(54) Title: MONOCLONAL ANTIBODIES SPECIFIC FOR TUMOR ANTIGENS

(57) Abstract

The present invention relates to tumor-specific monoclonal antibodies that recognize antigenic determinants of a membrane component characteristic of squamous cell carcinoma, and to hybridomas producing same. The invention further relates to a diagnostic kit comprising the tumor-specific antibodies. The invention also relates to the use of the tumor-specific antibodies in determining the presence of squamous carcinoma cells in a biological sample.
**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>Austria</td>
<td>ES</td>
<td>Spain</td>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
<td>FI</td>
<td>Finland</td>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>FR</td>
<td>France</td>
<td>MR</td>
<td>Mauritania</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>GA</td>
<td>Gabon</td>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
<td>GB</td>
<td>United Kingdom</td>
<td>NL</td>
<td>Netherlands</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>HU</td>
<td>Hungary</td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
<td>IT</td>
<td>Italy</td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>JP</td>
<td>Japan</td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
<td>KP</td>
<td>Democratic People's Republic of Korea</td>
<td>SE</td>
<td>Sweden</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>KR</td>
<td>Republic of Korea</td>
<td>SN</td>
<td>Senegal</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>LI</td>
<td>Liechtenstein</td>
<td>SJ</td>
<td>Soviet Union</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>LK</td>
<td>Sri Lanka</td>
<td>TD</td>
<td>Chad</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>LJ</td>
<td>Luxembourg</td>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>DE</td>
<td>Germany, Federal Republic of</td>
<td>LU</td>
<td>Luxembourg</td>
<td>US</td>
<td>United States of America</td>
</tr>
</tbody>
</table>
MONOClonAL antibOdIES SPECIFIC FOR TUMOR ANTIGENS

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates, in general, to monoclonal antibodies and, in particular, to monoclonal antibodies that recognize a tumor-specific antigen on squamous cell carcinomas.

Background Information

Squamous cell carcinoma is the most common form of cancer and yet little is understood concerning its heterogeneity as determined by surface phenotype, cell growth, or the influence of these characteristics on metastases. Recently, efforts at culturing human squamous cell carcinomas (SCC) and transplanting them into nude mice for in vivo models of SCC have been successful (Fogh, et al., J. Natl. Cancer Inst. 59:221-225, 1977; Kyriazis, et al., Cancer Res. 41:3995-4000, 1981; Easty, et al., Br. J. Cancer 48:772-785, 1981; and Baker, Laryngol. 95:43-56, 1985). These efforts have led to some initial identification of cell surface antigens (primarily glycolipid molecules) that are recognized by monoclonal antibodies and that may be useful in prognosis and, perhaps, therapy (Carey, et al., Otol. Head and Neck Sur. 91:482-491, 1983; Kimmel, et al., J. Natl. Cancer Inst. 76:9-17, 1986; Kato, et al., Cancer 40:1621-

Carey, et al. have identified a number of monoclonal antibodies specific for squamous cell carcinoma of the head and neck, but these antibodies also react to some extent with normal squamous epithelium. The antibody designated A9 is reported to react with 16 of 21 squamous tumors tested as well as 1 of 2 bladder carcinomas and 1 of 4 adenocarcinomas (Carey, et al., Otol. Head and Neck Sur. 91:482-491, 1983). Antibody, G10, reacts with a blood group antigen also expressed on normal squamous cells (Kimmel, et al., J. Natl. Cancer Inst. 76:9-17, 1986).

Another tumor antigen to which monoclonal antibodies have been produced, termed TA-4, has been described as a 48 KDa protein. This antigen is expressed at variable levels on cervical, vaginal, lung and esophageal tumors (33.3-47.1%) but its expression is more limited on tumors of other origins (10%) (Kato, et al., Cancer 40:1621-1628, 1977). TA-4 is found in the serum of patients bearing a tumor expressing this tumor antigen.

The development of monoclonal antibodies specific for antigens on SCC would provide a highly sensitive diagnostic, and also potentially therapeutic, tool. In tumor diagnosis, the use of such antibodies would result in improved diagnostic methods applicable to either tissue or serum samples. As therapeutic agents, monoclonal antibodies may be effective either alone or conjugated to chemotherapeutic agents, radioisotopes or toxins. The use of monoclonal antibodies as a therapeutic agent would require, however, that
expression of the antigen predominate on tumor (rather than normal) cells and that little or no circulating tumor antigen be produced which could complex with the antibody.

5

SUMMARY OF THE INVENTION

It is an object of the invention to provide hybridomas capable of producing monoclonal antibodies specific for antigenic sites on a membrane component characteristic of human SCC.

It is another object of the invention to provide monoclonal antibodies that recognize epitopes of a squamous cell carcinoma-specific antigen.

