Production and characterization of monoclonal antibodies identifying breast tumor-associated antigens

I. Keydar*, C. S. Chou*, M. Hareuveni*, I. Tarasfaty*, E. Sahar†, G. Selzer§, S. Chaitchik&&, and A. Hizi**

Departments of *Microbiology and †Biotechnology, G. S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel; ‡Department of Pathology, Sheba Medical Center, Tel Hashomer Hospital, Tel Aviv, Israel; †Department of Oncology, Tel Aviv Medical Center, Tel Aviv, Israel; and **Department of Histology and Cell Biology, Sackler School of Medicine, Tel Aviv, Israel

Communicated by Ephraim Katchalski-Katzir, October 17, 1988

ABSTRACT We have generated a mouse monoclonal antibody (H23) against the retrovirus-like particles (human mammary tumor virus) released in vitro by the human breast adenocarcinoma cell line T47D. This antibody reacts specifically with a glycoprotein with an apparent molecular mass of 68 kDa (gp68) that is detected in the growth medium of T47D cells as well as in pleural effusion fluids from breast adenocarcinoma patients. No detectable levels of this antigen could be observed in pleural effusions of patients with cancers other than of breast origin. The H23-related antigen was localized in the cytoplasm of breast tumor cells as well as on the cell surface of both T47D cells and metastatic cells from breast cancer patients. A survey of tissue from 812 patients was performed by using H23 in an indirect immunoperoxidase assay. The results showed that the antigen was detectable in 91% of all breast tumors tested. No cytoplasmic staining was observed in either normal tissues or nonbreast carcinomas. Only one of the benign breast tissues tested (out of a total of 56 samples of tissue) was positive for this antigen. Given the ability of this antibody to specifically detect breast tumor cells, H23 may be of importance in diagnosis and in clinical follow-up of patients for the detection of metastatic lesions by imaging and for therapy.

One out of 11 women in the Western societies will develop breast cancer during her lifetime. This neoplasia accounts for about one-quarter of all malignancies found in women (1). It is widely accepted that patients diagnosed at an early stage before their tumor becomes invasive have a significantly better prognosis. Therefore, it is extremely important to develop reagents enabling early diagnosis and prognosis, thus providing the clinicians with a tool for determining the mode of treatment.

Identification of factors involved in promoting malignant transformations will increase the prospects of early detection and possible prevention of the disease. One approach to this problem has focused on the association of human mammary adenocarcinomas with viral proteins and viral nucleic acid sequences. Most notably, studies were carried out with the proteins and nucleic acids of murine mammary tumor virus (MMTV). Thus, MMTV-related antigens as well as retrovirus-like particles have been identified in human breast tumors (2–7). The detection of such factors can be added to the numerous investigations reporting the existence of tumor-associated antigens with human breast cancer (8–11).

Previous studies have demonstrated the presence of retrovirus-like particles (human mammary tumor virus; HuMTV) released in vitro by T47D cells, a metastatic breast adenocarcinoma cell line established in our laboratory (12). A glycoprotein with an apparent molecular mass of ~68 kDa (gp68) was shown to be immunologically cross-reactive with the 52-kDa (gp52) major envelope glycoprotein of MMTV (13). To further investigate the relationship of these viral particles with human breast cancer, we generated mouse monoclonal antibodies (mAbs) to HuMTV.

We report here the production and characterization of these mAbs against HuMTV. One antibody was shown not only to be very specific to breast carcinomas but also to be able to recognize a very high percentage of breast malignancies.

MATERIALS AND METHODS

Production of Mouse mAbs. Immunization. Three-month-old BALB/c mice were injected three times subcutaneously at weekly intervals with 20 μg of purified HuMTV (14). The first injection was given in complete Freund's adjuvant and the subsequent two in incomplete Freund's adjuvant. Three days before hybridization, the mice were injected intravenously with 10 μg of HuMTV in isotonic phosphate-buffered saline (PBS).

Hybridization. Approximately 10⁸ splenocytes from the immunized mice were fused with 2.5 × 10⁷ mouse myeloma x63-Ag8.653 cells by using 50% (wt/vol) polyethylene glycol (Mw, 1000; Sigma) as described by Kohler and Milstein (15). After fusion the cells were suspended in RPMI 1640 selection medium [hypoxanthine/aminopterin/thymidine (HAT) medium] supplemented with 20% (vol/vol) fetal calf serum and seeded into six 96-well microtiter plates, at 4 × 10⁶ cells per 0.1 ml in each well. Twenty-four hours later an equal volume of RPMI 1640 HAT medium containing 20% (vol/vol) fetal calf serum was added. Starting 1 week after fusion, the cells were fed twice a week with HAT medium. Colonies appeared 2 weeks later. Supernatants from wells containing live colonies were screened for presence of mouse immunoglobulins by ELISA (see below). The positive colonies were further screened for the ability to recognize the HuMTV antigens by ELISA. Colonies producing anti-HuMTV antibodies were then cloned twice by the limiting dilutions method. The hybridomas in culture produce 1–2 μg of mouse IgG per ml of growth medium.

