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Monoclonal antibodies against antigens on breast cancer cells

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Summary. Of 360 mAb obtained in a cell fusion experiment with the spleen cells of a mouse immunized with a mixture of different human breast carcinoma cell lines, 30 mAb were selected which reacted more strongly with tumor cells than with (noncancerous) fibroblasts. These mAb were tested for reactivity with additional types of cancerous and noncancerous tissues. Two mAb showed high tumor selectivity, but the corresponding epitopes on individual tumor cells were heterogeneously expressed. The mAb will be evaluated for in vivo applications.
Key words. Murine monoclonal antibodies; tumor associated antigens; breast carcinomas.

Introduction

The advent of the mAb technology in 1975[5] renewed hopes of finding truly tumor specific antigens, bearing the promise of developing methods for tumor detection, localization and therapy. Despite efforts in many laboratories, however, antigens appearing exclusively on tumor cells have not yet been found. Early claims of tumor specificity, for example of mAb specific for carcinomas by recognition of an antigen named Ca[1,8], had to be abandoned after more extensive evaluation of the mAb reactivities with different types of normal human tissue[9]. Even if true tumor surface antigens were eventually to be found, they might not play a significant role in tumor localization and therapy, if the difficulties in finding them were a reflection of their low concentration. On the other hand, mAb reacting with malignant cells and restricted parts of normal tissue may be useful in spite of these normal tissue reactivities. In fact, such mAb have already proved useful, e.g. for radio localization of carcinomas of the colon[6] and of the breast[4]. Some of the unspecific background on such radioscintigraphs may be caused by normal cell reactivities of the mAb. The larger part of the background, however, is likely to be caused by unspecific effects, since significant background is also observed in nude mouse tumor models lacking normal tissue cross-reactivities. Furthermore, since such mAb usually do not react with all cells in any one tumor, mixtures (cocktails) of several mAb may have to be used. For therapy this may be further necessitated by the likelihood that even these antigens may be present at such low concentrations on cell surfaces that individual mAb cannot be effective[2]. Cocktails of mAb which react with restricted parts of normal tissue may furthermore have the advantage of

increased tumor selectivity if they reacted cumulatively with tumor cells but individually with different types of normal cells. Bearing these considerations in mind, the surface antigens of breast carcinoma cells were analyzed.

Materials and methods

1) Antigens

For immunization and/or for the primary screening the following human cell lines were used:

Four breast carcinoma lines: Hs0578T, established in 1971 by Dr A. J. Hackett at the Naval Biomed. Res. Lab., Oakland, Cal.; SK-BR-3, established in 1970 by Drs G. Trempe and L. J. Old; MCF-7, established in 1970 by Dr H. D. Soule at the Michigan Cancer Found., Detroit, Mich.; ZR-75-1, established in 1975 by Drs L. W. Engel and N. A. Young, at the NCI, Bethesda, Maryland. Hs0578T, MCF-7 and ZR-75-1 were karyotypically properly identified for us by Dr G. Staiger at our laboratories;

Two fibroblast lines: HF-LO, taken from a male patient was established by Dr B. Temminck, Kinderspital, Basel, Switzerland; HF-NF, of unknown origin, was obtained also from Dr B. Temminck.

2) Generation of the mAb

A 6-week-old female Balb c/J mouse was immunized by i.p. injection of whole cells: 10^7 Hs0578T+4 × 10^7 SK-BR-3 cells; 6 weeks later it was again given an i.p. injection of 2 × 10^7 cell of each of Hs0578T, SK-BR-3, ZR-75-1, MCF-7; finally, 7 weeks later this immunization was again repeated; however SK-BR-3 cells were not included because of a mycoplasma contamination; Three days later the spleen cells (2 × 10^8) were fused with an equal number of mouse myeloma cells (nonsecretor line PAI[15]), and distributed into 960 hybridoma cultures as described elsewhere[12].