It is a further object of the invention to provide a diagnostic method for identification of immunoreactive squamous carcinoma cells in a biological sample and a kit based thereon.

It is another object of the invention to provide a method of treating human SCC using monoclonal antibodies, and modifications thereof, that recognize a squamous cell carcinoma-specific antigen.

These objects, and others which will be apparent to those skilled in the art from the following detailed description, have been accomplished by providing novel monoclonal antibodies which are specific for membrane components characteristic of human SCC.

In one embodiment, the present invention relates to hybridomas, resulting from the fusion of myeloma cells and spleen cells, which produce tumor-specific monoclonal antibodies that form an immune
complex with antigenic determinants of membrane components characteristic of SCC.

In another embodiment, the present invention relates to tumor-specific monoclonal antibodies specific for antigenic determinants of membrane components characteristic of SCC.

In a further embodiment, the present invention relates to a diagnostic kit comprising

i) the above-described monoclonal antibodies, and

ii) a conjugate comprising a binding partner of the monoclonal antibody and a label; alternatively the kit can comprise a conjugate comprising the above-described monoclonal antibody and the label.

In another embodiment, the present invention relates to a method for determining the presence of squamous carcinoma cells in a biological sample suspected of containing such cells, comprising:

i) contacting the sample with the above-described tumor-specific monoclonal antibody under conditions such that binding of the monoclonal antibody to the antigenic determinant of the membrane component is allowed to occur, and

ii) detecting the presence of monoclonal antibody bound to the antigenic determinant, the extent of such binding being related to the presence of squamous carcinoma cells in the sample.

In yet another embodiment, the present invention relates to a conjugate for treating a patient having squamous cancer comprising:
i) the above-described monoclonal antibody, or binding fragment thereof, linked directly or indirectly to

ii) an agent capable of rendering a lethal or growth inhibiting event to cells of said squamous cell carcinoma.

In another embodiment, the invention relates to a method of treating a patient having squamous cancer comprising administering to the patient a therapeutically effective amount of the above-described conjugate.

In yet another embodiment, the invention relates to a method of diagnosing the presence of squamous carcinoma cells in a patient comprising: administering to the patient a conjugate comprising

i) the above-described monoclonal antibody, or binding fragment thereof, linked directly or indirectly to

ii) a moiety capable of being detected by a source external to the patient; and detecting the presence of the detectable moiety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Western blot of membrane proteins from tumor EE1 and tumor antigen purified from culture medium.

Figure 2: Immunoprecipitation of membrane proteins from HT 1376.
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to monoclonal antibodies specific for antigenic determinants present on a membrane component (apparently proteinaceous in nature) characteristic of human SCC. Monoclonal antibodies of the invention are, advantageously, of the IgM or IgG type. The antibodies of the invention are tumor-specific, that is, they are specific for an antigen expressed predominately on tumor (rather than adult normal) cells.

The monoclonal antibodies of the invention are produced by hybridomas, advantageously murine hybridomas, that can be prepared and selected as described in the Examples that follow. For example, cultured cells, or preparations thereof, derived from a human squamous cell carcinoma are injected into a mouse, advantageously a Balb/c mouse, after a time sufficient to induce an immune response, the mouse is sacrificed and the spleen cells are obtained and fused, advantageously, with myeloma cells, using known techniques. The resulting cells, which include the fused hybridomas, are allowed to grow in a selective medium, such as HAT-medium, and the surviving cells are grown in such medium using limiting dilution conditions. The cells are grown in a suitable container, e.g., microtitre wells, and the supernatants are screened for monoclonal antibodies having the desired specificity.

Monoclonal antibodies of the invention of the IgM type are exemplified by novel antibodies designated 1C11, and 10B2. Each of these monoclonal antibodies is specific for an antigenic site on a
membrane component characteristic of human SCC. When target cells are homogenized and
electrophoresed in a 7-15% gradient polyacrylamide
slab gel, and subjected to Western Blot analysis,
the antigen appears as a single band having a
molecular weight of about 87KDa (See Figure 1).
Immunoprecipitation studies, performed by adding the
antibody to a membrane preparation in which
antigenic sites are preserved, reveal additional
proteins of about 30 and about 114 KDa (see Figure
2) (monoclonal antibodies are sometimes unable to
recognize denatured antigens such as are found after
SDS polyacrylamide gel electrophoresis and Western
Blot analysis). Several possibilities exist to
explain these three species of antigen. These may
be different but antigenically related proteins, there may be a precursor product relationship
between these species, or the antigen may form
multimeric complexes. The latter two possibilities
are consistent with the closeness in multiplicity of
molecular weights.

Antibody 3A4 exemplifies an IgG antibody
of the invention. It recognizes an antigen of about
75 KDa as determined by immunoprecipitation (see
Figure 2).

