High Incidence of Mammary Tumors in Mice with Inherited Asplenia
Carriers for the Nude Gene

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ABSTRACT

A colony of mice suffering from dominant hemimelia associated with agenesis of the spleen has been developed and characterized during the past 7 years. The hereditarily asplenic (Dh/+ ) females were mated with mice homozygous (nu/nu) for hereditary athymia (nude) having a BALB/c background. BALB/c females heterozygous for the nu gene and with spleen (nu/+, +/+ ) have a moderate incidence (12%) of SMT, whereas nu/+, Dh/+ breeders have a drastic increase in the incidence of SMT to 46% when bred under identical conditions. Since all parent strains have a very low incidence of SMT, it appears that the spleen agenesis is a major factor accounting for an earlier and higher incidence of SMT in hereditarily asplenic (nu/+ , Dh/+ ) mice than in normal (nu/+ , +/+ ) siblings. The SMT express mammary tumor virus antigen(s) and possess estrogen, progesterone, and glucocorticoid receptors. The SMT rapidly metastasize and kill the host within 30 to 45 days. The BALB/c asplenic mice with SMT represent a unique model relevant to human breast cancer and for study of the function of the spleen in the development of solid tumors in general and of SMT in particular.

INTRODUCTION

Animal models of human diseases are valuable tools for investigative pathology and for developing relevant clinical treatments. Although the occurrence of SMT4 in mice, namely C3H and DBA strains, has been known since the beginning of this century [reviewed by Nandi and McGrath (25)], the relevance of murine SMT to the human counterpart has not yet been clearly established. For example, the interactions between viral and genetic factors in murine SMT have been studied extensively [reviewed by Hilgers and Bentvelzen (12)], whereas little is known of the role of such interactions in human mammary carcinoma. Furthermore, other factors such as the immunocompetence of the affected individuals and the role of sex hormones in the development of SMT are poorly understood.

We report herein the occurrence of SMT in an immunodeficient mouse model useful for the elucidation of the role of genetic, viral, immunological, and hormone interactions leading to the development of mammary carcinomas. Thus, hereditarily asplenic female mice heterozygous for both the nude (nu) gene and the gene for dominant hemimelia (Dh) offer a unique model for human breast cancer. Tumors in these mice occur naturally, have a high incidence (46%), rapidly metastasize, and express MT antigen(s). These mice were developed by mating athymic (nu/nu) nude males of BALB/c background with heterozygous asplenic females carrying the Dh gene (Dh/+ ). Although the parent strains have a low to moderate incidence of SMT, the BALB/c asplenic females heterozygous for both genes (nu/+, Dh/+ ) have a much higher incidence with an earlier onset of the SMT which, in turn, appear to depend on sex hormones for growth.

MATERIALS AND METHODS

Mice. A mutant strain suffering from hemimelia and asplenia was discovered by Searle (30, 31) among the breeding stock of luxoid mice (10). The abnormalities (which include skeletal anomalies, mainly of the hind limbs, and visceral alterations in addition to splenic agenesis) are inherited as an autosomal dominant trait. Since visceral defects in homozygous (Dh/Dh) mice result in death within a few days after birth, only heterozygous (Dh/+ ) mice are available for breeding and research. As a result of the absence of the embryonic epithelial anlage from which the spleen develops (11), these mice are the only mammals known to have a true inherited asplenia.

A breeding colony of mice with dominant hemimelia and asplenia has been maintained at the Memorial Research Center for more than 7 years (14, 17, 19, 21) and originated from hybrids (C57BL/6 X CBA F1) carrying the Dh gene from Jackson Laboratories, Bar Harbor, Maine.

A mutant strain of hairless (nude) mice was discovered by Flanagan (6) and was subsequently shown to be characterized by congenital athymia (26, 27), which is inherited via an autosomal recessive gene (nu). The development of our colony of hereditarily asplenic (nude) mice has also been described (3). To obtain vigorous breeders, 15-day-old nude males were reconstituted with a congenic thymocyte suspension (i.p.) equivalent to one-half of a normal thymus. The colony of athymic mice was maintained by breeding one reconstituted nude (nu/nu) male with 2 heterozygous (nu/+ ) females per cage. Our colony of nude mice was derived from that of Dr. Norman Reed from Montana State University, Department of Microbiology, Bozeman, Mont., and has been maintained on a BALB/c background for several generations.

