ABSTRACT

Monoclonal antibody B72.3 was generated using a membrane-enriched fraction of cells from a mammary carcinoma metastasis and has been shown previously to have a high degree of selective reactivity for human breast and colon carcinoma versus normal adult tissues. The reactive antigen has been shown to be a high-molecular-weight glycoprotein complex of approximately 220,000 to 400,000 and is termed tumor-associated glycoprotein 72 (TAG-72). We report here a dichotomy in the expression of TAG-72 in carcinoma biopsy material versus carcinoma cell lines. While 44% (25 of 56) of human breast carcinoma and 85% (16 of 20) of colon carcinoma biopsies express TAG-72 as assayed by radioimmunoassay or immunohistochemistry, only one of 25 breast cancer cell lines (MCF-7 [one variant]) and one of 18 colon cancer cell lines (LS-174T) express this antigen. Furthermore, TAG-72 expression in these two cell lines was shown to be a property of a low percentage of cells within each culture. Attempts to enhance TAG-72 expression in LS-174T cells by propagation on extracellular matrix proteins, such as collagen, laminin, and fibronectin, or in serum-containing or serum-free, hormone-supplemented medium proved unsuccessful. A pronounced increase in TAG-72 expression was observed, however, when the LS-174T cells were grown under culture conditions which promote three-dimensional growth. LS-174T cells grown in spheroid or suspension cultures demonstrated a 2- to 7-fold increase in TAG-72 antigen expression, while those grown on agar plugs demonstrated a 10-fold increase. When the LS-174T cell line was injected into athymic mice to generate tumors, the level of TAG-72 antigen increased over 100-fold, to levels comparable to those seen in the metastatic tumor masses from patients. Thus, spatial configuration of carcinoma cell populations is shown to influence the expression of a tumor-associated antigen and the subsequent surface binding of monoclonal antibody B72.3. The implications of these findings in the potential utility of monoclonal antibodies for the in vivo detection and destruction of carcinoma masses are discussed.

INTRODUCTION

MAb2 B72.3 was generated using membrane-enriched extracts of a breast tumor metastasis to the liver as immunogen (6). The antigen reactive with this antibody is a 220,000 to 400,000 high-molecular-weight, tumor-associated glycoprotein complex designated TAG-72 (13). Solid-phase RIAs showed differential reactivity of this MAb to approximately 50% of primary breast tumor biopsy extracts and no reactivity to several normal human tissues (6). Immunoperoxidase studies have demonstrated that B72.3 reacts with approximately 50% of breast carcinomas (22) and 85% (29) of colon carcinomas tested. In contrast, no reactivity was observed with normal adult human liver, spleen, heart, breast, uterus, lung, bone marrow, colon, stomach, salivary gland, lymph node, and kidney (22, 29). Initial studies to detect TAG-72 in monolayer cultures of established cell lines demonstrated that this antigen is expressed in only one of 3 breast and 0 of 3 colon carcinoma cell lines (6). In fact, the one positive breast carcinoma cell line, MCF-7, contained only a small percentage of reactive cells. Single cell cloning of the parental MCF-7 cell line failed to generate clonal lines stable for the production of high levels of TAG-72 (14). A disparity thus exists. The majority of breast and colon carcinoma biopsy material was positive for TAG-72, while cell lines of comparable origin were almost invariably negative.

The present study was therefore conducted to (a) define the extent of this dichotomy between antigen expression on biopsy material versus cell lines, (b) define those factor(s), if any, which might influence the expression of TAG-72 in vitro, and (c) establish an in vitro or in vivo model system for studying the expression, and subsequent purification and characterization of, TAG-72.

MATERIALS AND METHODS

MAbs

MAbs B72.3, B1.1, B6.2, and B139 were generated using membrane-enriched fractions of breast tumor metastases as immunogen (6). MAb B72.3 reacts with an M, 220,000 to 400,000 glycoprotein (13). B1.1 reacts with CEA, an M, 180,000 glycoprotein (7), and B6.2 reacts with an M, 90,000 glycoprotein present on breast and colon carcinoma cells (6, 8, 13). MAb B139 binds to an antigen found on all human cells (13). W6/32 is a MAb which recognizes HLA-A, -B, -C (3). MAb B72.3 was purified as described previously (22). Radiiodination of B72.3 was performed using a modification of the method described for MAb B6.2 (8) with 40 μg purified antibody, 20 μg iodogen, and 0.5 mCi 125I.

