Electron Microscope Study of Antigens in Cells of Mouse Mammary Tumor Cell Lines by Peroxidase-labeled Antibodies in Sera of Mammary Tumor-bearing Mice and of Patients with Breast Cancer

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SUMMARY

Sera of C3H/Z/Dm mice bearing spontaneous mammary tumors, sera of apparently normal C3H/Z/Dm mice at different ages, and a serum of an A/Dm mouse with spontaneous breast cancer were tested by the indirect immunoperoxidase method against cells of C3H/HeJ spontaneous mammary tumor line constantly producing type B or mouse mammary tumor virus particles. The sera were also tested against cells of C3H/He/TEX spontaneous mammary tumor line producing no virus particles. Sera of C3H/Z/Dm and A/Dm tumor-bearing mice and serum of an apparently normal C3H/Z/Dm mouse gave peroxidase labeling of immature and mature type B or mouse mammary tumor virus particles and also of budding type B particles. No peroxidase labeling of intracytoplasmic type A virus particles was observed. In addition, peroxidase labeling of certain parts (presumably, antigen formation sites) of plasma membrane of the C3H/HeJ mammary tumor cells was seen. Absorption of the positive mice sera with guinea pig kidney cells, C3H/Z/Dm mouse embryo cells, C3H/Z/Dm spontaneous mammary tumor cells, or with mammary tumor virus from milk of C3H/Z/Dm mice demonstrated that the immunoperoxidase reaction was due to specific antivirus antibodies. Positive sera failed to react with cells of the C3H/He/TEX mammary tumor line producing no virus particles.

A number of sera from patients with breast cancer and from their relatives were also tested by immunoperoxidase reaction against cells of the C3H/HeJ spontaneous mouse mammary tumor line. As in the case of positive mouse sera, some human sera gave peroxidase labeling of type B and budding virus particles. Absorption with suitable material (guinea pig kidney, sheep red blood cells, whole human embryo cells, breast tumor tissue homogenates, and a mouse mammary tumor virus preparation) demonstrated that the antibodies in human sera are specifically directed against mouse mammary tumor virus particles.

INTRODUCTION

It has long been assumed that mice are tolerant to MTV because of repeated failures to demonstrate transplantation immunity against mammary tumors induced in neonatally infected mice (8, 9, 21, 22, 34, 35). Antibodies that precipitate MTV have been reported in sera of mice neonatally infected with the virus, following immunization with extracts or implantation of MTV-containing tissues (5) and hyperimmunization with MTV preparations (3). Muller et al. (23), utilizing a micro-Ouchterlony technique, reported the spontaneous occurrence of precipitating antibodies against a soluble outer-membrane antigen of MTV particles or type B virions. Results of MHA, immunodiffusion, and FIF tests carried out in our laboratory have demonstrated antibodies to mouse mammary tumor cells present in sera of mice of several strains with or without spontaneous mammary tumors (10). Absorption tests indicated that these antibodies may be against tumor and/or viral antigens in these cells (10). The question remained as to whether these antibodies are directed against MTV particles present in these tumors. Previously reported immunoferritin studies (32) with sera of mice with spontaneous tumors have failed to localize MTV antigens in cells of spontaneous mouse mammary tumors. This is in contrast to the results of immunoferritin tests with heterologous anti-MTV rabbit sera, which gave ferritin labeling of MTV particles in mouse mammary tumor cells (30, 32).

Recently, Priori et al. (28) and Dmochowski et al. (10) reported the presence of antibodies in sera of patients with breast cancer, of some of their relatives, and of some normal donors which gave positive FIF and MHA reaction with cells of mouse mammary tumors producing both MTV or type B and murine leukemia virus or type C particles. Again, as in the case of antibodies in sera of mice with spontaneous mammary cancer, the question arose as to whether the antibodies in sera of patients are directed against tumor cells or mouse MTV particles. In preliminary experiments, the application of the immunoferritin tech-

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MATERIALS AND METHODS

Tissue Culture. Tissue cultures were grown in Eagle's medium supplemented with 20% fetal calf serum (15). One of the mammary tumor tissue culture cell lines was derived from a spontaneous papillary adenocarcinoma arising in a female C3H/HeJ mouse (10). Since initiation of this epithelial cell line more than 1 year ago, it has been a constant producer of numerous type B virus particles, intracytoplasmic type A virus particles, and a few type C virus particles. In addition to this cell line, a mammary tumor tissue cell line derived from a spontaneous mammary tumor of a C3H/He/TEX female mouse producing no virus particles was also used.