A colony of hereditarily asplenic BALB/c mice was developed at the Memorial Research Center by cross-breeding nu/nu...
males with asplenic (Dh/+) females as reported previously (17, 20, 22, 23). At present, the colony is maintained by breeding nu/nu males with asplenic females heterozygous for both the Dh and nu genes (nu/+, Dh/+) . Accordingly, the progeny of nu/nu × nu/+ males were scored as positive. After 2 washes in HBSS, the pellets were resuspended in HBSS coated charcoal (0.5%). The supernatant was incubated for 45 min at 4° and washed twice with HBSS and then the supernatant was centrifuged 30 min at 100,000 × g, and then the supernatant was counted in a Beckman scintillation counter at 45% efficiency. The cytosolic proteins were removed with a second 10-min treatment with dextran-coated charcoal. Supernatants containing the 3H-labeled steroid receptor complexes were counted in a Beckman LS 230 scintillation counter at 45% efficiency. The cytosolic proteins were measured by the assay of Lowry et al. (16) following a carboxymethylation treatment (29) to remove interference with reducing agents. Cytosolic estrogen receptors in SMT were measured by sucrose density gradient (34), using 5 × 10^-12 mol of 17β-[3H]estradiol (specfic activity, 110 Ci/mmoll New England Nuclear, Boston, Mass.) per 100 ml of cytosol with 2.5 × 10^-9 mol of diethylstilbestrol (500-fold excess) per 100 ml of cytosol used as a competitive inhibitor.

Progesterone receptors (1, 2) were measured using 5 × 10^-12 mol of [17-methyl-3H]R5020 (specific activity, 86 Ci/mmoll New England Nuclear) per 100 ml cytosol with 500-fold excess cold R5020 used as a competitive inhibitor.

Glucocorticoid receptors (8) were measured using 5 × 10^-12 mol of [3H]dexamethasone (specific activity, 33 Ci/mmoll New England Nuclear) with 7.5 × 10^-12 mol dexamethasone as a competitive inhibitor.

Sucrose density gradients (3.7 ml) were run (34) with 0.3-ml samples layered onto 5 to 30% sucrose gradients in 10 mmol Tris buffer plus 1.5 mmol disodium EDTA, pH 7.5. Gradients were centrifuged in a Beckman L2-65B ultracentrifuge for 18 hr at 300,000 × g, fractionated into 10-drop fractions, and counted in a Beckman scintillation counter. Hemoglobin was added as a marker protein (4.8S) to all gradients prior to centrifugation.

RESULTS

During a 3-year period, 126 of 269 asplenic (nu/+ , Dh+/+) BALB/c breeder females developed SMT, for a total tumor incidence of 47%. This number is quite high in relation to the incidence in BALB/c mice carrying the recessive nu gene (13%) or the Dh gene (9%) alone (Table 1). Female breeders have been maintained for periods up to 10 months of age. The age distribution of the appearance of SMT is shown in Table 2. Asplenic breeders (nu/+, Dh+/+) were observed to develop SMT as early as 6 months of age, and they had a maximum incidence at 10 months. In contrast, breeders of the nu/+ genotype developed no tumors prior to 10 months of age, and the maximum incidence occurred at 15 months. No SMT were observed in nu/+, +/+ or +/+ , Dh/+ or nu/+, Dh/+ virgins maintained in the same specific-pathogen-free environment for up to 14 months. Similarly, no SMT were seen in nu/nu, Dh/+ (fasat) virgins 1 to 4 months old.

The tumors were first noticed when they were approximately 0.5 cm in diameter, and they grew rapidly to 3 cm or more in diameter, ultimately resulting in the death of the animal within 4 months.

### Table 1

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Total no. of SMT</th>
<th>Incidence of SMT</th>
<th>Age (mos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>86</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Asplenic</td>
<td>89</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Normal</td>
<td>183</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Asplenic</td>
<td>289</td>
<td>46</td>
<td>10</td>
</tr>
</tbody>
</table>

* Percentages rounded to the nearest unit.
30 to 45 days. Histological examination of the tumors revealed that all were mammary adenocarcinomas. The lungs were regularly involved by the metastases. Occasionally, metastases were found in the brain, liver, and kidneys. Metastatic spread to other organs was most common with those tumors that reached a fairly large size.

The results of a search for MTV antigens are shown in Table 3. As can be seen, DMBA-chemically induced tumors do not express MTV antigens on their cell surfaces, whereas the MTV-positive SMT from the BALB/cfC3H strain had high percentages of fluorescent cells. All the SMT from the asplenic mice had positive fluorescent cells ranging from 29 to 81%, indicating the presence of viral antigen(s) in these SMT.