To obtain large amounts of mAbs, hybridoma cells were suspended in PBS and injected i.p. into pristane-sensitized BALB/c mice. The hybrid cells grew as ascitic tumors producing mAb-containing ascitic fluids. The tumor cells from these fluids were removed by centrifugation, immunoglobulins were purified (6), and their titers were determined by ELISA on HuMTV.

ELISA. ELISA was performed as described (16) using Nunc Immunoplates. The plates were coated either with 200 μl of HuMTV or rabbit anti-mouse immunoglobulins at 10

Abbreviations: MMTV, murine mammary tumor virus; HuMTV, human mammary tumor virus; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, pleural effusion.

*Permanent address: NDMC, P.O. Box 8244-7, Taipei, Taiwan.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
RESULTS

mAbs. Mouse mAbs against HuMTV were obtained and the clone, designated H23 producing IgG1, was chosen for further studies. mAb H23 was characterized by determining the following features: (i) Binding to HuMTV-related antigens, (ii) recognition of T47D cell surface and cytoplasmic antigens, (iii) binding to soluble antigens in body fluids of patients, and (iv) recognition of tumor cells in sections from biopsies of patients with breast adenocarcinoma.

Radioimmunoprecipitation of HuMTV Proteins. To characterize which proteins released in vitro by the T47D cells are recognized by mAb H23, 125I-labeled proteins were immunoprecipitated by mAb H23. The main protein species recognized by H23 was a polypeptide with a molecular mass of ~68 kDa (Fig. 1, lane 3). Being a glycoprotein (results not shown), it was designated gp68. On the other hand, P3, a nonrelevant mouse mAb, did not precipitate any detectable amounts of the HuMTV-related proteins (lane 4). In parallel we have also precipitated these proteins with R52, a rabbit polyclonal antiserum prepared against HuMTV (lane 1). In addition to the 68-kDa protein, this serum precipitated three other polypeptides of ~40, 18, and 14 kDa. A HuMTV protein with a molecular mass similar to that recognized by H23 and also designated gp68 was found (13) to react with antibodies against MMTV gp52. It is likely that these two 125I-labeled HuMTV proteins, 125I-labeled HuMTV proteins were immunoprecipitated with the following antisera. Lanes: 1, R52 (rabbit serum prepared against HuMTV); 2, normal rabbit serum; 3, H23 mAb; 4, a nonrelevant mouse mAb. All immunoprecipitates were analyzed by NaDodSO4/PAGE. Protein markers with known molecular masses were used to determine the molecular mass of the polypeptides precipitated (their positions are marked in kDa).

HuMTV 68-kDa proteins are very similar and possibly identical, as suggested by immunological cross-reactivity and peptide fingerprint analyses (unpublished data). Preliminary data indicated that H23 mAb is directed against epitopes located on the protein moiety of the glycoprotein and not on the sugar chains, since they also recognize the epitopes on peptides synthesized in vitro in Escherichia coli after the introduction of phages containing an expression library prepared from T47D cDNA (unpublished data).

Demonstration of Cell Surface Antigens. To investigate whether the antigenic epitopes recognized by H23 are expressed on the cell surface and whether this surface expression is preferentially associated with breast carcinoma cells, we did the experiment described in Fig. 2A.

Antibody binding to the cell surface was tested by flow cytometry on the following cell lines: (i) T47D, (ii) PC3 (derived from a human prostatic carcinoma), (iii) HuK (from normal human kidney) and (iv) Jurkat (a human lymphoma). It was found that the T47D cells are brightly stained with mAb H23, with a mean fluorescence intensity significantly higher than the background level detected on cells of the other lines (Fig. 2A).

Similar antigenic determinants were also detected on the surface of metastatic cells from pleural effusions (PEs) of breast cancer patients. In the PE sample, a subset of cells was intensely fluorescent, similar to the fluorescence of T47D cells (Fig. 2B). The intensely stained cells from the PE sample were sorted and were identified by morphology as carcinoma cells (data not shown). The nonstained cell fraction contained mainly leukocytes and erythrocytes.