Cells

Epithelial human mammary carcinoma cell lines (MCF-7, BT-20, BT-474, BT-483, ZR-75-1, ZR-75-27, ZR-75-30, MDA-MB-231, MDA-MB-330, DU-4475, T47D) and the HT-29 colon carcinoma cell line were obtained from the Breast Cancer Task Force, National Cancer Institute, NIH, Bethesda, MD. MCF-7 adenocarcinoma cell lines were also obtained from Dr. M. Lippman, National Cancer Institute [MCF-7 (ML)], Dr. S. Schaffie, National Cancer Institute [MCF-7 (SS)], and Dr. G. Cannon, Litton Bionetics, Rockville, MD [MCF-7 (GC)]. The following human cell lines were obtained from the Naval Biociences Laboratory, Oakland,
Mycoplasma and were negative. Many of the cell lines, including MCF-
fibroblast cells, were prepared using 5000 rads delivered by Co. Cells
7, LS-174T, and LS-180, were tested to verify the correct species of
their respective source. Cells lines were tested for the presence
obtained from Flow Laboratories, Rockville, MD. Chick embryo fibro-
the normal human fetal kidney (fibroblast) cell line, Flow-4000, was
thelioid colon carcinoma (LS-174T, LS-180, WIDR, COL0320DM,
were obtained from the American Type Culture Collection, Rockville,
were fibroblastic, while the majority of the breast carcinoma, colon
and muscle (HS0014M, HS0130M). The HS0612B1, HS0014M,
blasts were prepared from 9-day-old chick embryos. All cell lines were
maintained on the growth medium recommended by
CA: breast carcinoma (HS0578T, HS0228T, HS0897T, HS0275T,
HS0579T, HS0584T, HS0281T, HS0280T, HS0748T, HS0605T); colon
carcinoma (HS0675T, HS0293T, HS0255T, HS0844T); and normal
breast (HS0578Bst, HS0579Bst, HS0584Bst), kidney (HS0807K,
HS0715K, HS0761K), uterus (HS0769U), fetal testis (HS0181Tes), fetal
thyroid (HS0208T), fetal bone marrow (HS0074Bm), fetal spleen
(HS0203Sp), fetal brain (HS0129Br, HS0130Br), bladder (HS0612Bl),
and muscle (HS0014M, HS0130M). The HS0612Bl, HS0014M,
HS0769U, HS0807K, HS0715K, and HS0761K cell lines were
fibroblastic, while the majority of the breast carcinoma, colon
carcinoma, and normal breast cell lines obtained from the Naval Bio-
sciences Laboratory were epithelial. The following human cell lines
were obtained from the American Type Culture Collection, Rockville,
MD: epithelioid breast carcinoma (MDA-MB-134, MDA-MB-157); ep-
ithelioid colon carcinoma (LS-174T, LS-180, WIDR, COL0320DM,
COL0320HSR, COL0205, COL0201, SW520-3, SW1116, SW48,
SW1417, SW948, SW403, SW480); normal fibroblastic fetal skin (DS50,
DS51); normal fibroblastic fetal lung (MRC-5, WI-38); and normal fibro-
blastic colon (CCD-18CcOo, CCD-33CcOo, CCD-112 CON, COLO-20) human
colonic mucosa. Epithelioid human breast carcinoma cell lines (SW527,
SW613, SK-BR-3, Cama-1) were obtained from Dr. Jorgen Fogh of the
Sloan-Kettering Institute for Cancer Research, New York, NY. The PMC-
42 epithelioid breast carcinoma cell line was obtained from Dr. R. H.
Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia.
The normal human fetal kidney (fibroblast) cell line, Flow-4000, was
obtained from Flow Laboratories, Rockville, MD. Chick embryo fibro-
blasts were prepared from 9-day-old chick embryos. All cell lines were
maintained on the growth medium recommended by their respective source. Cells lines were tested for the presence of Mycoplasma and were negative. Many of the cell lines, including MCF-7, LS-174T, and LS-180, were tested to verify the correct species of origin by Dr. W. Peterson, Child Research Center of Michigan.

Preparation of Collagen, Laminin, and Fibronectin Substrata. Tissue culture dishes (60 mm) were coated with 25 μg of type I collagen (calf skin; Sigma), type IV collagen (mouse EHS sarcoma; BRL), laminin (EHS sarcoma; BRL), or human fibronectin (BRL) either alone or in various combinations as described previously (26, 27, 30). Cells were cultured on these substrata for 1 week prior to antigen assay by solid-phase RIA.

Preparation of Type I Collagen Gels. Type I collagen was prepared from acetic acid-solubilized adult rat tail tendons as described previously (9). Tissue culture dishes (100 mm) were coated with a 2- to 3-mm-thick adherent collagen gel as described previously (5, 21). Cells were cultured on collagen gels for 2 weeks prior to assay for antigen expression by solid-phase RIA.

Preparation of Basement Membrane. Intact basement membrane was prepared from confluent cultures of bovine corneal endothelial cells as described previously (10) following solubilization of the cells with sodium deoxycholate (20). Cells were cultured on basement membrane for 1 week prior to assay by live cell RIA.

Preparation of Feeder Layers. Feeder layers of Flow 4000 cells were prepared by treating cells with mitomycin C (20 μg/ml; Sigma) for 2 hr at 37° in the dark. The cells were then rinsed with complete medium and incubated with complete medium for an additional 2 hr at 37° prior to seeding the LS-174T cells. X-irradiated cultures of primary chick embryoblasts were prepared using 5000 rads delivered by Co. Cells were grown on feeder layers for 1 week prior to antigen quantitation by solid-phase or live cell RIA.