3) Solid phase antibody binding assay (SABA)

Binding of antibodies to cells attached to the surfaces of wells of Terasaki plates was determined. Terasaki plates were coated with breast cancer cells (MCF-7, Hs0578T, ZR-75-1) and fibroblasts (HF-LO, HF–NL) as follows. Breast cancer cells were detached from culture bottles with EDTA-solution pH 7.2 (0.035% w/v Na_2 EDTA, 0.2% glucose, 0.8% NaCl, 0.04% KCl, 0.001% phenol red, 0.18% $Na_2H\ BO_4 \cdot 2H_2O$; 0.07 g $NaH_2BO_4H_2O$) and suspended in normal growth medium (RPMI-1640+10% fetal calf serum). EDTA-solution was used rather than trypsin in order to minimize proteolytic degradation of surface proteins. However, the fibroblast lines could only be detached with trypsin-EDTA-solution (GIBCO, Buffalo, NY; inc.: 10 min at 37 °C). The cell suspensions were left standing for 10 min to allow settling of cell clumps. The remaining cells were then washed twice in PBS and finally suspended in PBS at 10^6 cells/ml.

Terasaki plates (Falcon, Oxnard, Cal., USA) were pretreated with a solution of 5% glutaraldehyde in H_2O for 30 min[16], and washed with tap water eight times and once with distilled water. 10 µl of cell suspension (10^4 cells) was pipetted into each well and the plates were left standing for 5 min. The plates were centrifuged 0.5–1 min at 700 rpm, with slow acceleration and deceleration, and fixed for 7 min in 0.25% glutaraldehyde in PBS. They were then washed very gently thrice in PBS and neutralized with 50 mM glycine in PBS for at least 20 min. The plates were then saturated with 3% BSA in PBS (containing NaN_3) for at least 30 min, washed thrice in PBS, and stored at 4 °C with wells filled with PBS, containing 0.02% NaN_3.

Plates were coated with human erythrocytes (50% female, B, Rh pos; 50% male, A, Rh neg.) by the same method using 20,000 cells/well. Finally, in the same way, plates were coated with 10,000 human lymphocytes/well that had been stimulated with Pokeweed Mitogen for 5 days in culture[10].

SABA was carried out by a modification of a method described elsewhere[5,6]. In brief: 10 µl of hybridoma supernatants were incubated in each well, usually overnight at RT; the fluid was removed by pouring PBS over the plate followed immediately by gentle flicking. This procedure was repeated thrice. 10 µl ^{125}I-sheep-antimouse-Ig Ab (approx. 30,000 cpm; in 0.5% BSA in PBS) were applied to each well for approx. 4 h at RT. Wells were then washed again as described above.

Plates were autoradiographed as follows: Radioautographs were produced with Kodak X-Omat AR films at −70 °C, using Cronex intensifying screen (DuPont). Microtiter plates, film and intensifying screen were sandwiched between two plastic boards and held together by rubber bands. Exposure times varied between 1 and 12 h. Exposed films were processed according to the manufacturers instructions.

4) Isotype determination

Isotypes were determined essentially as described elsewhere[14]. However, to avoid interference by possible peroxidase activity of target cells, mAb were collected in sheep-antimouse-Ig Ab (20 µg/ml) coated rather than target cell coated microtiter wells.

