or cancer cells of various origins (Blottiere *et al.*, 1991).

Noteworthy is the absence of reactivity of these Mabs with lymphoid cell lines. This is an important observation since localization of micrometastases in axillary lymph nodes have crucial prognostic implications in breast cancer patients (Salvadori *et al.*, 1990). In fact, ior-cama1 Mab has started to be used in the detection of breast cancer lymph node metastases and promising results have been obtained in this field (Tormo *et al.*, 1992).

Monoclonal antibodies generated by several groups have been used to detect micrometastatic lesions that are undetectable by conventional diagnostic procedures. The use MBr1 (Porro *et al.*, 1988), anti-keratin Mab LE61 and anti-CEA Mab 11.285.14 (Ghosh *et al.*, 1985) which recognize breast associated antigens but are negative on normal bone marrow cells demonstrated their ability to identify microfoci of neoplastic cells in bone marrow that can not be detected with conventional histology.

Several epithelial tumors, such as breast cancer, release antigens into body fluids which constitute diagnostic markers (Van Dalen, 1989; Robertson *et al.*, 1990). The ior-cama1 and ior-cama2 Mabs bind to antigens secreted at least to ascites fluid of breast cancer patients. The fact that ior-cama2 could be used for capture and AP-conjugated as detection system demonstrated that the epitope recognized by ior-cama2 is repeatedly expressed in the antigenic molecule.

The finding of Mabs reactive both with membrane and circulating antigens has been extensively reported by other researchers (Tondini *et al.*, 1989; Xing *et al.*, 1989).

The two Mabs included in this study immunoprecipitated molecules from breast carcinoma cell lines of completely different size. The ior-cama1 Mab identifies an epitope expressed not only in a large membrane antigen but also present in circulating antigens. The slight difference found in the molecular weight of this antigen in MCF-7 and MDA breast cancer cell lines could be explained due to the differential glycosilation levels present in this kind of molecules which has been reported previously (Hakomori, 1989). There are initial evidences that the

epitope recognized by ior-cama1 could be present in the structure of the CA 15-3 antigen (molecular weight 300-450 kDa) but the similarity between this Mab and those included in the solid-phase RIA CA 15-3 (115 D-8 and DF3 Mabs) (Kufe, 1988), should be tested in the future. In fact, the molecular weight of the antigen identified by ior-cama1 is quite different to that reported to the CA 15-3 antigen. In contrast, the epitope identified by ior-cama2 seems to be shared in a low molecular weight membrane complex antigen and in a large secreted molecule (MW 1.5×10^6 kDa), probably a mucin-like antigen. The detection of coexisting epitopes on different molecules confirms the presence of a close relationship among several mucin antigens. Mabs recognizing epitopes on both high- and low-molecular weight antigens, like the b-12 Mab, have been previously reported (Stahli *et al.*, 1988). In the case of the antigen recognized by ior-cama2 Mab, we found an apparent molecular weight determined by SDS electrophoresis in immunoprecipitation experiments that was markedly lower as compared with evaluations of the released antigen by column chromatography. The possibility of mucin aggregate formation that has been already described (Bieglmayer *et al.*, 1991) or the fact that ior-cama2 is recognizing a coexisting epitope present in two completely different antigens could be considered as possible explanations to this finding.

Further studies are necessary to determine the value of these Mabs in the diagnosis and/or prognosis of breast cancer.

ACKNOWLEDGEMENTS

This study was supported by SAREC (A Swedish-Cuban Scientific Exchange Program), funds at the Oncology Institute, Havana, Cuba, and grants from Karolinska Institute, Stockholm, Sweden.