Hybridomas designated 1C11, 10B2, and
3A4/73, producing antibodies designated 1C11, 10B2
and 3A4, respectively, were deposited with the
American Type Culture Collection, 12301 Parklawn
Drive, Rockville, MD, USA, on September 9, 1988, and
were given ATCC accession numbers HB 9819, HB 9820
and HB 9821, respectively.

The invention also relates to useful
binding fragments of the tumor-specific monoclonal
antibodies such as Fab or F(ab')₂. The antibody fragments are obtained by conventional techniques. For example, useful binding fragments can be prepared by digestion of the antibody using papain or pepsin.

While the above-specified examples of the antibodies of the invention are of the IgG and IgM classes and are from a murine source, this is not meant to be a limitation. The specified antibodies and antibodies functionally equivalent thereto (that is, capable of binding to the above-described membrane antigens characteristic of SCC) whether from a murine source, mammalian source, including human, or other sources, or combinations thereof, are included within the scope of this invention, as are antibodies of other classes such as IgA, IgE, and the like, including isotypes within such classes.

Various conventional methods exist for isolation and purification of the monoclonal antibodies, so as to free monoclonal antibodies from other proteins and other contaminants (see, for example, Goding, in Monoclonal Antibodies: Principals and Practice, Chapter 4, 1986; the entire contents of which document is hereby incorporated by reference and relied upon).

The present invention also relates to diagnostic methods employing the above-described monoclonal antibodies, or binding fragments thereof. In one such method, the presence of tumor cells in a biological sample is determined by contacting the sample with the tumor-specific monoclonal antibody of the invention under conditions such that binding of the antibody to squamous carcinoma cells present
in the sample occurs. The presence or absence of binding of the antibody to the cells of the sample (that is, immune complex formation) is detected using methods known in the art. For example, after contacting the cells of the sample with the monoclonal antibody of the invention, the cells can then be contacted with a labeled (that is, detectable) binding partner specific for the monoclonal antibody, under conditions such that binding occurs. The amount of label associated with the sample is then determined by known quantitative or qualitative means and is indicative of the presence (or absence) of tumor cells in the specimen.

The diagnostic methods of the invention find utility in the detection of a malignant condition, specifically SCC, for example, in the cut surface of biopsy specimens, in cultures initiated from tissue explants, and in serous fluids.

The invention also relates to a diagnostic kit for use in detecting the presence of squamous carcinoma cells in a biological sample, which kit is based, for example, on the method described above. In one embodiment, the diagnostic kit comprises (i) a tumor-specific monoclonal antibody or antibodies (or binding fragment(s) thereof) as defined above, and (ii) a conjugate of a specific binding partner for the monoclonal antibody and a label capable of producing a detectable signal. Reagents, such as ancillary agents, for example, buffering agents and protein stabilizing agents, e.g., polysaccharides and the like, can also be included. The diagnostic kit can further include, where necessary, other members of the signal producing system, of which
system the label is a member, agents for reducing background interference in a test, control reagents, and apparatus for conducting a test. In another embodiment, the diagnostic kit comprises a conjugate of a monoclonal antibody or antibodies of the invention and a label capable of producing a detectable signal. Ancillary agents as mentioned above may also be present.

It is anticipated that in addition to in vitro diagnostic use, the antibodies (or binding fragments thereof) will be used in vivo as an imaging diagnostic and/or therapeutic when conjugated to appropriate radioisotopes, or chemotherapeutic drugs (for example, toxins). The imaging of metastases and the extent of tumor progression can be detected, for example, using a gamma camera following injection of radiolabelled monoclonal antibody (Carrasquillo, et al. Cancer Treatment Reports 68:317-328, 1984; Bloomer, et al. Cancer 55:2229-2233, 1985). It is also anticipated that magnetic resonance imaging can be used to monitor metastasis and tumor progression following injection of an appropriately conjugated monoclonal antibody. The metastases may then be surgically removed following localization. In a similar fashion, antibodies labeled with an agent capable of inducing a lethal or growth inhibiting event (for example, radio- or chemotherapeutic-labeled antibodies) can be used to target tumors for therapy, dependent upon the dose of the labelled antibody delivered and the sensitivity of the tumor. Preliminary results of clinical trials using these approaches have been published (Carrasquillo, et al. Cancer Treatment Reports 68:317-328, 1984;

The following non-limiting Examples illustrate the invention in more detail.