5) Immuno-blot experiment

A cell mixture of the breast cancer lines MCF-7, Hs0578T and ZR-75-1 was lysed in a lysis buffer[17] containing 1% nonionic detergent NP-40 followed by centrifugation to remove nuclei and other insoluble material. The solubilized polypeptide antigens, 3 × 10^5 cell equivalents per slot, resolved by conventional 12.5% polyacrylamide electrophoresis in the presence of sodium lauryl sulfate (SDS), were transfered to nitrocellulose membranes as described by Towbin et al.[18], with slight modification[17]. The transfer buffer consisted of 0.025 M Tris, 0.192 M glycine, 20% (v/v) methanol, and 0.01% SDS. Blotting was done at RT for 90 min. at 1 A. Nitrocellulose membranes were then incubated in blocking buffer (3% BSA; 0.01 M Tris-HCl pH 7.4; 0.15 M NaCl; 0.02 % NaN_3) at 37 °C for 1 h with agitation. The blocking buffer was replaced with a fresh blocking buffer solution and left overnight at 4 °C. The membrane sheet was cut into 1 cm wide strips and each strip was incubated with a different hybridoma supernatant for 4–12 h at RT with rocking. Strips were then washed sequentially for 10 min each time in the following four solutions: 1) 0.01 M Tris-HCl pH 7.4+0.15 M NaCl (= Tris-saline); 2) 0.1% NP-40 in Tris-saline; 3) Tris-saline; 4) 3% BSA in Tris-saline. Bound antibodies were detected by incubating the membrane strips in ^{125}I-sheep-antimouse-IgG (0.4–1.0 × 10^6 cpm/ml) in Tris-saline buffer containing 3% BSA+3% normal sheep serum +3% horse serum

+0.02% NaN_3 for 3 h at RT with rocking. Membrane strips were then washed four to five times for 10 min each time in Tris-saline buffer containing 0.1% NP40 and 0.02% SDS, and once with H_2O, dried and exposed for autoradiography as described for microtiter plates.

6) Immunohistology

Fresh tissues from surgery or post mortem tissues were cut into cubes of 0.4 cm^2, and snap frozen by immersion into isopentane kept in a mixture of dry ice and acetone. Then 6-µm cryostat sections were cut. The air-dried sections were incubated with mAb culture supernatants or purified mAb in 0.5% BSA for 30 min at RT. After washing 15 min in PBS the sections were incubated for 30 min with fluorescein (FITC) labeled antibodies against mouse immunoglobulins (DAKO, distr. by Instr. Gesellschaft, ZH, Switzerland) diluted 1:10 in PBS. Finally, the sections were washed as before and mounted in glycerol buffer, pH 8.6 (0.7 parts glycerol plus 0.3 parts of a buffer composed of 14 g glycine + 0.7 g NaOH + 17 g NaCl + 0.1 g NaN_3 per l).

Results and discussion

Spleen and myeloma cells were fused and plated out in 960 culture wells. Growth was observed in about 360 wells. All but the 10% slowest growing cultures were screened for mAb reacting with three breast cancer lines used for immunization (MCF-7, Hs578T, ZR-75-1) and two fibroblast lines (HF-LO, HF-NF) in SABA (see 'materials and methods'). A typical autoradiogram is shown in figure 1. The top six horizontal lines show negative signals (background binding) as they can also be seen with fresh medium. The next two lines show the mAb b-12 reacting most strongly with MCF-7 (third row) and less with the other tumor and normal cells. The mAb in the next two lines reacts equally with all cells and was therefore not selected for further study. Approximately 60% of the hybridoma supernatants tested showed reactivity with at least one cell line. Of these, 30 mAb with some selectivity for tumor cells were chosen for further study (b-1 through b-30).

Some of these mAb were selected, even though their signals in SABA against tumor cells only slightly exceeded the signals against control cells (fibroblasts). However, when hybridoma supernatants were tested in SABA at three different dilutions (undiluted, 1:10, 1:100), for some mAb signals against fibroblasts decreased more quickly upon dilution than the signals against tumor cells. At supernatant dilutions (1:10 or 1:50) considered optimal according to the results of this titration experiment, the mAb were again assayed in SABA against the three tumor lines, the two fibroblast lines, erythrocytes, lymphoblasts and BSA (table 1). Under these optimized conditions of mAb concentration, about half of the mAb reacted clearly more strongly with at least one tumor line than with any of the normal cells (table 1), whereas in the screening some of these differences had been marginal. That the low reactivities of most mAb against normal cells, which were only clearly seen when the hybridoma supernatants were strongly diluted (table 1), represent the true situation, was confirmed by immunohistology experiments on tissue sections, where these mAb showed, for example, no reaction with spleen cells (see below). The mAb differed not only with regard to their reactivities with tumor cells compared to normal cells, but also in their reactivities with the different carcinoma lines. Some reacted preferentially with a single tumor line (e.g. b-1, b-8), others with two lines (e.g. b-13), still others equally with all three lines (e.g. b-22). These different reactivity patterns indicate recognition of different antigens. Also listed in table 1 are the mAb isotypes. The mAb were found to have κ light chains and either μ or γ_1 heavy chains.

