ABSTRACT

Using BALB/c nu/nu, BALB/c nu/nufC3H (BALB/c nu/nu mice raised by C3H/HeN foster mother), BALB/c thymus-engrafted BALB/c nu/nufC3H, BALB/c nu/+, and BALB/c nu/+fC3H mice, we examined what kinds of cells are carriers of blood-borne mouse mammary tumor virus (B-MMTV). A radioimmunoassay and an immunoperoxidase assay revealed the presence of MMTV-gp52 antigen in the mammary glands of all BALB/c nu/+fC3H and BALB/c thymus-engrafted BALB/c nu/nufC3H mice (more than 60 days old) but only of some BALB/c nu/nufC3H mice (more than 120 days old); those that possessed a significant number of functional T-cells. BALB/c nu/+ mice did not show the antigen expression at any age. Transfer experiments of cells or plasma from young (<12 weeks) BALB/c nu/nufC3H to BALB/c +/+ virgins revealed that cells besides T-cells can also become carriers of B-MMTV. This was confirmed by Southern blotting analyses; exogenous provirus DNA sequences were found in B-cells as well as T-cells of BALB/c nu/+fC3H mice. However, when young BALB/c nu/nu mice were inoculated with BALB/c nu/nufC3H blood, they did not show the MMTV-gp52 antigen expression. Transfer experiments of purified T-cells, B-cells, natural killer cells, and macrophages from BALB/c fC3H mice to BALB/c nu/nu mice revealed that only T-cells have the ability to transfer viral activity to the mammary glands.

These results suggest that B-MMTV is carried from the gastrointestinal tract to the mammary glands by lymphoid cells such as T-cells and B-cells, then transferred to the mammary gland cells by the T-cells.

INTRODUCTION

The in vivo life cycle of exogenous MMTV is still uncertain. MMTV is produced in the mammary gland of infected females and transferred to suckling pups as a milk factor. Intact MMTV B-particles can be observed on the surface of the intestinal epithelium but not in the cytoplasm (1). Destruction of the virus structure, therefore, seems to occur when the virus enters the cell, and MMTV “footprints” can be traced through viral antigens. Immunohistochemically, viral antigens are taken up in the intestinal epithelial cells by endocytosis in coated pits and channeled toward larger vacuoles by fusion with lysosomes (2). Vacuolar contents are released at the basolateral membrane. However, the antigen is not detected in the intercellular spaces of intestinal absorptive cells or in the lymph spaces below the basolateral membrane (2, 3).

A new method should be employed to determine the route of the virus from the gastrointestinal tract to the mammary gland cells. A number of investigators have reported that blood elements propagate MMTV from the digestive tract to the mammary gland (4). However, electron microscopic studies have failed to demonstrate B-particles in blood cells, indicating that MMTV is in a subviral state (5). Virus activity related to blood cells has been designated blood-borne MMTV (B-MMTV), as distinct from B-particle-associated mammary tissue-borne MMTV. The MMTV activity in the blood is found in cellular components but not in the plasma (6). This ability to infect was first thought to be associated with erythrocytes and later with nucleated hemic cells, i.e., leukocytes and/or hematopoietic stem cells (4). Lymphocytes have also been assumed to be the carriers (7). Recently, exogenous MMTV DNA sequences in lymphoid cells of BALB/cfC3H mice have been found (8), suggesting that lymphoid cells are the carriers of B-MMTV activity. However, it is not known which lymphoid cells are the actual carriers. Since B-MMTV is integral in the life cycle of MMTV, it is of great interest to determine the cells responsible for carrying the B-MMTV activity. The present study focuses on lymphoid cells and has been designed to determine using BALB/c nu/nufC3H mice which type of lymphocytes (T or B) are the carrier of B-MMTV.

MATERIALS AND METHODS

Mice. BALB/c +/+, BALB/c nu/+, BALB/c nu/nu, and C3H/HeN mice were purchased from CLEA Japan Inc., Osaka, Japan. The BALB/c nu/+fC3H and BALB/c nu/nufC3H mice were raised in our animal facility. In brief, a BALB/c nu/+ female was mated with a BALB/c nu/nu male and was kept on an elevated wire mesh platform to allow her babies to drop into a cage in which a C3H foster mother was kept. Subsequent generations were nursed by their natural mothers. All of these mice were kept under specific pathogen-free conditions.

Neonatal Thymus Graft. Whole or single thymus lobes of neonatal BALB/c mice were implanted under the shoulder skin of BALB/c nu/nufC3H mice (9).