REFERENCES

- ANSOTEGUI, I.J.; S.C. CHOW; M. JEDDI-TEHRANI; S. MELOCHE; A. PAWSON; R.P. SEKALY; T.W. MAK and H. WIGZELL (1991). Characterization of a monoclonal antibody directed against the phosphotyrosine kinase p56^{lck} in human T cells. *Scand. J. Immunol.* **33**:375-380.
- BIEGLMAYER, C.; T. SZEPESEI; B. KOPP; G. HOFFMANN; W. PETRIK; K. GUETTOUCHE; S. GRUNDLER; M. GREGORITS and M. STRASSER (1991). CA15.3, MCA, CAM26, CAM29 are members of a polymorphic family of mucin-like glycoproteins. *Tumor Biol.* **12**:138-148.
- BLOTTIERE, H.; S. PELHATE; J. AUBRY; M. KREMER; P. THEDREZ; C. SAI-MAUREL; J. DOUILLARD and J. CHATAL (1991). A new tumor associated antigen expressed on breast carcinomas, defined by monoclonal antibody BCA 227. *Canc. Res.* **51**:1537-1543.
- BRAY, K.R.; J. KODA and P.K. GAUR (1987). Serum levels and biochemical characterization of cancer associated antigen CA-549, a circulating breast cancer marker. *Cancer Res.* **47**:5853-5860.
- CERIANI, R.L.; E.W. BLANK; E.H. ROSENBAUM; D.B. ZEEV; R.S. LOWITZ; L. JOHANSEN and T. TRUJILLO (1990). Diagnostic ability of different human milk fat globule antigens in breast cancer. *Breast Cancer Research and Treatment* **15**:161-174.
- CORDELL, J.L.; B. FALINI; W.N. ERBER; A.K. GHOSH; Z. ABDULAZI; S. MCDONALD; A.F. PULFORD; H. STEIN and D.Y. MASON (1984). Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP) complexes. *J. Histochem. Cytochem.* **32**:219-229.
- DAVID, G.S. and R.A. REISFELD (1974). Protein iodination with solid state lactoperoxidase. *Biochemistry* **13**:1014-1021.
- ENGEL, L.W. and N.A. YOUNG (1978). Human breast cancer cell lines in continuous culture: A Review. *Cancer Res.* **38**:4327-4339.
- GHOSH, A.K.; W. ERBER; C. HATTEN; N. O'CONNOR, B. FALINI; M. OSBORN and D. MASON (1985). Detection of metastatic tumor cells in routine bone marrow smears by immunoalkaline phosphatase labelling with monoclonal antibodies. *Br. J. Haematology* **61**:21-30.
- HAKOMORI, S. (1989). Aberrant glycosilation in tumors and tumor-associated carbohydrate antigens. *Advances in cancer research* **52**:257-329.
- HENNEY, J.E. and V.T. DE VITA (1988). In: *Breast Cancer. Principles of internal Medicine* (eds. Petersdorf et al.) N.Y. pp. 788-795.
- IACOBELLI, S.; V. NATOLI; G. SCAMBIA; G. SANTEUSANIO; R. NEGRINI and C. NATOLI (1985). A monoclonal antibody (AB/3) reactive with human breast cancer. *Cancer Res.* **45**:4334-4338.
- KUFE, D.W., D.F. HAYES and M. ABE (1988). In: *Monoclonal antibody assay for breast cancer*. Institute Harvard Medical School, Boston, Massachusetts, pp. 67-99.
- LOCKER, A. P.; K. BIRRELL; J.A. BELL; R.I. NICHOLSON; C.W. ELSTON; R.W. BLAMEY and I.O. ELLIS (1992). Ki67 immunoreactivity in breast carcinoma: relationships to pronostic variables and short term survival. *Eur. J. Surg. Oncol.* **18**(3):224-229.
- MISEREZ, A.R.; I. GUNES; J. MULLER-BRAND; E. WALTHER; R. FRIDRICH and H. MACKE (1991). Clinical value of a mucin-like carcinoma-associated antigen in monitoring breast cancer patients in comparison with CA 15-3. *Eur. J. Cancer* **27**:126-131.
- OETTGEN, H.F. (1990). Biological agents in cancer therapy: cytokines, monoclonal antibodies and vaccines. *J. Canc. Res.Clin. Oncol.* **116**:116-119.
- OUCHTERLONY, O. and L.A. NILSSON (1978). Immunodiffusion and Immuno-electrophoresis. *Handbook of Experimental Immunology* (ed. D. M. Weir), Blackwell Scientific Publications, Oxford.
- PANCINO, G.; M.H. MORTADA; C. CHARPIN; E. OSINAGA; P. DE CREMOUX; B. BATAILLE; M.G. GOBERT; F.