Estrogen receptors measured with $17\beta^3$H]estradiol were found to range from 1.16 to 97.5 fmol/mg (10$^{-15}$ mol/mg) of cytosolic protein. Progesterone receptors were measured using $[17\text{-methyl-}3^H]R5020$, which is a progesterone derivative that does not bind to serum corticosteroid-binding globulin. Progesterone receptors ranged from 0.75 to 88.9 fmol/mg cystolic protein. Glucocorticoid receptors measured with $[3^H]$dexamethasone ranged from 62.93 to 212.34 fmol/mg cystolic protein (Table 4).

When SMT from asplenic mice were analyzed for estrogen receptor binding by the sucrose density gradient method, they showed both a 4S and a strong 8S component (Chart 1). The level of hormone receptors in SMT of hereditarily asplenic mice

**Table 2**

<table>
<thead>
<tr>
<th>Age (mos.)</th>
<th>Normal (nu/+, +/+ ) mice</th>
<th>Asplenic (nu/+ , Dh/+ ) mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>% with SMT</td>
</tr>
<tr>
<td>6</td>
<td>264</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>252</td>
<td>0.4</td>
</tr>
<tr>
<td>8</td>
<td>217</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>198</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>175</td>
<td>0.6</td>
</tr>
<tr>
<td>11</td>
<td>167</td>
<td>1.2</td>
</tr>
<tr>
<td>12</td>
<td>161</td>
<td>0.6</td>
</tr>
<tr>
<td>13</td>
<td>147</td>
<td>0.7</td>
</tr>
<tr>
<td>14</td>
<td>137</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>113</td>
<td>5.3</td>
</tr>
<tr>
<td>16</td>
<td>68</td>
<td>5.9</td>
</tr>
<tr>
<td>17</td>
<td>42</td>
<td>9.5</td>
</tr>
<tr>
<td>18</td>
<td>27</td>
<td>18.5</td>
</tr>
<tr>
<td>19</td>
<td>14</td>
<td>21.4</td>
</tr>
</tbody>
</table>

**Table 3**

**Presence of MTV antigen(s) in SMT of mice with hereditary asplenia**

Only values greater than 20% above normal rabbit serum controls are considered significant.

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Type of mammary tumor</th>
<th>Rabbit anti-MTV + FITC conjugate* (%)</th>
<th>Normal rabbit serum + FITC conjugate (%)</th>
<th>FITC conjugate alone (%)</th>
<th>No. of mice with significant levels of MTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>DMBA-induced</td>
<td>7% (3-11)%</td>
<td>6% (2-10)</td>
<td>0%</td>
<td>0/6</td>
</tr>
<tr>
<td>BALB/cfC3H</td>
<td>Spontaneous</td>
<td>77% (54-91)</td>
<td>6% (2-9)</td>
<td>0%</td>
<td>7/7</td>
</tr>
<tr>
<td>Asplenic BALB/c (nu/+, Dh/+ )</td>
<td>Spontaneous</td>
<td>58% (29-81)</td>
<td>7.0% (1-13)</td>
<td>0%</td>
<td>16/16</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Asplenic BALB/c mice (nu/+, Dh/+) have a significant increase in the incidence of SMT compared not only with the

**Table 4**

**Level of hormone receptors in SMT of hereditarily asplenic mice**

The mean and range of control cytosolic protein of 17 SMT was 6.48 (0.39 to 15.05) mg/ml.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>No. of SMT Analyzed</th>
<th>Mean ± S.E.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen</td>
<td>23</td>
<td>12.23 ± 4.23</td>
<td>0.306–97.5a</td>
</tr>
<tr>
<td>Progesterone</td>
<td>17</td>
<td>16.68 ± 5.18</td>
<td>0.75–88.9b</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>17</td>
<td>111.57 ± 30.80</td>
<td>27.00–212.34c</td>
</tr>
</tbody>
</table>

a Seventy-four %, 3 fmol/mg.

b Eighty-two %, 3 fmol/mg.

c Fifty-seven %, 100 fmol/mg.
conventional BALB/c strain but also with littermates homozygous for the recessive nu gene. If the heterozygous nu gene is involved in increased mammary tumorigenesis, it should be possible to demonstrate increased SMT incidence in other mouse strains carrying the gene. Since an increase of the incidence of SMT in nu/+ mice with a strain background other than BALB/c has not yet been reported, it appears that the agenesis of the spleen is the main cause of the high incidence of SMT in our model system. It should be stressed that SMT developed only in breeders. The Dh gene is lethal in the homozygous condition; hence female lassats (nu/nu, Dh/+ ) mice cannot be bred. Since no SMT were seen in up to 10-month-old virgins of genotypes such as nu/+, +/+; +/+; Dh/+; and nu/+, Dh/+ and owing to the short life span (3 to 4 months) of lasat mice, no SMT were to be expected in lasat mice. In fact, not a single spontaneous tumor has been found in nearly 900 lasat mice born in our colony (18).