The nature of the antigens recognized by these mAb on cells was analyzed by the immunoblot method (see 'materials and methods'). This method detects epitopes on proteins or glycoproteins unless they are irreversibly denatured by the procedure. Figure 2 shows the results with 6 mAb. b-8, b-12 and b-15 reveal the same characteristic high mol.wt (M_r around 350,000) double band, suggesting recognition of the same protein. b-14 seems to

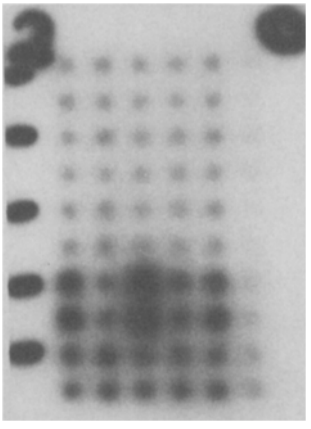

Figure 1. Autoradiograph of Terasaki plate used in screening. The column on the left was coated with MCF-7 cells, followed to the night by Hs0578T, ZR-75-1, HF-LO and HF-NF. The column at the right edge contained no cells. Each mAb was applied to two successive rows of wells.

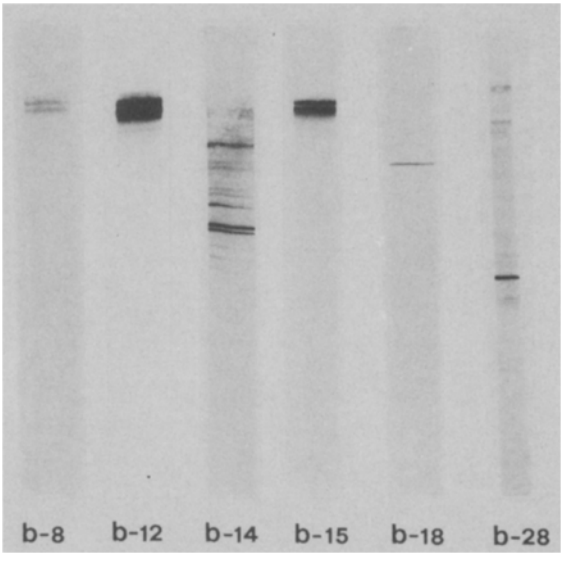

Figure 2. Immuno-blot analysis of six mAb with nonionic detergent-solubilized proteins from a mixture of MCF-7, Hs0578T and ZR-75-1 cells. For explanations see text and 'materials and methods'.

bind specifically to at least four components whose structural relationship is not clear. 15 other mAb did not reveal clear bands on such immunoblots. The majority of the IgM's gave high nonspecific background binding.

Finally, the mAb were analyzed by immunohistology on frozen tissue sections, both cancerous and normal. Sone of the more tumor-selective mAb are listed in table 2, together with some less specific ones for comparison. Reactions of mAb with sections of six normal tissues were either with the organ-specific cells of these tissues such as glandular cells in stomach, alveolar cells in lung, or tubular cells in kidney or with nonorgan-specific cells or structures (listed separately in table 2). Fluorescent staining of reactive cells either included the entire cytoplasmic area in addition to the cell surface (for the majority of the mAb) or was concentrated on the plasma membrane (e.g. for b-16, b-17, b-18, b-28). In table 2, these patterns are not distinguished and were marked + (or (+), to indicate a weak reactivity). Three mAb, b-8, b-12, b-15, in some noncancerous tissues stained extracellular material contained in the lumina only. This reactivity is marked 1 (or (1), weak) in table 2. In the kidney, for example, these three mAb only stained material contained in distal tubuli. It is unclear whether the surface membrane of epithelial cells lining these lumina is also stained.