Mouse Sera. All mouse sera were titrated by MHA and FIF tests, as described previously (15). Sera of the following mice were used: (a) Four C3H/Z/Dm female mice without spontaneous mammary tumors (3, 6, 9, and 12 months old). Sera of all 4 mice were negative by MHA and FIF tests against cells of the C3H/HeJ strain mouse mammary tumor culture producing type B virus particles. (b) C3H/Z/Dm mice bearing spontaneous mammary tumors. Serum from a female C3H/Z/Dm mouse bearing a mammary tumor was absorbed with passage A Gross leukemia virus preparation and used in the initial immunoperoxidase experiments. This serum was positive against C3H/HeJ mammary tumor cells by MHA (titer of 1:256) and by FIF (titer of 1:16) tests and was negative by immunodiffusion test. The other serum tested was composed of sera pooled from 5 female C3H/Z/Dm mice, all with spontaneous mammary tumors. This pooled serum was positive with C3H/HeJ mammary tumor cells by MHA (titer of 1:256) and by FIF (titer of 1:16). The same pooled serum was negative by MHA with the C3H/HeJ strain mouse tissue culture cells of mouse mammary tumor cell line which does not produce type B virus particles. Each of the 5 C3H/Z/Dm sera were positive by immunodiffusion tests against type B virus preparation from milk of C3H/Z/Dm mice. (c) An A/Dm female mouse with a spontaneous mammary tumor. Serum of this mouse was positive by MHA (titer of 1:512) against C3H/HeJ mammary tumor cells which produce type B virus particles and was negative against cells of the C3H/He/TEX mammary tumor line which do not produce type B or any virus particles. This serum was negative by FIF against cells of any of the cultures examined and was negative in an immunodiffusion test against virus preparation from C3H/Z/Dm strain milk.

All sera examined were diluted (1:16), before immunoperoxidase tests. The positive sera were further titrated at dilutions varying from 1:32 to 1:128 to ascertain the degree of reactivity with the different antigens.

Absorption of Mouse Sera. Absorption of the mouse sera (dilution, 1:16) was carried out at room temperature with GPK and C3H/Z/Dm mouse embryo tissue homogenates in equal proportions (w/v). Sera (dilution, 1:8) were absorbed with a high-speed pellet of milk from C3H/Z/Dm mice (proportion, 1 part serum to 9 parts of high-speed pellet preparation of milk). Sera (dilution, 1:8) were also absorbed with C3H/Z/Dm mammary tumor tissue homogenates (10) in equal proportions (w/v).

Sera of Patients with Breast Cancer. Sera from 5 breast cancer patients and sera from 2 of their relatives were used. All of the patients were bled a number of years postsurgery, ranging from 1 to 20 years, as shown in Table 1.

Absorption of Human Sera. Two sera, Nos. 40 and 43 (dilution, 1:8), were absorbed at room temperature with the following materials: GPK and breast tumor tissue homogenates (equal proportions w/v); whole human embryo tissue culture cells (0.3 ml of packed cells), used to absorb 0.1 ml of serum; a high-speed pellet from R111/Dm strain mouse milk (proportion, 1 part serum to 9 parts of milk); and washed SRBC (in equal portions v/v).

Preparation of the Peroxidase-conjugated Antibody. HPO was conjugated with globulin from goat antiserum against mouse 7S γ-globulin (γG) and against human IgG by the method of Avrameas (2), with glutaraldehyde as the coupling reagent. Isolation of the γG fraction from goat antiserum against mouse 7S γ-globulin and against human IgG (both obtained from Hyland Division, Costa Mesa, Calif.) was accomplished by repeated precipitation with ammonium sulfate followed by passage through a DEAE-cellulose column. HPO [Type VI; ca 320 units of purpuragillin per mg solid; Reinhardt-Zahl (R-Z): 3:2] was purchased from Sigma Chemical Co., St. Louis, Mo. Following conjugation, the remaining unconjugated HPO was removed by passing through a Sephadex G-200 column. Fractions of the conjugate were then pooled and condensed.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at surgery</th>
<th>Age at bleeding</th>
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<tbody>
<tr>
<td></td>
<td>(yr)</td>
<td>(yr)</td>
</tr>
<tr>
<td>13</td>
<td>43</td>
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<tr>
<td>25</td>
<td>47</td>
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<tr>
<td>40</td>
<td>68</td>
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</tr>
<tr>
<td>42</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>57 (1st bleeding)</td>
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</tr>
<tr>
<td>46</td>
<td>79</td>
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</tr>
<tr>
<td>56</td>
<td>60 (2nd bleeding)</td>
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to the original volume of goat antiserum and utilized as a stock solution containing approximately 4 mg of protein per ml and 2.2 × 10⁻³ mM of HPO. Before use, the stock solution was diluted 1:10.