Preparation of Spheroid and Suspension Cultures. Spheroids were prepared by seeding 10^6 LS-174T cells into 35-mm bacteriological Petri dishes as modified from Haji-Karim and Carlsson (12). Cells were cultured for 3 weeks in either improved minimal Eagle's medium containing 10% FCS or in serum-free FEIT medium. For suspension cultures, 2 x 10^6 cells were seeded in serum-supplemented or in serum-free FEIT medium. Cells were then grown for 2 weeks in spinner flasks. Cells were harvested and assayed for antigens by solid-phase RIA.

Growth of Cells on Agar. LS-174T cells were grown in organ culture by the procedure of Johnson et al. (17) and modified as follows: 3 x 10^6 cells in 10 μl of complete medium were deposited onto agar discs in 35-
mm cluster dishes (Costar). Agar discs were composed of 1% agar (Difco) in Earle's saline with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid without sodium bicarbonate and with gentamicin (100 μg/ml). To form discs, the mixture was autoclaved, and 2 ml were transferred by pipet into each well of a 16-mm 24-well cluster plate (Falcon No. 3008) and refrigerated overnight. Solidified agar discs were loosened and removed with a flat stainless steel spatula. The discs were then placed, miniscus side up, in the 35-mm wells. Three ml of medium were added to each well surrounding the agar plugs, and the dishes were subsequently incubated at 37° in 5% CO_2-95% O_2 for 21 days. The medium for the organ culture, Dulbecco's modified Eagle's medium with nonessential amino acids, bovine insulin (5 μg/ml), gentamicin (10 μg/ml), and 10% FCS, was replaced 3 times per week. Extracts of the tissues were prepared for antigen analysis.

Cell Extracts. Cell extracts were prepared from finely minced tissues or cell packs by homogenization for 2 to 3 min in 10 ml Tris-HCl (pH 7.2) containing 0.2 mm CaCl_2. The homogenate was subjected to pressure homogenization using a cell disruption bomb (Parr Instrument Co., Moline, IL) for 5 min at 1000 psi and then clarified at 1000 x g for 10 min. The supernatant was sonicated on ice for 1 to 2 min using 15-sec intervals (Branson sonifier; Setting 7). The sonicate was centrifuged at 10,000 x g for 10 min, and the supernatant was assayed for antigens by solid-phase RIA. Protein was determined by the method of Lowry et al. (19).

Solid-Phase RIA. Immuneactivity of MAB 722.3 was determined by solid-phase RIA using cell extracts. Five μg [in 50 μl of PBS containing calcium and magnesium (pH 7.2)] of the cell extracts were added to each well of 96-well microtiter polystyrene chloride plates and allowed to dry. To minimize nonspecific protein absorption, microtiter wells were treated with 100 μl of 5% BSA (w/v) in PBS and incubated for 1 hr at 37°. The BSA was removed, and varying amounts of antibody (in 50 μl) were added. After a 1-h incubation at 37°, or overnight at 4°, the unbound radiolabeled second antibody was removed by washing the plates with 1% BSA (w/v) in PBS. Radiolabeled goat anti-mouse IgG was then added (75,000 cpn in 25 μl). After an additional 1-h incubation at 37°, or overnight at 4°, the unbound radiolabeled second antibody was removed by washing the plates with 1% BSA in PBS. The bound cpn were counted by cutting the individual wells from the plate and measuring the radioactivity in a gamma counter. When radiolabeled Mabs were used, the incubation with the radiolabeled second antibody was omitted.

Live Cell RIA (for Cell Surface MAb Binding). Subconfluent monolayer cultures of established cell lines were detached from 75-cm tissue culture flasks using 0.1% trypsin containing 0.5 mM EDTA. Cells were seeded into 96-well flat-bottomed tissue culture plates (Linbro Scientific, Inc., Camden, CT), using the appropriate growth medium, at 5 x 10^4 cells/well and were incubated at 37° 18 to 24 hr in a humidified incubator containing 5% CO_2. All subsequent incubations were performed using identical conditions. The growth medium was then aspirated, and 100 μl of RPMI 1640 containing 10% (w/v) BSA and 0.08% (w/v) sodium azide were added to each well. After a 60-min incubation, the fluid was aspirated, and the plates were washed with RPMI 1640 with 1% (w/v) BSA and 0.08% (w/v) sodium azide (wash buffer). The wash buffer was then removed, and the plates were inverted to remove excess fluid. All additional washes were performed in this manner. Fifty μl of the appropriate concentration of MAb were then
added to the wells. After a 1-hr incubation, the unbound antibody was washed from the wells, and 75,000 cpm of 1251-goat anti-mouse IgG F(ab’)2 in 50 μl were added to each well. The plates were incubated for 1 hr and then washed 3 times with wash buffer. The wells were then examined for the presence of cells; more than 95% of the cells were present in all assays. To determine cpm bound, 50 μl of 2 N NaOH were added to each well. Cotton swabs were used to absorb the fluid from each well and then were counted in a gamma counter. Nonspecific binding [the average of the cpm obtained with wash buffer and NS-1 supernatant fluid (approximately 200 cpm)] was subtracted from the cpm obtained when MAb was used as primary antibody.