We have not found mAb reacting exclusively with tumor cells. The two mAb with the highest tumor selectivity, b-12 and b-15, however, reacted only with a limited number of normal tissues, and in these with minor substructures. Furthermore, some of these antigens are focally expressed on the luminal side of epithelial cells located on an intact basal membrane and may thus be poorly accessible to mAb from the blood compartment, whereas cells in a vascularized tumor may be more easily accessible. Thus, for in vivo applicatons (radio-imaging, therapy), b-12 and b-15 appear to be the most suitable of the mAb described here. For the following mAb some selectivity for tumor cell surface antigens and reactivity with high proportions of breast carcinomas has been reported: Br-1[7], HMFG-1 and 2[4], 10-3D2[11], F36/22[3], Ca1[1,8]. Their specificity, however, differs from that of b-12 or b-15 based on the nature of the antigen and/or the pattern of tissue reactivities.

All of these relatively specific mAb (b-12, b-15 as well as Br-1, etc.) share a disadvantage: none of these mAb reacts with all the tumor cells of any one breast tumor. Thus, the more tumor-specific an antigen, the less consistant appears to be its expression on the cells of this tumor. The heterogeneity of expression of these relatively tumor-specific epitopes thus appears to be something like an invariant, though not functionally significant feature of tumors. With other, less tumor-specific mAb, such as b-16, b-18 and b-28, this heterogeneity of tumors is not observed. Whether maximal selectivity or complete reactivity is the predominant desirable property for the selection of mAb for the intended in vivo applications remains to be evaluated.

Table 1. mAb binding (SABA) to a variety of cells; isotypes

mAb	SABA to M	H	Z	F	F′	E	L	B	Isotype
b-1	1	3	1	1	1	0	1	0	IgG
b-2	4	3	5	2	2	0	1	0	IgM
b-3	1	2	2	2	2	0	1	0	IgM
b-4	1	3	1	1	1	0	1	0	IgG
b-5	1	1	3	1	2	0	1	0	
b-6	1	1	1	1	1	0	1	0	
b-7	2	3	2	3	3	0	3	0	IgM
b-8	2	1	7	1	1	0	1	0	IgG
b-9	3	2	1	1	1	0	1	0	IgM
b-11	8	7	5	5	6	3	4	0	IgM
b-12	2	0	9	2	3	0	2	0	IgG
b-13	4	1	3	0	0	0	0	0	IgM
b-14	2	3	4	2	2	0	1	0	IgM
b-15	0	0	5	1	1	0	0	0	IgG
b-16	3	2	2	2	1	0	1	0	IgM
b-17	4	2	5	4	4	0	3	0	
b-18	2	1	2	1	1	0	0	0	IgG
b-19	1	1	2	1	2	0	1	0	IgG
b-21	1	1	1	1	1	–	–	0	IgG
b-22	2	2	2	2	2	–	–	0	
b-23	2	3	4	3	2	0	0	0	IgM
b-24	3	2	5	1	1	0	1	0	IgG
b-25	0	1	0	1	1	0	0	1	IgG
b-26	1	1	2	1	1	0	1	0	IgG
b-27	2	1	1	1	1	0	1	0	IgM
b-28	5	2	4	1	1	0	0	0	IgM
b-29	1	2	1	2	2	0	2	0	IgM
b-30	2	3	2	2	2	0	2	0	IgM

M = MCF-7, H = Hs0578T, Z = ZR-75-1, F = HF-LO, F′ = HF-NF, E = erythrocytes, L = lymphoblasts, B = BSA; assay: see 'materials and methods'; numbers reflect intensity of spots on autoradiograph (visual reading, arbitrary scale).