**Example 1**

**Development and Selection of Hybridomas**

**Establishment of Tumor Cell Lines:** Cell line EE1 was established from a recurrence of squamous cell carcinoma of the larynx obtained from a patient on chemotherapy at Tulane Medical Center. The tissue was obtained shortly after relapse, the primary tumor having been resected approximately 2 years earlier. Cell line SC1 was established from a primary squamous cell carcinoma of the larynx obtained from a patient at Tulane Medical Center. Both EE1 and SC1 cell lines were established from tumor pieces that were minced, and distributed into wells of a 24 well tissue culture plate. If needed, fibroblasts were eliminated by sequential treatment of the cell monolayer with 0.1 ml trypsin-EDTA, (Life Sciences) 0.125 g/ml, to which fibroblasts were more sensitive. Similarly, tissue was seeded into 25 cm² flasks and treated in the same fashion, but with 0.5 ml trypsin-EDTA. (Alternatively, the cell line could have been grown until fibroblasts, which were not transformed, ceased dividing.) Two bladder squamous cell carcinomas, Scaber and HT 1376, were obtained from the American Tissue Culture Collection, MD.
The cell lines were propagated in minimal essential medium (MEM) with Hanks salts (Irvine Biologicals) supplemented with 25 mM HEPES (Sigma Chemicals), 10% fetal bovine serum, 1 mM pyruvate, 0.1 mM nonessential amino acids and 0.05 mg/ml gentamicin (Irvine Biologicals). The cells were disrupted with trypsin-EDTA (Gibco Laboratories), then washed and reseeded in fresh medium weekly at 7.5 x 10^6 cells per ml. Cell lines can be grown in culture for one or more years, but some may not have an indefinite life span.

**Preparation of Hybridomas:** EEL cells were harvested by scraping, then washed several times in basic buffered salts (BSS). The tumor cells were irradiated (12,000 Rad) then injected intraperitoneally (ip) into BALB/c mice (1-10 x 10^6 cells per 0.5 ml). Mice were reinjected with tumor cells prepared in the same manner at 2-3 week intervals, subcutaneously or ip at least twice following the initial injection. Spleens were harvested three days following the final injection and fused with the SP/2 myeloma as described previously (Köhler and Milstein, *Nature* 256:495-497, 1975).

**Selection of Hybridomas:** Hybridomas were first screened for Ig production with anti-murine Ig biotinylated conjugate system (Vector Laboratories). Two µg goat anti-mouse Ig were coated onto each well of Linbro 96 well microtitre plates in coating buffer (1.599 g sodium carbonate, 2.93 g sodium bicarbonate and 0.2 g sodium azide per liter distilled water, adjusted to pH 9.6) for one hour at room temperature. Wells were washed in PBS-Tween (0.1% Tween-20) prior to addition of 50 µl hybridoma
supernatant and an equivalent amount of PBS-Tween. After incubation for 1 hour at room temperature, the manufacturer's procedure was followed for detection of monoclonal antibody in culture supernatants using a goat anti-mouse IgG alkaline phosphatase conjugated through the biotin avidin system (Vector Laboratories, ABC Kit).

Monoclonal antibodies that recognized red blood cells, from the patient that donated the tumor, were eliminated from consideration by hemagglutination using anti-mouse IgG secondary antibody. One hundred μl of a 2% dilution of red cells were added to each well of round bottom microtitre wells. Then, 10 μl hybridoma supernatant were added followed by 20 μl goat anti-mouse Ig (100 μg/ml in PBS). The mixture was incubated overnight at 4°C.

Ig positive hybrids were screened for reactivity against peripheral blood mononuclear cells (PBMC) and phytohemagglutinin (PHA) blasts, by a cellular ELISA (Stya, et al., J. Immunol. Methods 73:75-81, 1984), using cells from the same patient from which the tumor was obtained. PBMC at 2 X 10⁶ cells per ml were treated with 2 μg PHA for 3 days prior to screening. PHA-activated cells were first washed by centrifugation in basic salts solution (BSS). PBMC or activated PBMC were added at from 2 X 10⁴ to 5 X 10⁴ cells per well in a volume of 200 μl to Millipore cellulose acetate bottom microtitre plates. Reagents were removed and cells were washed by a vacuum filtration system (Millipore) as described (Stya, et al., J. Immunol. Methods 73:75-81, 1984). All other reagents were as described (Kyriazis, et al., Cancer Res. 41:3995-4000, 1981).
All monoclonal antibodies reacted strongly in this qualitative assay with no reaction with control cell lines. The addition of from 20,000 to 50,000 cells per well was tested in this system with reproducible results.

Monoclonal antibodies that reacted with normal cells from the patient were eliminated from consideration. In addition, monoclonal antibodies were screened by the cellular ELISA method on normal fibroblasts, established from a different patient. Monoclonal antibodies selected for further analysis were positive for reactivity with the EE1 cell line using the cellular ELISA method. Other positive cell lines are included in Table I.