RIA for Cells in Suspension

Single cell suspensions were prepared from monolayer cultures or tumors using 0.1% trypsin containing 0.5 m Eq EDTA. Cells were centrifuged at 800 x g for 5 min and resuspended in the appropriate growth medium at a concentration of 2 x 10^6 cells/ml. Cells (0.1 ml/well) were seeded into 96-well polyvinyl chloride microtiter plates and incubated at 37° for 1 hr on a microshaker (Dyynachem) placed in a humidified incubator containing 5% CO2. All additional incubations were conducted in an identical manner. The assay plates were then centrifuged at 1000 x g for 10 min, and the supernatant fluid was aspirated. The cells were washed twice in RPMI-1640 containing 1% BSA (w/v) and 0.08% (w/v) sodium azide. All washes were performed by centrifugation of the plates at 1000 x g for 10 min, followed by aspiration of the supernatant fluid and resuspension of the cells in wash buffer. The appropriate concentration of 1251-labeled primary antibody in 100 μl was then added, and the cells were incubated for an additional 1 hr. The unbound antibody was washed from the wells, and the cells were lysed with the addition of 50 μl of 2 N NaOH. Cotton swabs absorbed the fluid from each well and were counted in a gamma counter. Nonspecific binding [the average of the cpm obtained with a tumor antigen-negative cell line (approximately 200 cpm)] was subtracted from the cpm obtained with a tumor antigen-positive cell line.

Immunoperoxidase Assay

The avidin-biotin complex immunoperoxidase assay was performed on 5-μm sections from formalin-fixed, paraffin-embedded tissue sections as described previously (15, 22). An isotypic identical primary antibody was used as a negative control.

RESULTS

Reactivity of MAb B72.3 to Breast and Colon Tumors versus Normal Tissue. B72.3 reacts with 46% of mammary carcinomas tested by the immunoperoxidase assay on formalin-fixed sections and with 40% of a different group of mammary carcinomas assayed by solid-phase RIA of protein extracts (Table 1). B72.3 reacts with an even greater percentage of colon carcinomas: 82% of those assayed by the immunoperoxidase method and 2 of 3 of those tested by solid-phase RIA (Table 1). MAb B72.3 reacts, therefore, with a total of 25 of the 56 mammary carcinomas and 16 of the 20 colon carcinomas tested by both immunoperoxidase methods (Table 1; Refs. 22 and 29). No reactivity was observed to normal liver, spleen, kidney, ovary, or cervix by either assay (Table 1; Refs. 22 and 29).

Cell Surface Reactivity of MAb B72.3. In an attempt to further define the range of distribution of the B72.3-reactive antigen, TAG-72, MAb B72.3 was tested for reactivity to live cells in culture. Established human cell lines derived from carcinomas of the mammary gland and colon and also from normal tissue were assayed during log phase of growth using a live cell RIA for surface expression of TAG-72. As shown in Table 2, TAG-72 was present on the surface of only one (MCF-7) of 28 breast carcinoma cell lines. The reactivity observed with the MCF-7 cell line varied at different cell passages with some passages expressing no detectable TAG-72. Furthermore, 3 additional MCF-7 cell lines obtained from 3 different laboratories (M. L., S. S., G. C.) were consistently negative for TAG-72.

TAG-72 was detected on the surface of 2 of 19 colon carcinoma cell lines (Table 2). The 2 positive cell lines were both derived from the same culture of an adenocarcinoma of the colon, with the LS-174T being a faster growing variant of the LS-180 cell line (31). Immunohistochemical studies on the LS-174T and MCF-7 cell lines showed only a small percentage (usually less than 5%) of the cells binding MAb B72.3 (data not shown). As seen in Table 2, no reactivity was observed with MAb B72.3 to the surface of 26 normal human cell lines.

Effect of Substratum and Medium on Antigen Expression. A series of experiments were then conducted on LS-174T cells maintained on different substrates, or in serum-free medium, to determine if these modifications would modulate or enhance TAG-72 expression to the level detected in or on the mammary or colon carcinoma biopsy material (Table 3). In the experiments described below, the level of antigen expression was measured either by solid-phase or live cell RIA. The amount of antigen expressed on the cells propagated on different substrates or in serum-free medium was normalized against the level of antigen detected in cells grown on plastic tissue culture dishes for 1 week in 10% FCS-containing growth medium (control).

LS-174T cells maintained on live or X-irradiated chick embryo fibroblast or on mitomycin C-treated Flow 4000 cells showed no significant difference in the amount of TAG-72 (Table 3). The amount of TAG-72 measured on cells grown on tissue culture dishes coated previously with laminin, type I collagen plus fibronectin, or type IV collagen and laminin was comparable to the level of antigen being expressed on cells which had been grown on plastic in medium containing serum (Table 3). Some decrease in TAG-72 expression was seen using fibronectin, 5% FCS, or 20% FCS. These observations, however, were not studied further, since the primary object of our experiments was to define parameters that increase TAG-72 expression. As controls for these studies, the antigens detected by 3 other MAbs (B1.1, B6.2, and B139) were also monitored (Table 3).