Table 2. Immunohistology

	mAb: b- 2	7	8	12	13	15	16	17	18	24	28
Tumor tissues											
Mamma*	–	+	(+)	+	–	+	+	+	+	+	+
Mamma (CM-3)**	+	–		+	+	–	–	+	–	(+)	+
Colon (SLU)**	+	–		+	+	(+)	+	+	+	+	+
Melanoma (MEC)**	–	–		–	+	–	–	+	–	–	–
Normal tissues											
Thyroid	+	–		1	+	–	–	+	+	–	+
Stomach	+	+	1	1	+	(1)	+	+	+	+	+
Liver	–	–	(+)	–	–	–	+	–	+	–	+
Lung	–	+	–	(1)	+	(1)	+	+	+	–	+
Kidney	+	+	(+)	1	+	1	+	1	+	(+)	+
Spleen	–	+	+	–	+	–	–	–	–	+	–
Smooth muscle	–	–	–	–	–	–	–	–	–	–	–
Connective tissue	+	+	–	–	–	–	+	–	–	–	–
Endothelium	–	–	–	–	–	–	+	–	–	–	–
Sinus cells	–	–	–	–	–	–	+	–	–	–	–

For explanations see text and 'materials and methods'. *Surgical specimen (carcinoma solidum simplex). **Human tumor cell lines grown in nude mice (gift from Dr H.J. Staab, Friedrich-Miescher-Laboratorium, Max-Planck-Gesellschaft, Tübingen).

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Abbreviations. mAb = monoclonal antibody(ies); SABA = solid phase antibody binding assay; PBS = phosphate (15 mM, pH 7.2) buffered saline (150 mM NaCl); BSA = bovine serum albumine; RT = room temperature.

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Deletion mapping of a new gustatory mutant in *Drosophila melanogaster*

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Summary. A gustatory mutant of *Drosophila melanogaster* insensitive to the taste of salt has been isolated. Genetic crosses and a deletion mapping analysis show that this mutation, designated *gust-M_1* is located in the $93C_{3-6}$–$93D_{6-7}$ region of the third chromosome. *gust-M_1* is also insensitive to the taste of quinine sulfate. The behavior of this mutant may be explained by assuming that *gust-M_1* could be a mutation perturbing functions in the central nervous system affecting the responses to both compounds.
Key words. Behavior genetics; deletion mapping; taste; chemotaxis.

Introduction

In Diptera, the contact chemoreceptors that mediate feeding behavior are primarily located on both the tarsal segments of the leg and the labellum of the proboscis[6]. When these chemoreceptors are in contact with solutions of various sugars, a hungry fly generally extends its proboscis, whereas sugars mixed with salts elicit no proboscis extension.

During the last decade several researchers have isolated gustatory mutants of *Drosophila melanogaster*, which are deficient in their responses to sugars, salt or quinine[5, 10, 19, 20, 23, 24, 26]. Falk and Atidia[10] reported three mutations affecting the tolerance to the taste of salt. They were designated '*Lot*' and were located on the X-chromosome; the mutant flies showed reduced salt sensitivity. In particular, it was found that the mutation designated *'Lot-94'* is 'a mutation of a perception center, affecting the recognition of salt, with no detectable effect on that of sugar'[5].

Mutants insensitive to either NaCl or to quinine or to both compounds have also been described by Tompkins[24, 26]. Some of them are temperature-sensitive[25].

Siddiqi and Rodrigues have isolated mutations in an X-linked gene, *gust-A*, that block the responses of *Drosophila melanogaster* to a group of pyranose sugars[20]. It has been shown that the behavioral effects of this mutation are correlated with a loss of electrical responses in taste receptors. The mutation affects the chemoacceptors for pyranose sugars leaving the furanose acceptors intact. Mutants insensitive to NaCl and to quinine were also isolated by Siddiqi and Rodrigues[22]. They tested the electrophysiological responses of the mutants and found that the receptor responses of the mutants were indistinguishable from those of the wild-type. Therefore Siddiqi and Rodrigues think that 'the blocks produced by these genes are presumably central'[22].

Recently I have started a study of the taste responses of *ebony* flies, which show a number of behavioral abnormalities[3, 4, 8, 11–13]. Different alleles of *ebony* have been