**Immunoperoxidase Procedures.** The indirect immunoperoxidase technique was used. The cells were fixed in various ways to evaluate the effect of fixation on immunological reactivity and cellular ultrastructure. The best results were obtained when cells were prefixed for 30 min in cold neutralized 5% formalin (in PBS at pH 7.5) and post-fixed for 1 hr in a mixture of 0.5% glutaraldehyde and 3% formalin (in 0.05 M sodium phosphate buffer at pH 7.4). The cells were postfixed with conjugated antibody following incubation but prior to the peroxidase reaction. Glutaraldehyde almost totally eliminated the antigenic activity in this cell system when used prior to incubation with the antibody. The cellular ultrastructure was markedly damaged during the peroxidase reaction when cells were fixed in formalin alone.

Following prefixation, the cells were incubated (by mild shaking) together with the mouse or human sera (at dilutions mentioned above) for 3 hr at room temperature. After being washed 1 hr in PBS, the cells were incubated in HPO-labeled goat antimouse 7S γG for an additional 3 hr at room temperature. After being washed for 2 to 3 hr and postfixed in the glutaraldehyde:formalin mixture, the cells were incubated in incomplete Graham and Karnovsky’s medium (minus H2O2) (14) for 30 min. The cells were then transferred to the complete medium (diluted to one-half the concentration of the original) for an additional 30 min of incubation. The cells were again postfixed in 1% osmium tetroxide and subjected to standard embedding procedures (11).

As controls for these experiments, the following were included: incubation of the cells with HPO-labeled goat antimouse 7S γG, eliminating prior exposure to mouse sera in order to check for any nonspecific tagging by HPO-labeled antibody; treatment of cells with Graham and Karnovsky’s medium (minus H2O2) (14) for 30 min. The cells were then transferred to the complete medium (diluted to one-half the concentration of the original) for an additional 30 min of incubation. The cells were again postfixed in 1% osmium tetroxide and subjected to standard embedding procedures (11).

**Electron Microscopy.** Thin sections of Epon-embedded blocks containing pellets of cells subjected to the immunoperoxidase reactions were examined both before and after heavy metal staining. Untreated control cells were also processed and examined in the electron microscope.

**Light Microscopic Examination of the Immunoperoxidase Reaction on Cell Monolayers.** For comparison of the results obtained by immunofluorescence tests with the results of immunoperoxidase tests, the following mouse sera—1 composed of sera pooled from 5 C3H/Z/Dm female mice with spontaneous mammary tumors, 2 from normal C3H/Z/Dm female mice (6 and 9 months old), and 1 from an A/Dm female mouse with spontaneous mammary cancer—were tested by light microscopic immunoperoxidase method (18) against cells of 2 mammary tumor lines, C3H/HeJ and C3H/He/TEX. Cell monolayers were fixed for 10 min either with 5% formalin in PBS or with cold acetone and then washed and air dried. The monolayers were covered with a drop of mouse serum (in dilutions varying from 1:8 to 1:128) and then were incubated for 1 hr in a moist chamber. The monolayers were then washed with PBS and covered with HPO-labeled goat antimouse 7S γG and again incubated for 1 hr in a moist chamber. The cells were then washed prior to the addition of Graham and Karnovsky’s medium to the cells at room temperature for the 10 min required to obtain a peroxidase reaction. The slides were covered with glycerine and protected by a glass coverslip for light microscopy examination.

**RESULTS**

**General Morphology of Cell Cultures**

**C3H/HeJ Mouse Mammary Tumor Cell Line.** An electron microscopic examination of this cell line, processed by routine fixation and embedding, revealed that the cells contained numerous virus particles, such as intracytoplasmic type A and type B particles (9, 19, 33) displaying all stages of maturation and occasional type C virus particles. Only those details of ultrastructure of cells necessary for an understanding of the representative electron micrographs of immunoperoxidase labeled cells will be described.

Tissue culture monolayers viewed in bottles by light microscopy exhibited cells polygonal in shape. In the electron microscope, acinus-like structures consisting of a lumen surrounded by an epithelial wall were found. In the cytoplasm of cells, large and small vacuoles were frequently encountered. Some resembled secretion droplets and were composed of a homogenous material of intermediate electron density. Some vacuoles appeared to be continuous with extracellular spaces. Intracytoplasmic type A virus particles were found in clusters lining vacuoles in the cytoplasm or acinus-like lumen.

**C3H/He/TEX Mouse Mammary Tumor Cell Line.** In the light microscope, this cell line consisted of monolayers of networks of fibroblast-like cells. In the electron microscope, the cells had poorly developed cytoplasmic structures and were producing no virus particles.