Immunoperoxidase Assay. The ABC technique using anti-MMTV-gp52 serum was applied to paraffin-embedded sections that had been fixed in a 10% neutral buffered formalin solution. The method and the characterization of the antibody have been described in detail elsewhere (10).

Radioimmunoassay. Purified MMTV-gp52 was labeled with 125I by the chloramine-T method. The techniques used in the preparation of tissue extracts from the mammary glands, spleen, thymus, and bone marrow have been described elsewhere (11). Measurements are defined in pg/g of tissue extract.

Blood Cell Transfer Assay. Donors were 2- to 3-month-old BALB/c

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Received 9/10/87; revised 2/22/88, 6/29/88; accepted 8/2/88.

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1This work was supported in part by Grants-in-Aid for Cancer Research, 6101037 and 6201025, from the Ministry of Education, Science, and Culture, Japan (to A. T.). This work was also supported by the Japanese Ministry of Health and Welfare, a grant from the Naito Foundation, a grant from the Mitsubishi Foundation, a Grant-in-Aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, National Institutes of Health, the Science Research Promotion Fund of the Japan Private Promotion Foundation (1987), and a Grant-in-Aid for Cancer Research, 62015088, from the Ministry of Education, Science and Culture (to Su. I.).

2To whom requests for reprints should be addressed.

3The abbreviations used are: MMTV, mouse mammary tumor virus; nu/nu, congenital athymic nude; gp52, glycoprotein with a molecular weight of 52,000; NK, natural killer; IPA, immunoperoxidase assay; HAN, hyperplastic alveolar nodule; RIA, radioimmunoassay; B-MMTV, blood-borne mouse mammary tumor virus; Con A, concanavalin A.

Airo Tsubura,2 Munee Inaba, Shunsuke Imai, Akira Murakami, Naoki Oyaizu, Ryoji Yasunii/u, Yoko Ohnishi, Harutaka Tanaka, Sotokichi Morii, and Susumu Ikehara

Department of Pathology, Kansai Medical University, Moriguchi, Osaka 570 [A. T., M. I., N. O., R. Y., Y. O., S. M., Su. I.]; Department of Pathology, Nara Medical College, Kashihara, Nara 634 [Sh. I.]; and Institute for Virus Research, Kyoto University, Kyoto, Kyoto 606 [A. M., H. T.]; Japan

[ABSTRACT] Intervention of T-Cells in Transportation of Mouse Mammary Tumor Virus (Milk Factor) to Mammary Gland Cells in Vivo1

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These results suggest that B-MMTV is carried from the gastrointestinal tract to the mammary glands by lymphoid cells such as T-cells and B-cells, then transferred to the mammary gland cells by the T-cells.
gp52 antigen expression in the mammary glands, Osaka, Japan). The purity of T-cells was more than 95% when treated with monoclonal anti-Thy 1.2 antibody plus complement to deplete mature T-lymphocytes. The resultant population, which consists of more than 80% asialo Gm1-positive cells, was referred to as NK-cell-enriched population. In addition, we have confirmed that this population shows killing activity to YAC-1 cells. Macrophages were obtained from the plastic dish-adherent peritoneal exudate cells. The purity of macrophages was more than 90%, when analyzed by a cytofluorometer using anti-Mac 1 (M1/70) antibodies. Each cell count was adjusted to 10^6 cells/0.3 ml. These cell suspensions were i.p. injected to 3- to 5-week-old BALB/c nu/+, BALB/c nu/+, or BALB/c nu/nu females. Adult BALB/c mice served as hypophysial donors for hormonal stimulation of the host mammary gland. One pituitary gland was grafted to the right inguinal mammary fat pad. All mice were allowed either 5 or 8 weeks of hormone stimulation. Right inguinal mammary glands were removed together with the grafted pituitary gland and were processed for IPA. All mice with hormone stimulation for 8 weeks were checked using HAN assays 5 weeks after pituitary graft removal in wholemount preparations. Hormonal stimulation was also achieved by mating with BALB/c male mice, and IPA was performed during lactation.

Cytotoxicity Test. The cytotoxicity test was carried out, as previously described (15).

Mitogen Assay. The mitogenic reactivity was determined by measuring the incorporation of [3H]thymidine into DNA as previously reported (16).