Table I
Summary of Microtitre ELISA Assay on a Variety of Tumors and Normal Cells

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>3A4</th>
<th>1C11</th>
<th>10B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA Blasts</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PBMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EE1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SC1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCC25</td>
<td>nt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FADU</td>
<td>nt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HT 1376</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scaber</td>
<td>+</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>HEla</td>
<td>+</td>
<td>-</td>
<td>nt</td>
</tr>
<tr>
<td>SKMCE</td>
<td>nt</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SIHA4</td>
<td>nt</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

nt = not tested
These data show that monoclonal antibodies react with the two larynx (EE1 and SCl) as well as the two bladder squamous carcinomas (HT 1376 and Scaber) and other squamous carcinomas tested (FADU and SCC25). However, human fibroblasts from an unrelated patient were negative as was a cervical and lung squamous carcinoma (SIHA-4 and SKMES). Interestingly, HEla, a cervical carcinoma cell line, only reacts with 3A4, but not 1C11.

The screening procedures for identification of anti-tumor monoclonal antibodies resulted in the isolation of several antibodies of the IgM subclass and one of the IgG class that have been analyzed in detail. A total of 40 hybridomas were isolated.

Example 2

Detection of Plasma Membrane Bound Tumor Antigen

In order to determine whether monoclonal antibodies prepared and selected as described in Example 1 were specific for a cell surface antigen, cells from tissue culture were examined by immunoperoxidase staining. Fifty μl of cells were seeded onto Multiwell slides (Lux) at 7.5 X 10^6/ml for an overnight incubation. The cells were washed twice with modified PBS (PBS containing 20 mg/liter of thimerosal) then incubated with normal goat serum (10 minutes) to block nonspecific reaction of goat anti-murine IgM peroxidase conjugates. All reactions were carried out at room temperature in a humidified chamber. All subsequent reagents were added in 50 μl and the cells were washed 3-4 times with modified PBS in between each step. Monoclonal
antibody containing hybridoma supernatants, concentrated by 50% ammonium sulfate precipitation, were added at a 1:30 dilution (in PBS) for 20 minutes. The wells were again washed with modified PBS. Goat antimurine IgM peroxidase conjugated biotin-avidin reagents from Vector Laboratories were added (1:1000) for 15 minutes each followed by washing. Color was developed with 0.5 ml aminoethylcarbazole substrate (in N,N-dimethylformamide (120 mg in 15 ml) mixed with 9.5 ml 0.1M acetate and 50 μl H2O2) for 10 minutes. The treated cells were finally washed with water and dried or reacted in Mayers hematoxylin for 10 minutes, as a counter stain, prior to washing. The data in Table II summarize these results.

Table II

Summary of Peroxidase Immunohistochemical Staining on Tumors and Normal Cells

<table>
<thead>
<tr>
<th></th>
<th>MAB</th>
<th>EE1</th>
<th>SCI</th>
<th>Scaber</th>
<th>HT 1376</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10B2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

The negative controls included both irrelevant isotype matched monoclonal or mock addition of monoclonal antibodies.

Not all cells in the Scaber tumor were positive (approximately 50%). Most (70-80% in some experiments), but not all cells from an individual tumor were positive, indicating tumor antigen
expression may be cell-cycle dependent or controlled in some other fashion (for example, density).

Example 3

Characterization of Tumor Antigen

Western Blots:
To investigate the nature of the tumor antigen, the monoclonal antibodies of the invention were reacted in Western Blots produced from crude membrane preparations of tumor. Specifically, EEl cells were harvested from flasks by scraping, and washed in Tris buffered saline (25 mM Tris 0.15 M NaCl, pH 7.4, TBS). The cells were resuspended in TBS and homogenized in a dounce homogenizer. Nuclei were removed by centrifugation (400 x g x 10 min.) and the supernatant was centrifuged at 100,000 x g for 1 hr. to obtain a crude membrane preparation. Protein was determined by the method of Lowry (Lowry, et al., J. Biol. Chem. 193:265-275, 1951), modified for Tris buffers (Pierce Chemicals), and samples diluted into concentrated sample buffer for loading a final sample of 10 µg of membrane protein per well in a 7-15% gradient polyacrylamide slab gel. The proteins were electrophoresed at 30V overnight (Laemmli, Nature 227:680-685, 1970).

The proteins were electroblotted (BioRad Transblot) onto nitrocellulose at 70V for 4 hrs. The blot was treated with 1% bovine serum albumin (BSA) in PBS overnight, then washed 4 times with PBS-Tween for 5 minutes each. The blot was then incubated for one hour with monoclonal antibody-containing hybridoma supernatants or a 1:10 to 1:100 dilution of a 30X concentrate of hybridoma
supernatant made in PBS-Tween. This was followed by washing, then a similar incubation with goat anti-
mouse Ig peroxidase-conjugate (100 μg/ml in PBS-Tween). The blot was washed 3 times in PBS-Tween and once in PBS before addition of the developing reagent (60 mg of 4-chloro-1-naphthol dissolved in 100 ml methanol with 100 ml PBS and 50 μl of 30% H₂O₂ added).