**Light Microscopic Examination of Cell Monolayers Exposed to the Immunoperoxidase Reaction**

When monolayers of the C3H/HeJ mammary tumor cell line were treated with pooled serum from 5 C3H/Z/Dm female mice bearing spontaneous mammary tumors or from an A/Dm mouse with a spontaneous mammary cancer, diffuse or spotted cytoplasmic and perinuclear immunoperoxidase reaction was observed up to a serum dilution of 1:128. When the same cell monolayers were treated with serum from a normal C3H/Z/Dm female mouse (9
months old), a similar cytoplasmic staining was observed up to a dilution of 1:32 of the serum. No reaction was observed in the same cells treated with serum from a normal C3H/Z/Dm female mouse (6 months old). When monolayers of C3H/He/TEX mammary tumor cell line (which does not produce virus particles) were treated with the sera described above, no immunoperoxidase reaction was observed with any of the sera.

**Immunological Reactions of Mouse Sera Tested against Cells of C3H/HeJ Spontaneous Mammary Tumor Line**

A weak immunoperoxidase reaction was observed on virus particles associated with cells treated with serum from a normal 9-month-old C3H/Z/Dm female mouse, and no reaction was seen on the plasma membranes. There was no reaction on either the virus particles or plasma membranes with sera from 3-, 6-, and 12-month-old normal C3H/Z/Dm female mice. The negative result with sera of the 12-month-old mice may be due to the synthesis of virus in such large quantities that the antibody is absorbed in the mice.

Serum (diluted 1:16) from a mammary tumor-bearing C3H/Z/Dm mouse (absorbed with passage A leukemia virus) and pooled serum from 5 C3H/Z/Dm mice with tumors gave peroxidase labeling of extracellular type B virus particles (both mature and immature) and of budding type B virus particles (Figs. 1 and 2). No labeling was found on intracytoplasmic type A virus particles (Figs. 1 and 2).

Table 2

<table>
<thead>
<tr>
<th>Serum</th>
<th>Immunoperoxidase titer</th>
<th>Intracytoplasmic</th>
<th>Plasma membrane</th>
<th>MHA</th>
<th>ID&quot;</th>
<th>FIF</th>
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<tr>
<td>A/Dm spontaneous mammary tumor (303 days old)</td>
<td>1:16</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>1:32</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1:128</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C3H/Z/Dm with spontaneous mammary tumor (pooled from 5 mice; av. age, 225 days)</td>
<td>1:16</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>1:32</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>1:128</td>
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<tr>
<td>C3H/Z/Dm with spontaneous mammary tumor (absorbed with passage A leukemia virus)</td>
<td>1:16</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td></td>
<td>1:32</td>
<td>–</td>
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<tr>
<td></td>
<td>1:128</td>
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<tr>
<td>C3H/Z/Dm normal (No. 1) (9 mo. old)</td>
<td>1:16</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<td>1:32</td>
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<td></td>
<td>1:128</td>
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<tr>
<td>C3H/Z/Dm normal (No. 2) (6 mo. old)</td>
<td>1:16</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>1:32</td>
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<tr>
<td></td>
<td>1:128</td>
<td>–</td>
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<tr>
<td>C3H/Z/Dm normal (No. 3) (3 mo. old)</td>
<td>1:16</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>1:32</td>
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<td></td>
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<td>–</td>
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</table>

Peroxidase labeling was found intermittently and in some instances also continuously on cell membranes and on the walls of cytoplasmic vacuoles in areas where no virus budding was observed (Figs. 1 and 2). The reaction products deposited on virus particles were seen as electron-dense layers surrounding particle envelopes. These layers appeared to be located in an area corresponding to the surface spikes observed on type B virus particles processed by normal fixation and embedding techniques. Peroxidase labeling was particularly noticeable in areas of plasma membrane adjacent to intracellular type A virus particles (Figs. 1 and 2).

Serum (1:16 dilution) from an A/Dm mouse with spontaneous mammary tumor gave a similar reaction to that observed with serum of C3H/Z/Dm mice. The reaction occurred on type B virus particles and on certain parts of plasma membranes. The A/Dm mouse serum gave a much stronger reaction with the virus particles than that observed with serum from C3H/Z/Dm strain mice with mammary cancer.