An enhancement of TAG-72 expression was noted when LS-174T cells were grown on adherent type I collagen gels. Under these conditions, there was an approximate 2-fold increase in the level of TAG-72 (Table 3). Interestingly, there was also an

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Table 1

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Method of assay</th>
<th>No. of positive/total tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammary</td>
<td>Immunoperoxidase</td>
<td>19/41 (46)</td>
</tr>
<tr>
<td>Colon</td>
<td>Solid-phase RIA</td>
<td>6/15 (40)</td>
</tr>
<tr>
<td></td>
<td>Immunoperoxidase</td>
<td>14/17 (82)</td>
</tr>
<tr>
<td></td>
<td>Solid-phase RIA</td>
<td>2/3 (66)</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Solid-phase RIA</td>
<td>0/3</td>
</tr>
<tr>
<td>Spleen</td>
<td>Solid-phase RIA</td>
<td>0/4</td>
</tr>
<tr>
<td>Kidney</td>
<td>Solid-phase RIA</td>
<td>0/3</td>
</tr>
<tr>
<td>Ovary</td>
<td>Solid-phase RIA</td>
<td>0/1</td>
</tr>
<tr>
<td>Cervix</td>
<td>Solid-phase RIA</td>
<td>0/1</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of those positive per total number tested.
INFLUENCE OF SPATIAL CONFIGURATION ON TUMOR ANTIGEN EXPRESSION

Table 2
Reactivity of MAb B7-3 to surface of human carcinoma and normal cell lines
Reactivity was determined using live-cell RIA as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Origin of cell lines</th>
<th>Positive</th>
<th>Negative (&lt;500 cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>LS-174T (1600 cpm)</td>
<td>WIDR, HT-29, COL0320DM, COL0320HRS, COL0205, COLO201, SW603-3, SW1116, SW48, SW1417, SW1948, SW403, SW480, HS0644T, HS0675T, HS0293T, HS0255T</td>
</tr>
<tr>
<td>Normal</td>
<td>HS0578BST (breast), HS0579BST (breast), HS0584BST (breast), HS0181Tes (testis), HS0208Th (thymus), HS0748BM (bone marrow), HS02353p (spleen), HS0789Jt (uterus), HS0857K (kidney), HS0715K (kidney), HS0711K (kidney), HS0014M (muscle), HS0130M (muscle), HS0129Br (brain), HS0130Br (brain), HS0612BL (bladder), WI-38 (lung), MRC-5 (lung), DG50 (skin), DG51 (skin), Flow 4000 (kidney), CCD-19C0 (colon), HOCM (colon), CCD-33C0 (colon), COLO-20 (colon), CCD-112CON (colon)</td>
<td></td>
</tr>
</tbody>
</table>

a BCTF, Breast Cancer Task Force.
b MCF-7 (BCTF) was also negative at different passages.

Table 3
Effect of substratum and medium on antigen expression in the LS-174T cell line

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAB reactivity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic (control)</td>
<td>100 100 100 100</td>
</tr>
<tr>
<td>Substratum</td>
<td></td>
</tr>
<tr>
<td>CEF®</td>
<td>80 78 90 90 63</td>
</tr>
<tr>
<td>X-irradiated CEF®</td>
<td>73 75 77 79 89</td>
</tr>
<tr>
<td>Miltoycin C-treated Flow 4000®</td>
<td>71 100 NT NT</td>
</tr>
<tr>
<td>Basement membrane®</td>
<td>90 76 NT NT</td>
</tr>
<tr>
<td>Type I collagen gels®</td>
<td>180 225 NT NT</td>
</tr>
<tr>
<td>Fibronectin®</td>
<td>23 69 78 116</td>
</tr>
<tr>
<td>Laminin®</td>
<td>90 86 114 88</td>
</tr>
<tr>
<td>Collagen I + fibronectin®</td>
<td>87 70 94 92</td>
</tr>
<tr>
<td>Collagen IV + laminin®</td>
<td>78 95 117 99</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td>FEIT®</td>
<td>80 153 200 108</td>
</tr>
<tr>
<td>FEIT + 5% FCS®</td>
<td>64 139 184 100</td>
</tr>
<tr>
<td>EME + 2% FCS®</td>
<td>147 101 109 107</td>
</tr>
<tr>
<td>EME + 20% FCS®</td>
<td>48 125 149 112</td>
</tr>
</tbody>
</table>

a All experiments were performed with 100 ng of MAB B7-2, B1-1, and B6-2, and 10 ng of B139. All results are given as the percentage of control LS-174T cells grown on plastic.
b CEF, chick embryo fibroblast; NT, not tested; EME, Eagle’s minimal essential medium.
c Solid-phase RIA using 3 µg LS174T protein extract.
d Live cell RIA.
e Solid-phase RIA using 1.2 µg LS174T protein extract.
f Solid-phase RIA using 5 µg LS174T protein extract; cells grown in FEIT.
g Solid-phase RIA using 5 µg LS174T protein extract; cells grown in EME containing 2 mM L-glutamine and nonessential amino acids.

agitated in either low (2%) or high (20%) concentrations of serum. Under either situation, the amount of TAG-72 was not significantly different from the control cultures which were maintained in 10% serum.