Figure 1, shows the results of Western Blot staining with antibodies lC11 (1:100 dilution of a 10x concentrated by 50% ammonium sulfate precipitation). lC11 recognized an 87,000 dalton molecular weight species on EE1 membranes determined by molecular weight standards. An identical molecular weight membrane protein was similarly detected in membrane preparations derived from HT 1376 cells.

Affinity Purification:

A 100x concentrate of lC11 monoclonal antibody containing culture medium was prepared by ammonium sulfate fractionation (50%) and extensively dialyzed into coupling buffer for CnBr-activated sepharose (Sigma Chemical Co.) conjugation. An affinity column of monoclonal antibody was prepared by overnight incubation of the antibody with preactivated CNBr-Sepharose.

Culture medium from EE1 cells (100 ml after 7 days in culture) was concentrated by ammonium sulfate precipitation (70%) and dialyzed against PBS. This concentrate was applied to a monoclonal antibody affinity column prepared with lC11 antibody coupled to CnBr-activated Sepharose. The tumor antigen was eluted with 0.2 M glycine, pH
2.8, dialyzed against PBS, then analyzed by Western Blot using alkaline phosphatase-conjugated antimouse IgM developed with 5-bromo-4-chloro-3-indoly1 phosphate (Bio Rad). As seen in Figure 1 in the far right lane, a large band of approximately 87,000 daltons M.W. and some minor bands, possibly degradation products, are evident. The fact that the tumor antigen was purified on an affinity column with antibody 1C11 and the product was detected with antibody 10B2 indicates that these antibodies are specific for the same antigen. In conclusion, the 1C11 and 10B2 antibodies appear to be specific for the same 87 KDa antigen on squamous carcinoma cells.

Immunoprecipitation of Tumor Antigens:

Tumor antigens were immunoprecipitated from I-125 radiolabelled HT-1376 cells. A 225 cm² flask of cells grown to approximately 70% confluency was washed several times with PBS to remove any residual medium. Two ml of lactoperoxidase (1 mg/ml) was added to the flask followed by 2.5 mCi I-125 (ICN Pharmaceuticals, Irvine, CA) and 1 ml of H₂O₂ (1:1000 dilution of a 30% solution). After 20 min. at 37° C an additional 1 ml H₂O₂ was added for a second 20 min. incubation period. At the end of the incubation, the medium was removed from the flask and the flask was rinsed with PBS. The cells were scraped from the flask in 3 ml PBS and homogenized in a Dounce homogenizer (teflon/glass) for 7-8 strokes. The homogenate was centrifuged at 200 x g x 5 min. to remove nuclei, then the supernatant was rehomogenized a second time and nuclei again removed by centrifugation. The resulting supernatants from both nuclear spins were
combined and centrifuged at 20,000 x g x 20 min. and the pellet obtained was resuspended in 1 ml PBS containing 0.5% NP-40 and 1 μl phenylmethylsulfonylfluoride (PMSF) to inhibit protease activity.

A 10% suspension of prewashed Staph A cells (200 μl), in PBS, 0.5% NP-40, was added to the solubilized membrane preparation and incubated for 30 min. at 4°C. The Staph A cells were centrifuged at 500 x g x 15 min. and the pelleted cells were discarded. Aliquots of the supernatant were treated with 15 μl of monoclonal antibodies 3A4 (IgG, 1.5 mg/ml) or 1C11 (IgM), derived from Protein A purification or 50% ammonium sulfate precipitation of hybridoma culture medium (10-20 fold concentrated), respectively. Samples were incubated for 90 min. at 4°C before the addition of 150 μl of either goat anti-mouse IgG or IgM (1 mg/ml) for 3A4 or 1C11, respectively. The samples were incubated for an additional 1 hour. Finally, 50 μl of a 10% solution (w/v) of Staph A containing cells was added for 20 min. The samples were centrifuged at 500 x g x 15 min. and the pelleted cells were washed 3 times in PBS, 0.5% NP-40, 0.1% SDS, 0.1% DOC by centrifugation. The final pellet of cells was resuspended in electrophoresis sample buffer containing 2-mercaptoethanol and boiled for 3 min. Samples were loaded onto a 9% polyacrylamide SDS gel and electrophoresed overnight at constant voltage (30V). The gel was dried, then exposed to Kodak XAR-2 film for 14 days before development. The results are shown in Figure 2. Immune precipitation with antibody 3A4 revealed a protein band with molecular weight of approximately 74 KDa. In
contrast, the 1C11 antibody precipitated bands of approximately 30, 87, and 114 KDa. It, thus, appears that these antibodies recognize mutually exclusive antigens expressed on the surface of squamous cell carcinomas.