Results of the immunoperoxidase technique used in testing mouse sera against the C3H/HeJ tumor cell line are summarized in Table 2. Antibody titer against type B virus particles exceeded 1:128 in serum from an A/Dm mouse bearing a spontaneous mammary tumor (Fig. 3). Serum from a 9-month-old normal C3H/Dm mouse and the pooled serum from mammary-tumor bearing C3H/Z/Dm mice were positive with type B to a titer of 1:32. In general, the reaction between the labeled sera and plasma cell mem-

*HPO labeling* +++, peroxidase-labeled virus particles including budding of virus particles found in almost all cells in thin sections or showing a heavy deposition of 3,3'-diaminobenzidine; +, peroxidase-labeled virus particles found less frequently and showing weak deposition of 3,3'-diaminobenzidine or peroxidase labeling of plasma membrane at or near sites of virus particles budding.

*ID*, immunodiffusion.
branes, when present, diminished or disappeared with increasing dilution of the serum.

Absorption Tests of Mouse Sera

Table 3 shows that type B particles reacted with C3H/Z/Dm (9 month old) serum after absorption with GPK and C3H/Z/Dm embryo cells (Fig. 4). Both mature, immature, and budding (Fig. 5) type B particles reacted strongly with pooled serum from 5 C3H/Z/Dm mice bearing spontaneous mammary tumor following absorption with either GPK or C3H/Z/Dm embryo cells. There was no reaction following absorption of the pooled serum with C3H/Z/Dm mammary tumor cells or with C3H/Z/Dm mouse milk (Fig. 6). A weak reaction was observed at the plasma membrane following absorption with GPK and C3H/Z/Dm embryo cells, but no membrane reaction was seen following absorption with C3H/Z/Dm mammary tumor cells and C3H/Z/Dm strain mouse milk.

The results of absorption experiments (Table 3) with serum from an A/Dm mouse with spontaneous mammary tumor were comparable with those obtained with pooled serum from C3H/Z/Dm mice, with the exception that the reaction appeared somewhat stronger.

Immunoperoxidase Studies on Reactivity of Sera from Breast Cancer Patients with Mouse Mammary Tumor

In the preceding results, we reported that the immunoperoxidase technique is capable of demonstrating virion antigens in mouse mammary tumor tissue culture cells by the use of homologous sera of mice. These results led to an attempt in which the immunoperoxidase technique was applied to mouse mammary tumor tissue culture cells treated with sera from selected patients with breast cancer and from relatives of the patients. Sera that reportedly gave positive cytoplasmic and perinuclear fluorescence by the FIF test (27) were used in the experiments.

The same mouse (C3H/HeJ) spontaneous mammary tumor cell line was used in the experiment with human sera as was used in those with mouse sera.

The results of a preliminary study of human sera by immunoperoxidase technique are shown in Table 4. Three of the 8 sera tested (Nos. 40; 42, 1st bleeding; and 43) gave positive immunoperoxidase reactions with type B virus particles (mature, immature, and budding) and with certain areas of plasma membranes of cells of the mouse mammary tumor tissue culture (Figs. 7 and 9). Some of the type B virus particles displayed an uneven deposition of the reaction products. The immunoperoxidase reaction product deposition on the plasma membrane of cells was generally thinner in width and weaker in electron density than that seen on virus particles or on the budding particles. In general, peroxidase labeling of cell membranes could be detected only in unstained sections. The immunoperoxidase reaction pattern on both the virus particles and on the plasma membrane was similar to that observed in the same cell culture treated with several mouse sera.

Absorption Tests with Human Sera

Absorption of 2 positive sera (Nos. 40 and 43) with either GPK, SRBC, or whole human embryo cells failed to remove peroxidase labeling of type B virus particles (Fig. 8). Absorption of 2 positive human sera with high-speed pellet preparation of RII/Dm milk containing type B particles or human breast cancer cell homogenates removed the labeling of type B virus particles.

Control Experiments

No peroxidase labeling was found on virus particles or on cell organelles in cultures incubated with HPO-labeled goat antimouse 7S γG or in the cultures treated with Graham and Karnovsky's medium without prior exposure to either mouse or human sera and conjugated antisera. Reaction products were found in the intercristate space of mitochondria of some cells only when the cells had not been postfixed in glutaraldehyde during processing for immunoperoxidase. Such reaction products in the mitochondria are believed to be the result of cytochrome oxidase activity. Exposure of cells to HPO-labeled normal goat γG following incubation with serum of strongest reactivity obtained from an A/Dm female mouse bearing spontaneous mammary cancer did not result in peroxidase labeling of any structures including type B virus particles.

DISCUSSION

This study was undertaken in an attempt to determine whether sera of apparently normal mice and of mice bearing spontaneous mammary tumors contain specific antibodies to type B virus particles. It was necessitated by previous observation of antibodies to mouse mammary tumor cells in sera of mice with or without spontaneous mammary tumors as demonstrated by MHA, FIF, and immunodiffusion tests (10). The results of these studies (10) left the specificity of antibodies in sera of the mice examined uncertain, although absorption tests indicated that these antibodies may at least in part be directed against type B virus particles or their antigens (10).