Effect of Cell Configuration on Antigen Expression. LS-174T cells were cultured in serum-supplemented (10% FCS) or serum-free FEIT medium on bacteriological Petri dishes to prevent cell attachment and to facilitate the formation of spheroids or organoids (12, 36). Cells were also grown in suspension cultures to promote the formation of cell aggregates. Expression of TAG-72, as well as CEA, was quantitated by solid-phase RIA after 2 to 3 weeks and compared to the level of antigen expressed in cells grown in monolayer cultures in the presence of serum or in serum-free FEIT medium (Chart 1). In serum-free FEIT medium (Chart 1A), there was a 2-fold increase in TAG-72 levels in cells maintained as spheroids or in suspension culture as compared to cells grown on plastic tissue culture dishes. This enhancement in antigen expression was observed at the different amounts of protein extracts which were tested in the solid-phase RIA. A 3- to 4-fold increase in the level of CEA, as detected by MAb B1.1, was also observed in the spheroid and suspension cultures. Growing cells for an extended length of time as monolayers or to confluency did not alter TAG-72 expression.

In serum-containing medium, the effect of growing LS-174T cells as spheroids was more dramatic on TAG-72 expression (Chart 1B). There was approximately a 6- to 7-fold increase in the level of TAG-72, and a 3- to 4-fold increase in the amount of CEA, as compared to the amount of these antigens in cells grown on monolayers on plastic.

Studies were then undertaken to determine if additional variations in cellular configuration during growth could increase TAG-72 expression above the level observed when LS-174T cells were grown as spheroids. LS-174T cells were grown as monolayers on plastic culture flasks or on agar plugs as described in "Materials and Methods." Monolayer cultures, assayed using the immunoperoxidase cytospin method (14), showed approximately 1% of the cells expressing TAG-72. In contrast, LS-174T cells were also propagated in either low (2%) or high (20%) concentrations of serum. Under either situation, the amount of TAG-72 was not significantly different from the control cultures which were maintained in 10% serum.

The propagation of various cells in serum-free, hormone-supplemented medium has been shown to be efficacious for the expression of certain differentiative functions (2, 35). When LS-174T cells were maintained in serum-free FEIT medium or supplemented with 5% FCS, there was no significant effect on the expression of TAG-72 (Table 3). However, B6.2 binding was increased approximately 2-fold, while that of B1.1 was only slightly increased by 40 to 50%. LS-174T cells were also propagated in either low (2%) or high (20%) concentrations of serum. Under either situation, the amount of TAG-72 was not significantly different from the control cultures which were maintained in 10% serum.

Effect of Cell Configuration on Antigen Expression. LS-174T cells were cultured in serum-supplemented (10% FCS) or serum-free FEIT medium on bacteriological Petri dishes to prevent cell attachment and to facilitate the formation of spheroids or organoids (12, 36). Cells were also grown in suspension cultures to promote the formation of cell aggregates. Expression of TAG-72, as well as CEA, was quantitated by solid-phase RIA after 2 to 3 weeks and compared to the level of antigen expressed in cells grown in monolayer cultures in the presence of serum or in serum-free FEIT medium (Chart 1). In serum-free FEIT medium (Chart 1A), there was a 2-fold increase in TAG-72 levels in cells maintained as spheroids or in suspension culture as compared to cells grown on plastic tissue culture dishes. This enhancement in antigen expression was observed at the different amounts of protein extracts which were tested in the solid-phase RIA. A 3- to 4-fold increase in the level of CEA, as detected by MAb B1.1, was also observed in the spheroid and suspension cultures. Growing cells for an extended length of time as monolayers or to confluency did not alter TAG-72 expression.

In serum-containing medium, the effect of growing LS-174T cells as spheroids was more dramatic on TAG-72 expression (Chart 1B). There was approximately a 6- to 7-fold increase in the level of TAG-72, and a 3- to 4-fold increase in the amount of CEA, as compared to the amount of these antigens in cells grown on monolayers on plastic.

Studies were then undertaken to determine if additional variations in cellular configuration during growth could increase TAG-72 expression above the level observed when LS-174T cells were grown as spheroids. LS-174T cells were grown as monolayers on plastic culture flasks or on agar plugs as described in "Materials and Methods." Monolayer cultures, assayed using the immunoperoxidase cytospin method (14), showed approximately 1% of the cells expressing TAG-72. In contrast, LS-174T cells

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Chart 1. Effect of cell configuration on TAG-72 and CEA expression. LS-174T cells were grown in serum-free F10T medium (A) or in improved minimal Eagle's medium supplemented with 10% FCS (B). MAb (100 ng) of B72.3 (circles) or of B1.1 (triangles) were used to measure antigen expression in cell extracts by solid-phase RIA. Extracts were prepared from cells grown in monolayer for 3 days (open symbols), as spheroids for 3 weeks (closed symbols), or from spinner cultures for 2 weeks (semiclosed symbols).

Propagated on agar plugs formed 3-dimensional tissue-like growths which, when prepared for histological sections and assayed using the immunoperoxidase method, showed extensive cytoplasmic as well as extracellular staining with MAb B72.3 (Fig. 1). Solid-phase RIA of cell extracts confirmed that cultures grown in agar plugs contained approximately 10 times more TAG-72 than monolayer cultures (Chart 2A). Levels of CEA, as detected by MAb B1.1, and HLA-A, -B, -C, as detected by MAb W6/32 (3), were not appreciably altered by the growth of LS-174T cells on agar plugs (Chart 2, A and B).