Immunoprecipitation of Antigens from Body Fluids of Breast Cancer Patients. To further extend the in vitro studies with H23 to human material, we did the experiment described in Fig. 3. Proteins from PE fluids from a breast cancer patient and a lung carcinoma patient were used. A glycoprotein chain with an apparent molecular mass of 68 kDa was recognized by the H23 in the breast cancer-derived proteins and not in the lung carcinoma-derived proteins. In addition, polypep-
Fig. 2. Quantitation of H23 binding to the surface of cells. Approximately 10^6 pelleted cells were incubated for 60 min with 100 μl of the biotinylated mAb H23 (100 μg/ml), washed extensively, and incubated with 100 μl of FITC-conjugated avidin at 100 μg/ml. Fluorescence intensity per cell (expressed in arbitrary units) was measured by flow cytometry. (A) Mean fluorescence intensity of cells grown in vitro. T47D was derived from a human breast adenocarcinoma; PC3 was from a human prostatic carcinoma; HuK was from human kidney; Jurkat was from a T lymphoma. T47D control was stained with avidin-FITC alone. (B) Fluorescence distribution of cells collected from the PE of a patient with breast adenocarcinoma (PE+) compared to T47D cells and to control PE+ cells stained with avidin-FITC omitting mAb H23.

Fig. 3. Radioimmunoprecipitation of 125I-labeled proteins from human PEs. The proteins from PEs of either an infiltrating duct carcinoma patient (PE+) or a nonbreast cancer patient with a lung carcinoma (PE−) were precipitated with 50% saturated ammonium sulfate at 4°C followed by iodination with 125I. The 125I-labeled proteins were immunoprecipitated with the following antisera. Lanes: 1 and 8, normal rabbit serum; 2 and 7, R52 rabbit anti-HuMTV; 3 and 6, G401, a goat antiserum against HuMTV; 4 and 5, mAb H23. The precipitates in lanes 1−4 are from PE− and in lanes 5−8 are from PE+. Nonrelevant mouse mAbs (P3) did not give any detectable bands (data not shown). The labeled proteins in the immunoprecipitates were analyzed by NaDodSO4/PAGE.

Paraffin sections of tissues derived from 812 biopsies were tested by using mAb H23. The number of stained cells per tumor section varied as well as the intensity of staining per cell. Fig. 4 Lower shows an example of the positive staining observed in sections taken from an infiltrating duct carcinoma of the breast. The intensity of staining was pronounced in some tumor cells, less in others, and absent in many of the cells.

Table 1 lists all the cases tested. The great majority of the breast adenocarcinomas analyzed from 390 patients were of the infiltrating duct carcinoma type. The other histological types examined in the study were intraductal, infiltrating lobular, medullary, and tubular carcinomas. Of the breast adenocarcinomas tested, 532 (90.6%) showed clear evidence of a positive reaction with H23. All the 29 (100%) metastatic breast tissues were also positive, suggesting a somewhat higher incidence of antigen detection in breast tumor metastases. Histological type of the tumor did not seem to correlate with either detection of antigen or the number of cells stained. The 58 breast carcinomas in which antigen was not detected were of various histological types and stages of the disease. Thus, no characteristic pattern could be established for this group.

None of the 45 normal breast tissues tested (derived from reduction mammoplasties or normal tissue adjacent to the tumors) showed cytoplasmic staining. The 56 benign breast tumors tested were of the following types: fibrocystic disease, fibroadenomas, papillomatosis, sclerosing adenosis, and epitheliosis. Only one of the benign tumors tested, an epitheliosis, showed a positive cytoplasmic reaction. None of the 70 carcinomas other than breast showed a positive reaction. Not one of the 22 normal tissues tested (including colon, thyroid, and stomach) showed any detectable reaction.

**Discussion**

In the present report, we describe the production of mouse mAb H23 prepared against HuMTV—the retroviral-like par-
particles released in vitro from the human breast carcinoma cell line T47D.

The particles were designated retroviral-like because of their density in sucrose gradients, their single-stranded RNA content, and their reverse transcriptase activity (14). We do not have evidence to date that the protein recognized by mAb H23 is coded by viral genes; therefore, the gp68 antigen might also be a tumor-associated protein that copurified with the particles. In T47D cells, as well as in breast tumor cells, H23 recognizes antigenic determinants localized in the cytoplasm, as well as on the cell surface.

The results obtained with the 619 breast tumors (including primary and metastatic tumors) tested by immunoperoxidase are impressive. In 90.6% of the tumors, an antigen was detected in the tumor cell cytoplasm (Table 1). None of the sections of normal tissues and carcinomas of other organs displayed this type of staining. Only one tumor, an epithelioid, from all the benign breast tissue tested showed a positive reaction. Since this patient was lost in the follow-up study, we do not know if the positive reaction is indicative of the recognition of a precancerous lesion. These results prompted us to undertake a retrospective study of epitheliosis of the breast (unpublished results).