The results of recent studies on sera from patients with breast cancer have demonstrated the presence of FIF antibodies in sera of some patients with breast cancer, their relatives, and of some normal donors against antigens in cells of mouse mammary tumor cell cultures (10, 27). Absorption tests failed to provide convincing evidence as to the nature of these antibodies, whether directed against tumor or virus antigens shown to be present in the sera of patients with breast cancer (10, 27). Sera from breast cancer patients reportedly neutralize the mouse mammary tumor virus (type B virus particles), while control sera did not neutralize the virus (6). These observations appeared of interest and significance in view of the observation of virus particles, morphologically similar to those known to cause mouse mammary tumors, in human breast cancer (11, 12) and human milk (13, 20).
Table 3
Reactivity of mouse sera against C3H/HeJ spontaneous mammary tumor cells following absorption as determined by immunoperoxidase technique

<table>
<thead>
<tr>
<th>Serum obtained from</th>
<th>Absorbed with</th>
<th>Dilution</th>
<th>Type A virus</th>
<th>Type B virus</th>
<th>Plasma membrane</th>
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<tr>
<td>C3H/Z/Dm normal (9 mo. old)</td>
<td>GPK</td>
<td>1:16</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>C3H/Z/Dm embryo</td>
<td>1:16</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>C3H/Z/Dm MMT</td>
<td>1:8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C3H/Z/Dm milk</td>
<td>1:8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3H/Z/Dm with MMT (av. age, 225 days)</td>
<td>GPK</td>
<td>1:16</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>C3H/Z/Dm embryo</td>
<td>1:16</td>
<td>-</td>
<td>+</td>
<td>±</td>
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<tr>
<td></td>
<td>C3H/Z/Dm MMT</td>
<td>1:8</td>
<td>-</td>
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<td>A/Dm with MMT</td>
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<tr>
<td></td>
<td>C3H/Z/Dm embryo</td>
<td>1:16</td>
<td>-</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>C3H/Z/Dm MMT</td>
<td>1:8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C3H/Z/Dm milk</td>
<td>1:8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a ++, peroxidase-labeled virus particles found in almost all the cells in thin sections or showing a heavy deposition of 3,3'-diaminobenzidine; +, peroxidase-labeled virus particles found less frequently and showing weak deposition of 3,3'-diaminobenzidine; ±, peroxidase labeling of some parts of plasma membrane (see text).

Table 4
Results of tests of human sera against C3H/HeJ spontaneous mammary tumor cells by immunoperoxidase technique

<table>
<thead>
<tr>
<th>No. of sera examined</th>
<th>Serum no.</th>
<th>Patient no.</th>
<th>Diagnosis</th>
<th>Age (yr)</th>
<th>Serum dilution</th>
<th>HPO labeling*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>NA</td>
<td>No BC</td>
<td>NA</td>
<td>1:8</td>
<td>FIF plasmic</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>NA</td>
<td>BC</td>
<td>NA</td>
<td>1:8</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>NA</td>
<td>No BC</td>
<td>NA</td>
<td>1:8</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>47644</td>
<td>BC</td>
<td>NA</td>
<td>1:8</td>
<td>+</td>
</tr>
<tr>
<td>5-1</td>
<td>42</td>
<td>76798</td>
<td>BC</td>
<td>50</td>
<td>1:8</td>
<td>++</td>
</tr>
<tr>
<td>5-2</td>
<td>42</td>
<td>76798</td>
<td>BC</td>
<td>50</td>
<td>1:8</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>25546</td>
<td>BC</td>
<td>NA</td>
<td>1:8</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>75969</td>
<td>BC</td>
<td>NA</td>
<td>1:8</td>
<td>+</td>
</tr>
</tbody>
</table>

*Tests reported previously (27).
*+++, peroxidase-labeled virus particles found in almost all cells in thin sections or showing a heavy deposition of 3,3'-diaminobenzidine; +, peroxidase-labeled virus particles found less frequently and showing weak deposition of 3,3'-diaminobenzidine or peroxidase labeling of plasma membrane at or near sites of virus particle budding.