Level of Expression of TAG-72 in LS-174T Cells in Vitro versus In Vivo. To determine if tumors generated from the LS-174T cell line and thus possessing a 3-dimensional cell configuration would show enhanced expression of TAG-72, LS-174T cells were inoculated s.c. into athymic mice (nu*¡nu*). Protein extracts of the resulting tumors as well as of the cultured monolayer cell line were then assayed for reactivity with 125I-labeled B72.3 IgG.

As a positive control, extracts of the breast tumor metastasis that was used as immunogen in the generation of this antibody were reacted with 125I-B72.3 in solid-phase RIA. As can be seen in Chart 3A, the radiolabeled B72.3 reacted strongly with the immunogen. No reactivity of the MAb was seen to similar extracts of normal human liver (Chart 3A). Extracts of the LS-174T cell line and the subsequent LS-174T tumors grown in athymic mice were then tested for reactivity with the radiolabeled B72.3 for detection of TAG-72. As illustrated in Chart 3B, B72.3 showed significant binding to the extracts made from the LS-174T tumors. In fact, the level of TAG-72 in the extracts of the LS-174T tumors was slightly higher than that found in the metastasis used as the original immunogen to generate the MAb or in any of the LS-174T cell cultures the conditions of which had been modified (Table 3). The radiolabeled B72.3, however, showed very low reactivity to extracts of the LS-174T cell line (Chart 3B). When a vast excess of unlabeled B72.3 is added to extracts of the LS-174T cell line, low levels of TAG-72 could be detected. The amount of TAG-72 that is found in the LS-174T tumors is approximately 100 times the level found in extracts prepared from the cell line. This is in contrast to the results obtained with control MAb B1.1 (anti-CEA), which showed greater binding to the LS-174T cell line than to the LS-174T tumor extract (Chart 3C).

Experiments were conducted to determine if repeated passage of the LS-174T tumors in athymic mice would produce an alteration in levels of TAG-72 expression. Primary tumors were
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Chart 2. Expression of TAG-72, CEA, and HLA in LS-174T cells grown on agar versus monolayers. Using a solid-phase RIA, 100 ng of purified MAb IgG were reacted with increasing amounts of cell extracts of LS-174T cells grown on agar (□, △, ■) or LS-174T cells grown as monolayers (O, △, △). A, MAb B72.3 (□, △) and MAb W6/32 (△, △); B, MAb B1.1 (■, △).

Chart 3. Expression of TAG-72 (using MAb B72.3) and CEA (using MAb 1.1) in LS-174T cells grown in culture and as tumors in athymic mice. Approximately 4 ng of 125I-B72.3 were added to increasing amounts of protein extracts of a human breast tumor metastasis to the liver (□) and to normal human liver (○) (A), LS-174T cells grown in culture (○) and as a tumor in athymic mice (□) (B), and 125I-B1.1 (approximately 2 ng), a MAb reactive with CEA that was used in a solid-phase RIA with increasing amounts of protein extracts of LS-174T cells grown as a tumor in athymic mice (□) or LS-174T cells grown in monolayer culture (○) (C).

Chart 4. Surface expression of TAG-72 in LS-174T cells grown in culture or as tumors in athymic mice. Using a live-cell-suspension RIA, increasing amounts of 125I-labeled MAb B72.3 were tested for binding to a single-cell suspension of LS-174T cells grown as a tumor in athymic nu/nu mice (□) and as a monolayer on plastic (○). Approximately 20 × 10^3 cpm (10-fold more) of the 125I-labeled antibody bound per 5 × 10^5 cells (Chart 4).

DISCUSSION

The initial observation that there was a significant degree of TAG-72 expression in mammary and colon carcinoma biopsy material versus cell lines led us to attempt to delineate factors which might be important in modulating the expression of this antigen. It has been demonstrated previously that fibroblasts are critical components of the extracellular matrix can function in a permissive fashion to facilitate cell proliferation and differentiation in vivo (5, 9, 10, 12, 16, 20, 21, 24, 26, 27, 30, 34, 37). In fact, type I collagen gels and some combinations of defined medium and serum did appear to slightly enhance TAG-72 levels on monolayer cultures of LS-174T cells. The most striking increases in TAG-72 expression were observed, however, when LS-174T cell were grown in culture systems designed to promote 3-dimensional growth. Spheroid and suspension cultures increased TAG-72 levels by 2- to 7-fold, while agar cultures gave a 10-fold enhancement. The role of spatial configuration on TAG-72 expression may be related to the differences in cell cluster size that the different culture methods produce. For example, LS-174T cells form clusters of up to several hundred cells in spheroid and suspension cultures, while LS-174T cells grown on agar plugs form a single solid mass of perhaps 1 to 10 million cells. This suggests that these aggregates may have to reach some critical mass or number of cells before TAG-72 expression is appreciably enhanced. Alternatively, there may be a cooperative effect in cell-to-cell interactions in these structures in vitro which may closely parallel the situation in vivo in the tumors with respect to facilitating antigen expression. In this respect, Rutsky et al. (25) have reported that the LS-174T cell line forms organoid structures resembling the patient's original tumor and produces μg quantities of CEA when grown in an artificial hollow-fiber apparatus. In fact, histological sections of LS-174T cells grown on agar plugs (Fig. 1) demonstrate the presence of glandular structures and columnar epithelial cells, which closely resembles the morphology of LS-174T cells grown as tumors in mice (data not shown) as well as those cells grown in hollow fibers.
appears, therefore, that the level of expression of TAG-72 in vitro can in part be controlled or determined by the 3-dimensional configuration of these cells during growth and their concurrent differentiation into organized structures resembling those found in tumor tissue in vivo. This is substantiated by the observation that LS-174T cells grown on agar plugs also exhibit abundant neutral and acid mucin formation as determined by Alcian blue-periodic acid-Schiff staining (data not shown). The production of copious quantities of mucin has been reported previously for both the LS-174T and LS-180 cell lines (32). The present study demonstrates that spatial configuration may serve as an important factor which may modulate antigen expression in a mixed population of tumor cells. Spatial configuration may be acting here by either amplifying the expression of TAG-72 on cells which already express this antigen or by inducing the appearance of TAG-72 on a negative subpopulation of tumor cells.