Restriction Endonuclease Digestion and Southern Blotting Analyses. T-cells and B-cells were separated from the spleen of mammary tumor-bearing multiparous BALB/c nu/+/F3H mice (9- to 13-month old) and multiparous BALB/c nu/nu/nu females (16-month-old). MuMT73, an epithelial cell line which was derived from spontaneous mammary tumors bearing multiparous BALB/c nu/+/F3H mice (9- to 13-month old), was used for a positive control while BALB/c nu/nu females served as a negative control. Each cellular DNA (10 /ig) was isolated, digested with PstI, subjected to electrophoresis on a 0.8% agarose gel, and transferred to nitrocellulose (18). The nitrocellulose filter was then hybridized with a 32P-labeled probe specific for the gag-pol fragment of MMTV(C3H) (8).

Mammary Tumor Incidence and Latency. Multiparous BALB/c nu/nu/nu, BALB/c nu/+/F3H, and BALB/c nu/+/ which survived more than 200 days were sacrificed when carrying visible mammary tumors.
TRANSPORTATION OF EXOGENOUS MMTV IN VIVO

Table 2 Correlation between MMTV-gp52 antigen expression in the mammary glands and mitogen responsiveness in spleen cells of BALB/c nu/+fC3H, BALB/c nu/nu with neonatal thymus grafts, and BALB/c nu/nu/fC3H mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age (days)</th>
<th>Thy-1.2* spleen cells (%)</th>
<th>cpm x 10^-3 (mean ± SD)</th>
<th>St*</th>
<th>IPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c nu/+fC3H</td>
<td>162</td>
<td>5.6 ± 0.0</td>
<td>96.1 ± 9.4</td>
<td>220.8 ± 13.2</td>
<td>17.0</td>
</tr>
<tr>
<td>BALB/c nu/fC3H + thymus graft</td>
<td>206</td>
<td>8.6 ± 0.3</td>
<td>19.6 ± 2.4</td>
<td>162.6 ± 9.9</td>
<td>2.3</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>71</td>
<td>0.4 ± 0.2</td>
<td>1.7 ± 1.2</td>
<td>0.8 ± 0.0</td>
<td>3.7</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>78</td>
<td>0.6 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>0.7 ± 0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>82</td>
<td>0.7 ± 0.4</td>
<td>1.6 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>157</td>
<td>7.6 ± 0.4</td>
<td>6.1 ± 1.3</td>
<td>31.6 ± 0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>162</td>
<td>4.8 ± 0.2</td>
<td>4.2 ± 0.3</td>
<td>34.8 ± 2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>238</td>
<td>8.2 ± 2.7</td>
<td>9.9 ± 1.3</td>
<td>82.9 ± 5.1</td>
<td>1.2</td>
</tr>
<tr>
<td>BALB/c nu/nu/fC3H</td>
<td>310</td>
<td>6.7 ± 0.7</td>
<td>11.4 ± 0.0</td>
<td>70.7 ± 5.2</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Stimulation index.

Table 3 IPA and HAN assay for MMTV-gp52 expression in the mammary glands of BALB/c +/+ virgins inoculated i.p. with peripheral blood cells, spleen cells, or plasma of BALB/c nu/+fC3H or BALB/c nu/nufC3H mice

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>Peripheral blood</th>
<th>Spleen cell</th>
<th>Plasma</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nu/+</td>
<td>7/10³</td>
<td>7/10³</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Nu/nu</td>
<td>3/10</td>
<td>1/10</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Triple-diluted peripheral blood (0.3 ml) inoculation.

Table 4 Immunoperoxidase assay for MMTV-gp52 antigen expression in the lactating mammary glands of female BALB/c nu/nu or BALB/c nu/+ inoculated i.p. with peripheral blood of 2 to 3-month-old BALB/c nu/+ or nu/nu/fC3H mice

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient*</th>
<th>[Nu/+]</th>
<th>[Nu/nu]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nu/+</td>
<td>14/16</td>
<td>12/13</td>
<td></td>
</tr>
<tr>
<td>Nu/nu</td>
<td>13/15</td>
<td>0/12</td>
<td></td>
</tr>
</tbody>
</table>

* Number of positive mice/number of mice examined.

Table 5 Immunoperoxidase assay for MMTV-gp52 antigen expression in the mammary glands of BALB/c nu/nu virgins inoculated i.p. with splenic T-cells, B-cells, or peritoneal macrophages of 6- to 7-month-old BALB/c nu/+fC3H mice

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>T-cell</th>
<th>B-cell</th>
<th>NK cell</th>
<th>Macrophage</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nu/nu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient*</th>
<th>[T-cell]</th>
<th>[B-cell]</th>
<th>[NK cell]</th>
<th>[Macrophage]</th>
<th>[Control]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nu/nu</td>
<td>7/10³</td>
<td>0/10</td>
<td>0/10</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* 1 x 10⁶ cells in 0.3 ml RPMI 1640 or 0.3 ml RPMI 1640 alone.