Thus, there appeared to be evidence available for the presence of antibodies (MHA, FIF, or immunodiffusion) in the sera of mice and humans directed either against their own mammary tumors (10, 27, 28) or against mammary tumors of heterologous species (10, 27). Recently, in sera of patients with breast cancer, the presence of an antigen related to mouse mammary tumor virus antigen has been reported (24). The presence of antibodies in sera of women with breast cancer reacting specifically with an intracellular antigen of MTV has also been reported (25). The application of the immunoferritin technique has failed to demonstrate the nature of the antibodies present in the sera of mice (32). It became obvious that other techniques or methods may have to be used in order to present convincing evidence of the nature of the antibodies present in the sera of mice and humans with breast cancer.

The immunoperoxidase method (26) has been successfully applied to the study of a number of cell-virus systems (16–18, 29, 31). In view of these findings, an attempt was made to apply this method to the study of the nature of antibodies in the sera of mice with spontaneous breast cancer and to those in sera of patients with breast cancer.

Application of the immunoperoxidase technique has resulted in the demonstration of antibodies in the sera of mice with spontaneous mammary cancer and in the sera of some apparently normal mice which label with peroxidase both type B and budding type B virus particles. These antibodies are directed against type B virus particles present in cells of mammary tumors of mice of their own and other strains. The specificity of the antibodies was demonstrated by absorption with suitable material such as GPK, mouse embryo cells, mouse mammary tumor cells, and mouse MTV.
preparations. The positive immunoperoxidase reaction of these sera found in certain places on the cell membrane and on or near budding type B virus particle, again based on the results of absorption studies, appears to be due to virus antigen formation sites. The absence of immunoperoxidase reaction on intracytoplasmic type A virus particles does not appear to be due to poor penetration of antibody into the cytoplasm, since type B virus particles close to type A virus particles in areas where cell membranes were disrupted were tagged with the immunoperoxidase reaction products. Furthermore, type B virus particles present in cytoplasmic vacuoles were found to be labeled with peroxidase. Tanaka and Moore (32) concluded from an immunoferritin study using rabbit antisera against mouse (MTV) that intracytoplasmic type A virus particles are not related antigenically to type B virus particles. However, Shigematsu et al. (30) later reported that a specific antigenic relationship exists between type A and B particles when mouse mammy tumor tissue culture cells were tested by the indirect immunoferritin method using rabbit anti-MTV sera. They also noted that anti-MTV antisera, when tested in serial dilutions after absorption in vivo, had a comparatively higher titer for type B virus particles and a lower titer for intracytoplasmic type A virus particles. This observation was interpreted as possibly due either to the lower antigenicity of type A virus particles or to the presence of additional antigens in type B virus particles. Lack of antibody against intracytoplasmic type A virus particles in mouse sera as found by the immunoperoxidase method may be due to the weak antigenicity of type A particles. This assumption may be supported by the observation of the presence of a comparatively lower titer of antibody against type B virus particles in sera of mice compared with that present in sera of heterologous animals as shown by the MHA and FIF tests (10).

No deposition of peroxidase reaction products was found inside the type B virus particles on examination of unstained thin sections. Abelson et al. (1) reported that, in cells infected with lymphocytic choriomeningitis virus, antibodies could not penetrate into the virus particles unless the cells were previously treated with formaldehyde:alcohol. The absence of peroxidase labeling of the nucleoids inside the virions in the present experiments may be due to lack of antibodies against the nucleoid antigens in the sera of mice rather than to poor penetration of the antibody. This hypothesis is supported by the observation that in thin sections in which cell membranes with budding virus particles were fragmented, the peroxidase reaction products were found only on outer surfaces of the budding sites and not around the nucleoid or the cytoplasmic side of the plasma membranes of cells. These observations seem to correlate with the findings reported by Muller et al. (23) that the antibodies present in mammary tumor-bearing and tumor-free mice are directed against the outer membrane of type B virus particles. It is conceivable that the internal antigens of type B virions may not be antigenic in the mouse immune system in the natural condition. This speculation appears to be compatible with the hypothesis that the intracytoplasmic type A virus particles are the precursor of type B virus particles (4, 7, 9).

By means of the immunoperoxidase technique the presence of antibodies to the MTV particles was demonstrated in both isologous and homologous tumor tissues. The antibodies to MTV are present not only in sera of mice with spontaneous mammary tumors but also in sera of some apparently normal mice. Only further studies may demonstrate the importance of age on the appearance of anti-MTV antibodies in sera of mice of various inbred strains. There is little doubt that the immunoperoxidase technique is a sensitive method for demonstration of apparently weak antibodies in sera of hosts with spontaneous neoplasia.