**Example 4**

**Immunoperoxidase Staining of Tissue Sections**

Ten to thirty micron sections from paraffin blocks of formalin fixed tissues were prepared by microtome sectioning, and placed onto slides coated with albumin according to standard procedures. The sections were deparaffinized, treated with iodine (1 g/100 ml xylene) and rehydrated by standard procedures in xylene, ethanol and finally, distilled water. If necessary, the slides were treated with trypsin (0.0125 g in 12.5 ml PBS) for 5 minutes or less. All procedures were carried out in a humidified chamber at room temperature. Normal serum of the same species as the secondary antimouse Ig reagent was incubated for 20 minutes (Vector Laboratories instructions and reagents) on each section to block nonspecific reaction of the secondary reagent. Serum was drained prior to addition of monoclonal antibody (diluted 1:5 in PBS/0.05% Tween) which was incubated for 1 hour on tissue sections. After 3 rinses in PBS/Tween, the slides were treated with 3% H$_2$O$_2$, in methanol for 20 minutes to block endogenous peroxidase. The sections were then treated with the Vector ABC kit reagents according to manufactures instructions. The Diaminobenzidine (DAB) substrate was used for color development in a 5-20 minutes
incubation (0.023 g DAB, 1 ml H₂O, in 50 ml PBS). Counterstaining was performed with the hematoxylin reagent and the slides were then dehydrated prior to mounting with cover slips.

The staining of fixed tissue sections from a variety of squamous carcinomas of the head and neck resulted in 11 of 11 positives for 10B2, 22 of 22 for 1C11 and 12 out of 18 staining positively with the 3A4 monoclonal antibody. Two of the fixed tumors that were not positive using the 3A4 antibody, did in fact stain weakly. While the use of the 1C11 antibody resulted in some staining of normal epithelium, the extent of staining was much greater on tumor tissue. On frozen sections the antigen recognized by 3A4 is present on normal tissues, but quantitatively greater on tumor.

When fixed tissues are used, several potential problems may occur. First, antigenicity is sometimes lost in formal in fixation. The fact that these antibodies stain formalin fixed tissues is important with respect to their usefulness as in vitro diagnostics. It may also mean that in some tissues antigenicity may be diminished or lost and the extent of staining could be underestimated.

Second, some artifactual staining occurs in formal in fixed tissues.

Much of the staining observed in the above-noted sections from fixed tissue was focal and associated only with carcinoma, arguing against nonspecific staining.

Importantly, of 17 tumors positive for 1C11, all but three were also positive for 3A4 staining. This emphasizes the potential usefulness
of these reagents used together as both a diagnostic and therapeutic.

**Example 5**

Immunohistochemical staining of paraffin block tissue sections, performed as described for figure 1, revealed staining of some cell other than squamous carcinoma. In the pituitary, adrenocorticotropic hormone (ACTH) secreting cells also stained positive with antibody 1C11. In addition, glandular cells in the genital tract (cervix) and intestinal tract were staining positive with 1C11. Some staining of normal larynx on frozen sections was noted. Overall 1C11 antigens are more limited to larynx in the suprabasal layers, but were not found on squamous epithelium from bronchus or esophagus or squamous epithelium from other organs. However, not all normal larynx samples were positive.

* * * * *

The foregoing invention has been described in some detail by way of examples for purposes of clarity and understanding. It will be obvious to those skilled in the art from a reading of the disclosure that various combinations in form and detail can be made without departing from the scope of the invention. It will also be clear to those skilled in the art that the antibodies of the invention can be used to isolate the antigens for which they are specific.
WHAT IS CLAIMED IS:

1. A hybridoma resulting from the fusion of a myeloma cell and a spleen cell, which hybridoma produces a tumor-specific monoclonal antibody specific for an antigenic determinant of a membrane component characteristic of squamous cell carcinoma.

2. The hybridoma according to claim 1 wherein said myeloma cell is derived from a mouse.

3. The hybridoma according to claim 2 wherein said myeloma cell is SP/2.

4. The hybridoma according to claim 1 wherein said spleen cell is derived from a mammal immunized with squamous cell carcinoma cells or cell fragments.

5. The hybridoma according to claim 4 wherein said mammal is a mouse.

6. The hybridoma according to claim 1 wherein said membrane component has a molecular weight of about 87,000 daltons by Western Blot from one dimensional SDS polyacrylamide gel electrophoresis and said membrane component displays molecular weights of about 30,000, about 87,000 and about 114,000 daltons by immunoprecipitation.

7. The hybridoma according to claim 1 wherein said membrane component has a molecular weight of about 75,000 daltons by immunoprecipitation.
8. A hybridoma designated 1C11, ATCC accession no. HB 9819.