RESULTS

Age-dependent Increase in Expression of MMTV-gp52 Antigen in BALB/c nu/nu/fC3H. We examined the expression of MMTV-gp52 antigen in various organs using RIA and IPA. As shown in Table 1, BALB/c nu/+ mice did not show the antigen in any organs, whereas BALB/c nu/+fC3H mice showed the antigen in the thymus, bone marrow, and mammary glands, and BALB/c thymus-engrafted BALB/c nu/nu/fC3H mice exhibited the antigen in the spleen and mammary glands. The positive rate of the MMTV-gp52 antigen in RIA was high in the mammary glands but low in the spleen, bone marrow, and thymus. The level of MMTV-gp52 in the mammary glands, which was measured by RIA, varied considerably from mouse to mouse (26 to 3 x 10⁶ pg/µl). In contrast, IPA was more sensitive and the results more consistent than those of RIA, although IPA does not allow quantitation of viral antigens. In histological examinations including in situ hybridization and immunoperoxidase staining for MMTV-gp52 were performed.
agreement with the results of St. George et al. (19), lactating BALB/c nu/+)C3H mice showed MMTV-gp52-positive alveoli clusters surrounded by antigen-negative alveoli in the mammary glands (Fig. 1). This uneven distribution of viral antigen might reflect the wide range in the RIA data or false negative results in RIA. BALB/c nu/nufC3H mice (60–120 days) did not show the antigen in RIA, whereas BALB/c nu/nufC3H mice (121–250 days) did, although the percentage of Thy 1.2* cells was low. Fig. 2 more clearly shows an age-dependent increase in MMTV-gp52 antigen expression of mammary glands as estimated by IPA; the viral antigen was detected in the mammary glands of BALB/c nu/nufC3H mice from about 120 days of age.

Relationship between Viral Antigen Expression in Mammary Glands and T-Cell Functions. The next step was to determine whether or not there is a correlation between the viral antigen expression and T-cell functions. As shown in Table 2, all BALB/c nu/+)C3H mice and BALB/c nu/nufC3H with thymus grafts (5 mice, respectively) showed MMTV-gp52 antigen expression and high responsiveness to T-cell mitogens (phytohemagglutinin and Con A). Young BALB/c nu/nufC3H mice (3 mice; <120 days) exhibited neither MMTV-gp52 antigen expression nor response to T-cell mitogens, whereas old BALB/c nu/nufC3H mice (4 mice; >120 days) showed MMTV-gp52 antigen expression and significant responsiveness to Con A, although the percentage of Thy 1.2* cells was low. We therefore concluded that there is a correlation between MMTV-gp52 antigen expression and T-cell functions (but not percentage of Thy 1.2-positive cells).

Transfer of Viral Activity. We examined whether viral activity can be transferred by cells or plasma. As shown in Table 3, inoculation of peripheral blood or spleen cells from BALB/c nu/nufC3H as well as BALB/c nu/+)C3H mice (2–3 months old) caused the MMTV-gp52 antigen expression in the mammary glands of BALB/c +/+ virgins, although the incidence in HAN assays was relatively low compared with IPA. Plasma inoculation however did not cause any viral activity. To exclude the influence of host T-cells on the expression of the antigen, lactating mammary glands of young BALB/c nu/nu recipient were used. As shown in Table 4, the BALB/c nu/nu mice showed MMTV-gp52 antigen expression in the mammary glands when inoculated with BALB/c nu/+)C3H blood (but not BALB/c nu/nufC3H blood). Furthermore, as shown in Table 5, only young BALB/c nu/nu mice which had been inoculated with exogenous MMTV-infected T-cells (but not B-cells, NK cells, or macrophages) exhibited the MMTV-gp52 antigen expression in the mammary glands. These results indicate that T-cells are essential for MMTV-gp52 antigen expression, although cells other than T-cells can also be carriers of B-MMTV.