In addition to the peroxidase labeling of budding type B virus particles, a positive reaction was also observed on certain parts of plasma membrane of mammary tumor cells treated with either mouse or human sera (Tables 2 to 4). Following absorption with GPK or mouse embryo cells, the peroxidase reaction on plasma membrane was greatly decreased, which may indicate the presence of Forssman-like and tissue antigens on the cell surface. However, the complete removal of all detectable activity by absorption of the mouse sera with MTV preparations, which by electron microscopy did not reveal cell membranes or mammary tumor cells, indicates the presence of virus and/or tumor antigens on the surface of the examined cells. The possibility of the presence of cell membrane material in purified virus preparations cannot be excluded. Thus, both virus and tumor antigens present on the cell membranes may be responsible for the results.

The presence of anti-MTV antibodies in the sera of some breast cancer patients as shown by peroxidase labeling of type B virus particles with these human sera is of interest. The results of suitable absorption tests of the positive human sera with GPK, SRBC, whole human embryo cells, breast tumor tissue, or MTV preparations indicate specificity of the observed immunoperoxidase reaction of human sera with mouse MTV particles. In the small number of human sera examined, no correlation could be seen between the FIF and the immunoperoxidase reaction. Some human sera positive by FIF reaction were negative by immunoperoxidase test, while in some mouse sera the opposite was observed. The small number of mouse and human sera tested so far does not permit any interpretation of the findings yet made. It is of interest to note that one of the human sera (Table 4, No. 42) was originally positive by FIF and immunoperoxidase tests at 1st bleeding. It was found negative by both techniques after 2nd bleeding of the patient (3 years later). The significance of this finding in relation to the state of health of this patient cannot be assessed at the present time, but it may indicate that antibodies to MTV in sera of some patients appear to fluctuate. Further studies of serum of this patient and of others may help to elucidate the possible relationship of the MTV antibodies in human sera to the progression of neoplasia.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to W. Clydell Williams for excellent technical assistance.
REFERENCES


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Fig. 1. Appearance of C3H/HeJ spontaneous mammary tumor tissue culture cell treated with serum from C3H/Z/Dm mouse with spontaneous mammary tumor (absorbed with passage A leukemia virus). Surface of budding type B virus particles and immature type B virus particles (single arrows) are labeled by immunoperoxidase. No reaction can be seen on intracytoplasmic type A virus particles (double arrows). × 45,000.

Fig. 2. Part of Fig. 1 at higher magnification. Immunoperoxidase labeling of immature type B virus particle (single arrow) and of plasma membrane around intracytoplasmic type A particles (double arrows) may be seen. × 80,000.

Fig. 3. Part of cytoplasm of C3H/HeJ spontaneous mammary tumor cell treated with serum from A/Dm mouse with spontaneous mammary tumor (diluted 1:128). Immunoperoxidase reaction can be seen on the surface of mature type B virus particles (single arrow) and budding type B virus particles (double arrows). No reaction with peroxidase is present on intracytoplasmic type A particles (triple arrows). × 60,000.

Fig. 4. Part of cytoplasm of C3H/HeJ strain spontaneous mammary tumor cell treated with serum from normal C3H/Z/Dm mouse (9 months old) after absorption with either GPk or C3H/Z/Dm embryo cells. Type B virus particles are labeled by immunoperoxidase. × 60,000.

Fig. 5. Peroxidase-labeled budding type B virus particles (arrow) in C3H/HeJ strain spontaneous mammary tumor cells treated with pooled serum from 5 C3H/Z/Dm mice with spontaneous mammary tumor (absorbed with C3H/Z/Dm mouse embryo). × 60,000.

Fig. 6. Part of cytoplasm of C3H/HeJ strain mammary tumor cells treated with pooled serum from 5 C3H/Z/Dm mice (absorbed with C3H/Z/Dm mammary tumor cells). No immunoperoxidase labeling of type B virus particles is seen. × 60,000.

Fig. 7. Part of cytoplasm of cell of C3H/HeJ strain mammary tumor line treated with serum from a breast cancer patient. Arrow, budding type B virus particle labeled by immunoperoxidase reaction. × 80,000.

Fig. 8. Immature type B virus particles in cells of C3H/HeJ strain mammary tumor line treated with serum from a breast cancer patient and absorbed with guinea pig kidney cells. Immunoperoxidase labeling of the particles (arrows) can be seen. × 80,000.

Fig. 9. Part of intercellular space of C3H/HeJ strain spontaneous mammary tumor cell treated with serum from a breast cancer patient. Immunoperoxidase reaction can be seen on mature type B virus particles (arrows). × 80,000.
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Electron Microscope Study of Antigens in Cells of Mouse Mammary Tumor Cell Lines by Peroxidase-labeled Antibodies in Sera of Mammary Tumor-bearing Mice and of Patients with Breast Cancer

Munemitsu Hoshino and Leon Dmochowski


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