- CALVO and A. ROSETO (1991) Two monoclonal antibodies identify antigens preferentially expressed on normal human breast cells versus breast cancer cells. *Hybridoma* **10**(2):241-253.
- PERRONE, F.; S. MENARD; M.G. DA DALI; F. LEONI; S. DI PALMA; C. PATRIARCA; F. RILKE and M.I. COLNAGHI (1990). Expression of two antigens defined by monoclonal antibodies in normal, benign and malignant human mammary tissues. *Tumori* **76**:525-529.
- PORRO, G.; S. MENARD and E. TAGLIABUE (1988). Monoclonal antibody detection of carcinoma cells in bone marrow biopsy specimens from breast cancer patients. *Cancer* **61**:2407-2411.
- ROBERTSON, J.F.R.; D. PEARSON; M.R. PRICE; C. SELBY, R.A. BADLEY; J. PEARSON; R.W. BLAMEY and A. HOWELL (1990). Assessment of four monoclonal antibodies as serum markers in breast cancer. *Eur. J. Cancer* **26**:1127-1132.
- SALVADORI, B.; P. SQUICCIARINI; D. ROVINI; S. OREFICE; S. ANDREOLA; F. RILKE; L. BARLETTA; S. MENARD and M.I. COLNAGHI (1990). Use of monoclonal antibody MB1 to detect micrometastases in bone marrow specimens of breast cancer patients. *Eur. J. Cancer* **26**:865-867.
- SHURBAJI, M.; R.A. BEVERIDGE; D.W. CHAN; R.C. ROCK and F.P. KUHAJDA (1989). CA 549: Immunohistochemistry and serum levels in breast carcinoma and other neoplasms. *Ann. Clin. Lab. Science* **19**:408-414.
- SKILTON, R.A.; H.M. EARL; M.E. GORE; R.A.J. MCILHINNEY; B.A. GUSTERSON; R. WILSON; R.C. COOMBES and A.M. NEVILLE (1990). Characterization of monoclonal antibodies reactive with normal resting, lactating, and neoplastic human breast. *Tumor Biol.* **11**:20-38.
- SKOOG, L.; A. MACIAS; E. AZAVEDO; J. LOMBARDEO and C.B. KLINTENBERG (1986). Receptors for EGF and oestradiol and thymidine kinase activity in different histological subgroups of human mammary carcinomas. *Br. J. Cancer* **54**:272-276.
- SOOMRO, S. and S. SHOUSA (1992). Monoclonal Antibody B72.3 immunostaining of breast carcinoma. *Arch. Pathol. Lab. Med* **116**:32-35.
- STAHLI, C.; M. CARAVATTI; M. AESCHBACHER; C. KOCYBA; B. TAKACS and H. CARMANN (1988). Mucin-like carcinoma associated antigen defined by three monoclonal antibodies against different epitopes. *Canc. Res.* **48**: 6799-6802.
- STEIN, R.; D.M. GOLDENBERG and M.J. MATTES (1991) Normal tissue reactivity of four anti-tumor monoclonal antibodies of clinical interest. *Int. J. Cancer* **47**:163-169.
- THOR, A.; J. SIMPSON; N. OCHUCHI; P. HORAN; C.A. SZPAK; W.W. JHONSTON and J. SCHLOM (1988). Monoclonal antibodies and human carcinomas: Diagnostic and experimental applications. In: *Advances in Immunohistochemistry* (ed. Ronald A. Delellis), Raven Press, New York.
- TONDINI, C.; D. F. HAYES and D. W. KUFÉ (1989). Circulating tumor markers in breast cancer. *Cancer Hematology/Oncology Clinics of North America* **3**(4):653-673.
- TORMO, B. R.; M. ALFONSO; E. RENGIFO; S. FRANCO and A. CHONG (1992). Immunoperoxidase staining with ior-camal monoclonal antibody in the detection of breast cancer lymph node metastases. *Biotechnologia Aplicada* **9**(3):259-262.
- VAN DALEN, A. (1989). Tumor markers in breast cancer. *Annales Chirurgiae et Gynaecologiae* **78**:54-64.
- VAZQUEZ, A. M.; B. TORMO; A. VELANDIA; R. GISCOMBE; I. J. ANSOTEGUI; M. JEDDI-TEHRANI; R. PEREZ; H. MELLSTEDT and P. BIBERFELD (1992). Characterization of the colorectal antigen ior-c2. *Hybridoma* **11**(2):245-251.
- VOLLER, A.; D. E. BIDWELL and A. BARTLETT (1976). Microplate enzyme-immunoassays for immunodiagnosis of virus infections. In: *Manual of Clinical Immunology*. Chapter 69 (eds. N. Rose and H. Fridman), Pub. American Soc. for Microbiology 506-512.
- XING, P. X.; J. J. TJANDRA; S. A. STACKER; J. G. TEH; C. H. THOMPSON; P. J. MCLAUGHLIN and I. F. C. MCKENZIE (1989). Monoclonal antibodies reactive with mucin expressed in breast cancer. *Immunol. Cell Biol.* **67**:183-195.
- YEDEMA, K. A.; P. KKENEMANS; T. WOBBS; G.J. VAN KAMP; H.W. DE BRUIJN; C.M. THOMAS; L.F. MASSUGER; C.P. SCHIJF; G. G. BON; J. B. VERMORKEN; F. VOORHORST and J. HILGERS (1991). Carcinoma associated mucin serum markers CA M26 and CA M29: Efficacy in detecting and monitoring patients with cancer of the breast, colon, ovary, endometrium and cervix. *Int. J. Cancer* **47**:170-179.
- ZENKLUSEN, H.R.; C. STAHLI; F. GUDAT; J.V. OVERBECK; J. ROLINK and Ph.U. HEITZ (1988). The immunohistochemical reactivity of a new anti-epithelial monoclonal antibody (MAb b-12) against breast carcinoma and other normal and neoplastic human tissues. *Virchows Archiv. A Pathol. Anat.* **413**:3-0..LS1