A variety of apparently diverse factors also appear to influence the selective expression of different antigens. For example, MAb B1.1 binds to CEA, which is expressed on the LS-174T and LS-180 cell lines (32). In contrast to the results observed with TAG-72, a 3- to 4-fold increase in CEA was found when LS-174T cells were grown in suspension or spheroid cultures, but no increase was detected on cells which were propagated on agar or on cells which were obtained from tumors in nude mice. The selective effect of factors such as spatial configuration on antigen expression agrees with the findings of Raz and Ben-Ze’ev (23), who demonstrated that there was a reduction in the availability of some cell surface proteins to external iodination after growing B16-F1 melanoma cells as spheroids. Other factors have also been shown to be involved in controlling the level of other antigens in carcinoma cell populations. For example, using MAb B6.2, we have reported previously the influence of cell cycle on the level of detectable tumor-associated antigens (14, 18). Specifically, the Mr 90,000 glycoprotein reactive with MAb B6.2 which binds to a majority of breast carcinomas is expressed more during S phase; other antigens have been shown to be more evident during different phases of the cell cycle (14, 18, 28).

MAb B72.3 reacts with a high-molecular-weight glycoprotein bound predominantly on breast and colon carcinoma cells, TAG-72. Several MAbs, including those that recognize tumor-associated glycoproteins, have been useful in determining the role of and in purifying the antigen with which they react (1, 4, 33). Since TAG-72 is located on the surface of cells, and since its expression is markedly enhanced by cell-to-cell contact and spatial configuration, one possible role for this antigen may be in the adhesion of tumor cells to each other and/or to their substratum. Growth of LS-174T cells on agar and as tumors in athymic mice should prove to be useful in in vitro and in vivo model systems not only to study other factors which may be involved in the expression of TAG-72, but also to aid in the characterization of this antigen and the determination of its function in tumor cell populations.

The use of carcinoma cell lines as model systems for tumor antigens should be approached with caution, considering the knowledge that antigenic heterogeneity and modulation are permissive factors which are operative in vitro as well as in vivo. We have demonstrated previously that there is a selective reduction in TAG-72 levels in MCF-7 cells as a function of cell passage levels (14). We have also observed that the same MCF-7 cell line obtained from 4 different laboratories expresses 4 distinct antigenic phenotypes (14). Furthermore, several clones derived from the same MCF-7 cell line fail to maintain a stable antigenic phenotype during repeated passage in vitro. Many, in fact, eventually reverted to the antigenic phenotype of the original parent cell line (14). These observations tend to support the conclusion that an established cell line may not accurately reflect the antigenic characteristics of the original tumor or tissue from which it was derived. This is not surprising in view of the difficulty in establishing carcinoma cell lines and the extensive selective pressure exerted on cell populations toward that end. Furthermore, the dynamic nature of antigenic heterogeneity and modulation may make this the rule rather than the exception. It is for this reason that biopsy material may be more favorable than cell lines as a source of immunogen for the generation of certain MAbs.

The antigenic heterogeneity and modulation in carcinoma cell populations may have profound implications in the use of some MAbs in patient management. Antigen expression on primary tumors could differ from that of metastases from the same patient. Antigens present on a tumor at the time of biopsy may also modulate over the course of several weeks, months, or years. Factors such as vascularization, size of the tumor, as well as other environmental elements could be involved in this antigenic modulation. The problem of using MAbs in diagnostic and therapeutic procedures in light of antigenic heterogeneity and modulation may possibly be overcome by the use of groups of MAbs, so that all tumor cells react with at least one of the MAbs. Alternatively, coupling of MAbs to isotopes decaying via high-energy transfer with short-range radiation could lead to the killing of not only the tumor cell expressing the tumor-associated antigen, but also of nearby tumor cells which do not. Another possibility is the administration of agents, such as recombinant interferon, to patients to enhance tumor-associated antigen expression prior to use of MAbs. In vitro studies on the use of alpha clone A interferon to enhance the expression of tumor-associated antigens on breast and colon carcinoma cell lines support this latter hypothesis (11).

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