10. A hybridoma designated 3A4/73, ATCC accession no. HB 9821.

11. A monoclonal antibody derived from the hybridoma according to claim 8, or binding fragment thereof.

12. A monoclonal antibody derived from the hybridoma according to claim 9, or binding fragment thereof.

13. A monoclonal antibody derived from the hybridoma according to claim 10, or binding fragment thereof.


15. The monoclonal antibody according to claim 14 wherein said membrane component has a molecular weight of about 87,000 daltons by Western Blot from one dimensional SDS polyacrylamide gel electrophoresis and said membrane component displays molecular weights of about 30,000, about 87,000 and about 114,000 daltons by immunoprecipitation.
16. The monoclonal antibody according to claim 14 wherein said membrane component has a molecular weight of about 75,000 daltons by immunoprecipitation.

17. The monoclonal antibody according to claim 14 wherein said antibody is of the IgM class.

18. The monoclonal antibody according to claim 14 wherein said antibody is of the IgG class.

19. A diagnostic kit comprising:
   i) at least one monoclonal antibody according to claim 14, and
   ii) a conjugate comprising a binding partner of said monoclonal antibody and a label.

20. A diagnostic kit comprising a conjugate comprising:
   i) at least one monoclonal antibody according to claim 14, and
   ii) a label.

21. A conjugate for treating a patient with squamous cell carcinoma comprising:
   i) the monoclonal antibody, or
   ii) an agent capable of rendering a lethal or growth inhibiting event to cells of said squamous cell carcinoma.
22. A method of treating a patient with squamous cell carcinoma comprising administering to said patient a therapeutically effective amount of said conjugate according to claim 21.

23. A method of diagnosing the presence of squamous carcinoma cells in a patient comprising administering to said patient a conjugate comprising:
   i) the monoclonal antibody, or binding fragment thereof, according to claim 14, linked directly or indirectly to
   ii) a moiety capable of being detected by a source external to said patient, and detecting the presence of said detectable moiety.

24. A method of determining the presence of squamous carcinoma cells in a biological sample, comprising:
   i) contacting said sample with at least one tumor-specific monoclonal antibody specific for an antigenic determinant of a membrane component characteristic of said squamous carcinoma cells, under conditions such that binding of said monoclonal antibody to said antigenic determinant occurs, and
   ii) detecting the presence of said monoclonal antibody bound to said antigenic determinant, said binding being related to the presence of squamous carcinoma cells in said sample.
25. The method according to claim 24 wherein said membrane component has a molecular weight of about 87,000 daltons by Western Blot on one dimensional SDS polyacrylamide gel electrophoresis and said membrane component displays molecular weights of about 30,000, about 87,000 and about 114,000 daltons by immunoprecipitation of surface labelled cells.

26. The method according to claim 24 wherein said membrane component has a molecular weight of about 75,000 daltons by immunoprecipitation.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>10B2</th>
<th>130,000</th>
<th>90,000</th>
<th>43,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C11</td>
<td>1C11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 1**

**FIG. 2**

**SUBSTITUTE SHEET**
I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(S): A61K 39/395; C07K 15/28; C12P 21/08; G01N 33/574.
US Class: 530/387; 424/85.8; 530/389; 424/85.91; 435/7; 436/518; 436/523.

II. FIELDS SEARCHED

Minimum Documentation Searched

<table>
<thead>
<tr>
<th>Classification System</th>
<th>Classification Symbols</th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>530/387, 389, 391, 806, 808, 828; 424/85.8, 85.91; 435/7, 240, 27; 436/518, 523, 528, 531, 536, 540, 542, 548, 808, 813.</td>
</tr>
</tbody>
</table>

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Computer database searches on CAS-Online, Biosis, and APS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, 11 with indication, where appropriate, of the relevant passages 12</th>
<th>Relevant to Claim No. 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US A, 4,569,788 (MULSHINE ET AL), issued 11 February 1988, see columns 1 to 5 especially.</td>
<td>1-6, 14, 15, 24, 25</td>
</tr>
<tr>
<td>Y</td>
<td>Cancer Research, Vol. 46, issued July 1986 (USA), Kimmel et al., &quot;Altered Expression in Squamous Carcinoma Cells of an Orientation Restricted Epithelial Antigen Detected by Monoclonal Antibody A9&quot;, see pages 3614 to 3623.</td>
<td>1-20, 23-26</td>
</tr>
<tr>
<td>X</td>
<td>US A, 4,708,862 (BALDWIN), issued 24 November 1987, see columns 1 to 5.</td>
<td>1-26</td>
</tr>
</tbody>
</table>

* Special categories of cited documents: 10
  A document defining the general state of the art which is not considered to be of particular relevance
  E earlier document but published on or after the international filing date
  L document which may throw doubts on priority claim(s) of which is cited to establish the publication date of another citation or other special reason (as specified)
  O document referring to an oral disclosure, use, exhibition or other means
  P document published prior to the international filing date but later than the priority date claimed

IV. CERTIFICATION

Date of the actual completion of the international search: 20 February 1990
Date of mailing of this international search report: 2 MAR 1990

International Searching Authority: ISA/US

Signature of Authorized Officer: Jeff Kushan