OCCURRENCE OF ANTIBODY AGAINST INTRACYTOPLASMIC A-PARTICLES OF MOUSE MAMMARY TUMOR VIRUS IN SERA FROM BREAST CANCER PATIENTS*1

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Acetone-fixed smears of DBA/2 mouse leukemia cells that produce clusters of intracytoplasmic A-particles (pronucleocapsids of mouse mammary tumor virus) were employed as an indirect immunofluorescence system to detect the antibody to A-particles in human sera. With positive test sera, specific fluorescence was easily detectable as discrete cytoplasmic granules at the site of A-particle clusters. The antibody was found in 26 (60%) out of 43 breast cancer patient sera and 4 (25%) of 16 mammary fibroadenoma patient sera, while only 4 (11%) out of 37 control woman sera were antibody-positive. In the case of breast cancer patients, occurrence of the antibody was not specifically related to a particular type of tumor histology. In a considerable number of positive cases, the antibody tended to disappear within various lengths of time after surgical operation of the breast cancer.

An increasing number of reports are being published suggesting that mouse mammary tumor virus (MTV) or its related virus is somehow involved in human mammary carcinogenesis. Some authors have observed MTV gene products at various levels in human breast cancer tissues, blood serum, or milk (for references, see reviews of Dmochowski5 and Moore11)). Other reports have been concerned with immunological host responses such as antibody formation against MTV3,4,6,5) and cellular immunity to MTV-related antigens.2) Among the latter, those of Müller and his co-workers12`15) indicate that sera from breast cancer patients contain an antibody to intracytoplasmic A-particles. Intracytoplasmic A-particles (hereafter referred to as A-particles) have long been assumed to be precursors of B-particles (mature virions) of MTV.1) This assumption was first substantiated by our demonstration24) of common antigens in purified A- and B-particles, which was later confirmed by other authors18,19,28). In further work, we were recently able to disclose that three major internal components of B-particles are generated through enzymic cleavage from a common precursor polypeptide of A-particles. Thus, the antibody to A-particles is directed toward the internal components of MTV.

The present paper describes the occurrence of antibody to A-particles in the sera of Japanese women in association with proliferating disorders of mammary glands. This antibody was detected by a new system of indirect immunofluorescence using acetone-fixed smears of A-particle-forming mouse leukemia cells. This work has been reported in a preliminary form elsewhere.16,17,22,23)

MATERIALS AND METHODS

Human Sera Sera from 44 breast cancer patients admitted to the Department of Surgery of this University were collected within 1 week after surgical operation of the breast cancer. This work was supported by Grants-in-Aid for Cancer Research (Nos. 001529 and 101548) from the Ministry of Education, Science and Culture.
before or after surgery. In 20 cases, sera were further examined at various intervals after surgical operation. Sera of mammary fibroadenoma patients were obtained from 16 out-patients on or immediately before the day of biopsy. Control sera were those from 37 women who were either healthy or suffering from diseases (mostly trauma) other than those of mammary gland. The three positive reference sera (two from breast cancer patients and one from a mastopathy patient) used were a gift from Dr. M. Müller, Pathologisches Institut, Medizinische Akademie "Carl Gustav Carus," Dresden, Germany.

Preparation of Antisera and Labeled Antibodies Rabbit antisera against A-particles were the same as those used in our previous studies.\(^{22,23}\) A rabbit antiserum to human IgG was prepared by a method described earlier.\(^{24}\) \(\gamma\)-Globulin fractions prepared from these sera were conjugated with fluorescein isothiocyanate (FITC) and then purified by the method of Kawamura.\(^{25}\) It was confirmed by the direct method that this conjugate specifically stained antibody-forming cells of human lymph nodes but showed no affinity to A-particle clusters in mouse leukemia cells. FITC-labeled goat anti-rabbit IgG antibody was purchased from Boehringerwerke AG, Marburg Lahn, Germany.

Absorption of Sera and Labeled Antibodies All materials were routinely absorbed with the same volume of centrifuged pellet of BALB/c mouse tissue homogenate plus one-half volume of 7% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature for 1 hr and kept at 4°C overnight. These were then clarified by centrifugation at 100,000g for 60 min. In some cases, sera were absorbed \(in vivo\) in BALB/c mice.\(^{18}\) All materials were further absorbed with the same volume of packed sheep red blood cells three times each for 60 min at room temperature, followed by similar absorption with packed C57BL mouse red blood cells. Finally, sera were diluted to 10 times the original volume and used at this dilution unless otherwise indicated. Labeled antibodies, however, were used at 10-20 fold dilution. For specific absorption, positive human sera were absorbed with the same volume of A- or B-particle suspension (1-2 mg protein/ml). A- and B-particles were dissolved in \(8M\) urea and dialyzed against PBS before absorption.