There are many reports describing mAbs directed against human breast carcinomas antigens (8–11). None of these reports describe a 68-kDa glycoprotein as the major tumor cell-associated antigen. For example, Edwards et al. (18) reported the identification of a 43-kDa glycoprotein by mAb. This protein was detected in 59% of breast carcinomas and 75% of metastatic nodes. This mAb reacted also with 20% of benign breast tumors but did not detect normal breast tissue. However, this mAb also reacted with 57% of nonbreast carcinomas tested (with the highest incidence in endometrial and colon carcinomas), suggesting only a moderate specificity to breast tumors. Higher specificity to breast tumors was reported (19) for mAb B72.3, which reacts with a high molecular mass membrane glycoprotein in 84% of all invasive ductal carcinomas tested. This antigen is detected also in a few benign breast tumors (with the exception of apocrine metaplasia) and is probably oncofetal in nature, since it reacts with fetal gastrointestinal tissue (19, 20). Thus the specificity of H23 to breast carcinomas and the sensitivity as high as that

**Fig. 4.** Immunoperoxidase staining of human breast tumor cells with mAb H23. (Upper) T47D cells grown in suspension. (Lower) Infiltrating duct carcinoma of the breast. (Methylene blue counterstain. Upper, ×400; Lower, ×400.)
Table 1. Indirect immunoperoxidase staining of human malignant and normal tissues with mAb H23

<table>
<thead>
<tr>
<th>Sample</th>
<th>Positive/total tested, no./no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal breast tissue</td>
<td>0/45</td>
</tr>
<tr>
<td>Normal tissue (other than breast)</td>
<td>0/22</td>
</tr>
<tr>
<td>Benign breast tissue</td>
<td>1/56</td>
</tr>
<tr>
<td>Breast adenocarcinomas</td>
<td>532/590</td>
</tr>
<tr>
<td>Breast metastases to other organs</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>19/19</td>
</tr>
<tr>
<td>Ovary</td>
<td>5/5</td>
</tr>
<tr>
<td>Lung</td>
<td>4/4</td>
</tr>
<tr>
<td>Bone</td>
<td>1/1</td>
</tr>
<tr>
<td>Carcinomas of other organs</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>0/26</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/4</td>
</tr>
<tr>
<td>Ovary</td>
<td>0/3</td>
</tr>
<tr>
<td>Skin</td>
<td>0/3</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0/3</td>
</tr>
<tr>
<td>Lung</td>
<td>0/2</td>
</tr>
<tr>
<td>Prostate</td>
<td>0/1</td>
</tr>
<tr>
<td>Liver</td>
<td>0/1</td>
</tr>
<tr>
<td>Uterus</td>
<td>0/9</td>
</tr>
<tr>
<td>Stomach</td>
<td>0/9</td>
</tr>
<tr>
<td>Bladder</td>
<td>0/9</td>
</tr>
</tbody>
</table>

The total number of tissue samples tested was 812.

obtained with this antibody provides a powerful reagent for the detection of breast carcinomas.

Since we have shown here that gp68 is localized not only in the cell cytoplasm and membrane but also released into body fluids, H23 can be used for screening and monitoring the levels of the soluble gp68 in plasma. Indeed, we have found that high levels of gp68 in the patient’s plasma correlates with the progression of the disease (unpublished data).

Our previous findings with anti-MMTV gp52 antibodies and indirect immunoperoxidase staining showed that if antigen is detected at the time of mastectomy, the prognosis of the patient is unfavorable (6, 7). In contrast, staining by H23 on paraffin sections does not seem to have a prognostic significance, since it is observed in almost all cases. Whether the number of cells stained in a given tumor tissue or the intensity of staining per cell has any prognostic value remains to be investigated.

Our studies with anti-MMTV gp52 also implicated a 68-kDa glycoprotein as the antigen cross-reacting with MMTV gp52 (13). This protein is very similar if not identical to the gp68 recognized by H23. In contrast, we could not detect binding of H23 to any MMTV proteins (data not shown), suggesting that H23 is directed against gp68 epitopes that are not shared with MMTV gp52. This supports the idea, suggested earlier by us (13), that there is only a restricted similarity between the gp68 and MMTV gp52. In spite of the fact that the gp68 recognized by the anti-MMTV gp52 and the gp68 recognized by H23 are very similar, there is a substantial difference between the incidence of their detection in human breast tumors by the two antibodies. The most likely explanation is that H23 has a higher affinity and/or that specific epitopes recognized by H23 are more accessible and possibly more stable in the procedure used in preparation of the tumor tissue for immunohistochemistry. Therefore, a combination of the two antibodies may be useful both for diagnostic and for prognostic purposes.

The selective expression of the gp68 in breast adenocarcinomas raises the question of the possible involvement of this protein in the biology of this malignancy. Analysis at the molecular level may shed light on this important issue.

We thank Dr. A. Frensdorff for critical comments and for reading the manuscript, Mrs. R. Smutza and S. Karby for excellent technical assistance, Mr. A. Shuv for performing the gel photography, and Miss G. Hoffnung for preparing the manuscript. This work was supported in part by Teva Pharmaceutical Industries, Ltd., The Simko Chair for Breast Cancer Research, and by a grant from Mr. Toby Green, London.