The finding of high percentages of SMT in the asplenic (nu/+ , Dh/+ ) BALB/c breeders lends support to the fundamental role of the spleen in the control of mammary tumorigenesis. The spleen is the largest lymphopoietic organ interposed into the systemic circulation, and it accounts for 90 to 95% of the antibody produced after antigenic stimulation by i.v. or i.p. routes (28). Also, the data collected in the past 5 years indicate that the agenesis of the spleen in mice results in a diminished T- and B-cell synergy for antibody synthesis (4, 21, 32). The serum level of interferon of hereditarily asplenic (Oh/+ ) mice is significantly (<0.001) below that of normal littermates with Newcastle disease virus. However, the level of serum interferon is nearly normal in asplenic mice given transplants of spleen cells at birth (14). More important is the finding that spleen has a regulatory role on other lymphoid tissue and that the lack of the spleen during embryogenesis resulted in an impairment of the T-cell subpopulations [reviewed by Welles and Battisto (33)]. The enlargement of the spleen is a common reactive phenomenon accompanying the growth and metastatic spread of many tumors, whereas metastases are extremely rare in the spleen (5). In addition, a factor which induces splenomegaly in normal animals has been partially purified from the spleen of tumor-bearing mice (7). Thus, these studies suggest that one of the important functions of the spleen is the regulation of cellular and/or humoral immune reactions controlling the onset and progression of malignant growth in mammals.

It is important to emphasize that in previous work (15) we have demonstrated that BALB/cCrGl mice, which are free of MTV and have a low incidence of SMT, possess lymphocytes capable of responding in cell-mediated immune assays (i.e., blastogenic transformation and migration inhibition) to MTV antigen(s). This natural immunity is in contrast to the findings observed with the MTV-positive BALB/cIcScH mice, which develop SMT at high incidence levels and possess splenocytes nonsignificantly reactive to MTV. Thus, it appears that there is a strong correlation between the presence of natural immunity in spleen cells and a low incidence of breast tumors, and the high incidence of SMT in asplenic BALB/c mice seems to reinforce the validity of such a correlation.

When SMT from hereditarily asplenic (nu/+ , Dh/+ ) mice and normal (nu/) littermates were analyzed for estrogen receptor binding, they showed both a 4S and a strong 9S component. This pattern is typical of the receptor binding found in many human breast cancer tumors and in breast tissue from pregnant or lactating rodents (13). Estrogen receptors from uterine tissue typically show only the BS receptor form and are much less labile than the mammary receptor. In addition, the presence of progesterone and glucocorticoid receptors suggests that most of the SMT examined were well-differentiated hormone-sensitive tissues. Further studies with antihormones, ablation, or steroidogenesis inhibitors are needed to determine what proportion of these SMT may actually be hormone dependent. In DMBA-induced breast cancers, a large percentage of the tumors are hormone dependent and contain high levels of hormone receptors for both estrogen (9) and progesterone (1). However, these mammary tumors do not produce metabolites such as those arising from SMT of asplenic mice.

The hereditarily asplenic mouse bearing SMT provides a useful and relevant model for the study of human breast cancer. Mouse mammary tumors have been used for studies on hormone dependence and on genetic and epidemiological aspects of the disease. In more recent years, they have become a major basis of studies on B-particles, 70S RNA, and reverse transcriptase, expanding the scope of knowledge about the role of viruses in this neoplasia (12). We believe that the availability of asplenic mice with a high incidence SMT is an important tool for experiments designed to investigate the function of the spleen in the development of solid tumors in general and of mammary tumors in particular. A study is under way to observe the incidence of SMT in hereditarily asplenic mice (nu/+ , Dh/+ ) neonatally transplanted with syngeneic spleen cells as reported previously (14, 19).

REFERENCES


High Incidence of Mammary Tumors in Mice with Inherited Asplenia Carriers for the Nude Gene

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