Cell Smears for Immunofluorescence Studies Smears of transplanted DBA/2 mouse leukemia cells were employed for indirect immunofluorescence studies. Mice of this strain and spontaneous neoplasias arising among them were characterized elsewhere.\(^{26,27}\) The original leukemia developed spontaneously and was found to produce an abundance of A-particles. The leukemia cells have since been maintained in cell culture, and cells from this culture, when inoculated subcutaneously, grew into a local tumor. Single-cell suspensions were prepared by teasing this tumor tissue gently through a stainless steel screen in cold PBS. After filtration through packed glass wool and washing with PBS, cells were suspended in PBS containing 7% BSA at a concentration of 10° cells/ml, and smeared on a fluorescence-free object glass. Smears were air-dried, fixed with cold acetone for 10 min, and used immediately or stored at -20°C until use. No morphological or immunological deterioration took place within 2 months of storage at this temperature.

Indirect Immunofluorescence Microscopy Approximately 25 µl of test sera was applied to a restricted area (10 × 10 mm) of a smear after moistening with PBS. The smear was kept in a humidified chamber at 4°C overnight. After gentle washing twice with PBS, about 25 µl of an appropriately labeled antibody was applied to the same area of this smear and stored at 37°C for 2 hr. The smear was then washed with and mounted in PBS, and examined in a Zeiss RA34 fluorescence microscope.

Control Experiments A series of experiments were carried out to confirm reaction specificity in the present system. These were (1) positive controls using unlabeled rabbit anti-A-particle antisera followed by labeled anti-rabbit IgG antibodies, (2) a series of blocking tests in which (a) unlabeled rabbit anti-A particle antisera were applied before the test sera, and (b) appropriate unlabeled anti-IgG antibodies were inserted between test sera and labeled anti-IgG antibodies, (3) specific absorption tests in which positive test sera were absorbed with A- or B-particle samples, and (4) negative controls using smears of spontaneous DBA/2 mouse leukemia cells with no A-particle formation.

Viral Particles A-particles used for the absorption were isolated from transplanted DBA/2 mouse leukemias by the method of Tanaka et al.\(^{28}\) which has recently been further improved.\(^{29}\) B-particles purified from RIII mouse milk were a kind gift from Dr. Dan H. Moore, Department of Microbiology, Hahneman Medical College, Philadelphia, U.S.A.

Electron Microscopy Thin sections of transplanted DBA/2 mouse leukemia were prepared by the conventional technique and examined in a JEM 100B electron microscope.
RESULTS

Ultrastructure of A-Particle-forming DBA/2 Mouse Leukemia Cells

A thin-section electron micrograph of leukemia cells is shown in Photo 1. The architecture of these cells was relatively simple. A-particles were found, usually in a single cluster, in the cytoplasm of almost all leukemia cells, and there are no other structures comparable in size, shape, and site with A-particle clusters. Other viral particles such as extracellular B- or C-type particles, budding particles at the plasma membrane, and intracisternal A-particles were hardly encountered.

Indirect Immunofluorescence Observations with Rabbit Anti-A Particle Antisera (Positive Control)

A-particle clusters were easily identified as specifically fluorescing discrete granules in the cytoplasm of leukemia cells (Photo 2). Neither nuclear nor diffuse cytoplasmic fluorescence was observed. This peculiar feature of specific fluorescence is in good agreement with the electron microscopic findings mentioned above, and also with observations by direct immunofluorescence of spontaneous DBA/2 mouse leukemia cells we reported previously.25)

Immunofluorescence Observations with Human Sera

All of the following observations were made with sera absorbed in vitro because there has been no confirmation that in vivo absorption is better in terms of eliminating nonspecific reactions. Essentially the same results as with rabbit antisera mentioned above were obtained with sera from some breast cancer patients (Photo 3), indicating that these sera contained an antibody against A-particles. The three reference German sera provided by Dr. Müller also gave positive reaction in the present system. Distribution of the antibody among Japanese women is shown in Table I. The antibody was detected in 26 (60%) out of 43 breast cancer patients and in 4 (25%) out of 16 mammary fibroadenoma patients while only 4 (11%) were antibody-positive out of 37 control women.

<table>
<thead>
<tr>
<th>Age (yr) groups</th>
<th>Mammary disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>21~ 40</td>
</tr>
<tr>
<td>41~ 40</td>
<td>60</td>
</tr>
<tr>
<td>61~ 40</td>
<td>Total</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>6/9</td>
</tr>
<tr>
<td>Mammary fibroadenoma</td>
<td>1/2</td>
</tr>
<tr>
<td>Control</td>
<td>2/18</td>
</tr>
<tr>
<td></td>
<td>2/12</td>
</tr>
<tr>
<td></td>
<td>0/7</td>
</tr>
<tr>
<td>4/37</td>
<td></td>
</tr>
</tbody>
</table>

Denominators indicate the number of patients examined and numerators, the number of positive cases.