Southern Blotting Analyses. As shown in Fig. 3, both lane 4 (extract from T-cells from BALB/c nu/+)C3H mice) and lane 5 (extract from B-cells of BALB/c nu/+)C3H mice) displayed not only 5.0- and 5.4-kilobase endogenous fragments but also the 4.0-kilobase (—) genomic band (exogenous MMTV band) as seen in lane 6 (extract from MuMT73, a cell line originating from spontaneous mammary tumors of BALB/cfC3H mice). In contrast, lane 1 (extract from BALB/c liver), lane 2 (T-cells of BALB/c nu/+ mice), and lane 3 (B-cells of BALB/c nu/+ mice) lacked the 4.0-kilobase band, which is specific for exogenous MMTV.

Mammary Tumor Incidence and Latency in BALB/c nu/+, BALB/c nu/+)C3H and BALB/c nu/nufC3H Mice. All mice were normally bred females (maximum three pregnancies). The incidence and mean latency of mammary tumors after introducing milk factor to BALB/c nu/+ or BALB/c nu/nu mice were 31/51 (60.8%) and 284 ± 66 days in BALB/c nu/+ mice, and 5/13 (38.5%) and 338 ± 50 days in BALB/c nu/nu mice. Histological examination revealed that all were mammary adenocarcinomas, which expressed MMTV-gp52 antigen. Although two mammary tumors were found in BALB/c nu/+ mice (2/13 = 15.4% and 379 ± 30 days), they were MMTV-gp52 antigen-negative mammary sarcomas.

DISCUSSION

Nude mice have been thought to exhibit no T-cell functions despite the presence of some Thy 1.2-positive cells (20). However, it has recently been demonstrated that they possess functional T-cells, the number of which increases with age (15, 16, 21). In the present study, we examined the expression of MMTV-gp52 antigen in the mammary glands of BALB/c nu/nu mice in relation to T-cell functions. As shown in Table 1 and Fig. 1, an age-dependent increase in the expression of the antigen in BALB/c nu/nufC3H mice was observed. In addition, we have found that there is a correlation between the antigen expression and T-cell functions (but not percentage of Thy 1.2-positive cells), as shown in Table 2. These results strongly suggest that functional T-cells are essential to the expression of MMTV-gp52 antigen in the mammary glands.

To examine whether T-cells are the only carriers of B-MMTV, transfer experiments were carried out. As shown in Table 3, inoculation of peripheral blood and spleen cells (but not plasma) from young BALB/c nu/nufC3H mice caused the MMTV-gp52 antigen expression in the mammary glands of BALB/c +/+ virgins. However, if there are no T-cells in vivo, no viral transmission occurred (Table 4). Table 5 shows that only MMTV-infected T-cells (but not B-cells, NK cells, or macrophages) can induce MMTV-gp52 antigen expression in the mammary gland of BALB/c nu/nu mice. These results indicate that cells other than T-cells can also be carriers of B-MMTV, but that T-cells are essential for transmission of B-MMTV to the mammary gland cells in vivo.

MMTV DNA sequences in lymphoid cells have been documented; several B-lymphocyte cell lines of BALB/c origin have endogenous MMTV DNA sequences (22), and splenic B-cells express MMTV-related antigen(s) (23–26). It has been demonstrated that T-cell lymphomas of BALB/c origin amplify endogenous MMTV sequences (27). BALB/c mice have been shown to possess one subgenomic-size and two genomic-size MMTV proviruses, but exogenous MMTV has not been found in these strains (28–30). A method to discriminate between the exogenous and endogenous MMTV DNA sequences is therefore necessary. Recently, Liegler and Blair have demonstrated the exogenous MMTV DNA sequences in lymphoid cells of BALB/cfC3H mice using the DNA hybridization method (8). Using this same method, we have clearly demonstrated the exogenous MMTV DNA sequences in both T-cells and B-cells of BALB/c nu/+)C3H mice.

In conclusion, both T-cells and B-cells are B-MMTV carriers, but T-cells are essential for the transfer of B-MMTV activity to mammary gland cells. There are two possibilities: (a) T-cells carry B-MMTV the whole way from the gastrointestinal tract to the mammary gland cells, and (b) B-cells first carry B-MMTV in the gastrointestinal tract and then transfer it to T-cells. Since MMTV B-particles are not produced in blood cells (including T-cells), unencapsulated RNA and DNA may be transferred by cell-to-cell contact. However, the possibility that additional factors such as cytokines are involved in the transfer...
of B-MMTV activity to mammary glands still remains. We are now attempting to elucidate the exact transfer mechanism of MMTV activity from the gastrointestinal tract to the mammary gland cells.

REFERENCES


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