<table>
<thead>
<tr>
<th>Histological Type of Mammary Cancer</th>
<th>No. of cases examined</th>
<th>No. of positive cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrating carcinoma in situ</td>
<td>3</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Infiltrating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>papillotubular</td>
<td>11</td>
<td>5 (45)</td>
</tr>
<tr>
<td>medullary tubular</td>
<td>14</td>
<td>9 (64)</td>
</tr>
<tr>
<td>scirrhous</td>
<td>10</td>
<td>7 (70)</td>
</tr>
<tr>
<td>lobular</td>
<td>2</td>
<td>1 (50)</td>
</tr>
</tbody>
</table>

This overall distribution pattern was reproducible in a single age group of 21~40 years. In brief, occurrence of an antibody is intimately related with proliferating disorders of mammary glands.

In the analysis of 40 breast cancer cases, the antibody did not appear to be specifically correlated with a particular type of tumor histology (Table II). Similarly, no preference was confirmed with respect to clinical stage or blood group (not shown).

Results of the follow-up studies are summarized in Fig. 1. Although the length of observation periods was not uniform, it appears that surgical removal of breast cancer results in disappearance of the antibody in most cases; out of 13 breast cancer patients who were antibody-positive at the time of operation, 10 (Group 1, Cases 1~10) became negative within 1 year or so after operation, while 3 (Group 2, Cases 11~13) remained positive. Three patients who were initially
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Fig. 1. Follow-up studies on anti-A particle antibody after surgical operation of breast cancer

antibody-negative (Group 3, Cases 14–16) became positive within 1 year after operation, but two of them (Cases 14 and 15) were again negative at later stages. In remaining 4 cases (Group 4, Cases 17–20), the antibody was persistently negative. The follow-up studies have not been completed yet but what can be said at present is that all members of Group 1 are apparently healthy while 3 of Group 4 are either dead of advanced tumor or suffering from tumor recurrence.

Reaction Specificity Positive human sera when used after having been absorbed with disrupted A- or B-particles gave completely negative results. Blocking tests of both types 2(a) and (b) mentioned earlier resulted in either marked reduction or disappearance of specific fluorescence. No positive reactions were observed with A-particle-negative leukemia cells treated with an appropriate combination of test sera and labeled antibody. All these seem to confirm the reaction specificity of indirect immunofluorescence in the present system.

DISCUSSION

The indirect immunofluorescence system employed in the present system has successfully detected antibody against A-particles in some sera from Japanese women. Reaction specificity was well defined by a series of control experiments, and participation of Forssman antibody is unlikely since all sera were exhaustively absorbed with sheep red blood cells. The present observations are in good agreement with those of Müller et al.,12–15 as well as of several others.3,7 It is interesting to note, however, that in the present study, antibody incidence among breast cancer patients (60%) is considerably higher than that (32.9%) described by Müller et al.15 It remains to be investigated whether this reflects a difference in ethnic background. At the same time, however, difference in the detection system employed should also be taken into consideration, that is, the specific fluorescence of a single large cluster of A-particles found in the cytoplasm of most leukemia cells allows an easier, more reliable identification than is possible with frozen-sectioned mammary tumors employed by other authors.

A question has immediately arisen as to what the significance of the antibody among humans is. In the mouse system, in which the relationship between MTV and mammary carcinogenesis is already established, we disclosed that antibody against A-particles is rather ubiquitous.10,23 A more restricted distribution of the antibody was also reported by Müller et al.27 It remains unknown, however, whether and to what extent the mouse system can be employed as a model for human cases.

To promote studies a step further, our efforts are being concentrated on identifying the A-particle antigen(s) (internal antigen(s) of MTV) toward which human antibody is directed.

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ANTIBODY TO MTV IN HUMAN SERA

REFERENCES

1) Bernhard, W., Cancer Res., 18, 491～509 (1958).

EXPLANATION OF PLATE

Photo 1. A thin section electron micrograph of transplanted DBA/2 mouse leukemia cells. A large cluster of A-particles is seen in the cytoplasm. ×11,000

Photo 2. Indirect immunofluorescence with rabbit anti-A particle antiserum. Each cell contains a specifically fluorescing granule at the site of A-particle cluster in the cytoplasm. In one cell, such a granule appears in the nuclear region due to superimposition. ×800

Photo 3. Indirect immunofluorescence with serum from a breast cancer patient. Findings are identical to those in Photo 2. ×800