Detection by Immunodiffusion of Mouse Mammary Tumor Virus in Milk Samples and Correlation with Tumor Development

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SUMMARY

Milk from female mice infected with the mammary tumor virus (MTV) was examined in immunodiffusion for MTV virion antigen. Very few of the milk samples from females in the first lactation contained detectable virus; the incidence of positive samples increased in succeeding lactations. No correlation was observed between the early development of a mammary tumor and the presence of detectable amounts of MTV virion antigen in the milk of the first and second lactations. However, the presence of detectable MTV in the later lactations was correlated with the development of a mammary tumor during the experimental period.

INTRODUCTION

The mouse mammary tumor virus (MTV) is normally transmitted from mother to offspring via the milk during the suckling period, and foster-nursing susceptible mice on infected lactating females is a recognized procedure for transmitting the virus experimentally (6). There is some evidence that the amount of virus produced by an infected female may increase with the parity of the female; more tumors developed in the offspring from late litters than in those from early litters in breeding experiments reported by Bittner (2, 3). In parallel with these observations on the transmission of infective MTV, Nowinski and associates (12, 13) reported that they were sometimes unable to detect a soluble MTV-associated antigen in immunodiffusion tests of the milk of MTV-infected females with fewer than 3 litters, although they could detect the antigen in the milk of females with more litters. In the present report, an immunodiffusion procedure has been used to detect the presence of MTV virions in milk samples. Milk from each lactation of MTV-infected breeding females was examined for the presence of MTV. The immunodiffusion results were then correlated with the eventual development of MTV-induced mammary tumors in the females.

MATERIALS AND METHODS

A colony of 43 BALB/cF3HCrgl females was mated with BALB/cF3H males at 2.5 months of age. As each female became pregnant, she was isolated from the breeding cage. The female was kept with her babies until they were five weeks old, at which time she was returned to the breeding cage; a similar procedure was followed for each pregnancy. When a mammary tumor developed in one of the females, she was removed from the breeding colony, and the date the tumor was first observed was recorded. The tumor-bearing females were then used in other experiments, and no further data on them were accumulated for this experiment.

At each lactation, each female was milked when her babies were approximately 2 weeks old. The babies were removed from the female in the late afternoon, and she was milked the following morning by means of a pulsating suction device, after which she was returned to her babies. Usually 1 ml of milk was collected from each female. A sample of approximately 0.1 ml of the fresh milk was refrigerated and tested in immunodiffusion within 24 hours. Occasionally samples were not available for testing because of technical difficulties. A recurrent problem was termination of the lactation prior to sample collection because of cannibalization of the litter by the female.

The immunodiffusion assay for MTV was used to detect the MTV virion antigen in the milk samples (4). The assay as developed and currently used in this laboratory detects antigen associated with the MTV type B virus particle (1). The virion itself is the antigen unit (5); it can be detected by electron microscopy in the precipitate line which develops after reaction of the particles with specific rabbit antiserum (9). The participation of the virion in this precipitate line has been further documented by ferritin-labeling studies (7). Thus, use of this assay procedure enabled us to detect the type B particle in the milk samples.

Preparation of the rabbit antisera used in the immunodiffusion assay has been described previously (4, 5, 8). Each rabbit received 4 weekly intramuscular injections of tissue extract (totaling 2–4 gm equivalents of tissue) and was bled 1 week after final injection. The resulting sera were frozen until needed. Most of the rabbits were immunized with MTV partially purified by differential ultracentrifugation of mammary tissue extracts. Thus, the antisera contained not only antibodies against MTV but also antibodies against some normal mouse tissue components. However, as noted below, the procedure used in the assay permitted easy identification of any specific reaction involving MTV virions. The specificity of these antisera, and their use in...
MTV-free BALB/c milk; it was found that antisera from rabbits immunized with either MTV-free or MTV-infected mammary tissue extracts in this experiment, and only those sera which did not react in immunodiffusion with the milk component prior to precipitation line resulting from the presence of the milk component, whereas other precipitate lines found in this system do not appear if such absorbed antiserum is used. Further, the characteristic MTV precipitate line is not produced if antisera from rabbits immunized with MTV-free mammary tissue extracts are used in the immunodiffusion plates, whereas the precipitate lines in the vicinity of the antiserum wells are found under such conditions. The tests carried out in this experiment were constantly monitored for specificity by the use of antiserum against MTV-free mammary tissues, as well as by comparison of the test samples with control antigen preparations known to contain MTV virions.

All milk samples were tested in at least two immunodiffusion plates, against at least two antisera known to be reactive against MTV virion antigen, and, for control purposes, against at least one antiserum known to contain no antibodies against MTV.

RESULTS AND DISCUSSION

As can be seen in Table 1, the number of BALB/cF3H females secreting detectable MTV virion antigen in their milk increased with each lactation. Only 4 of 33 females had clearly detectable MTV antigen in the milk of their first lactation. Samples of milk from an additional 2 females reacted very weakly in immunodiffusion; a barely detectable MTV-specific precipitate line was observed in reaction with a strong antiserum. Thus, a total of 6 of 33 samples of milk from females in their first lactation (18%) were positive for MTV virion antigen. Of the 32 samples collected during the second lactation, 9 were strongly positive for MTV antigen in immunodiffusion and 1 was weakly positive. Thus, the incidence of positive samples in the second lactation was 10 of 32 tested, or 31%. The incidence of positive samples increased even further at the third lactation. Eleven of 18 samples tested were strongly positive for MTV virion antigen during the third lactation (61%). Only a few samples were available for testing during the fourth and fifth lactations. Four of the 9 samples of milk from the fourth lactation were strongly positive for MTV antigen (44%).

These data indicate that there is an increase with increasing parity in the amount of MTV which is secreted in the milk of MTV-infected females. This is in agreement with previous experiments in which MTV was assayed indirectly by observation of tumor development in the offspring (2, 3) or by detection of soluble MTV-associated antigen (10, 12, 13).

It must be emphasized that negative results in the immunodiffusion tests reported herein do not necessarily indicate that

<table>
<thead>
<tr>
<th>Lactation</th>
<th>No. of milk samples tested</th>
<th>No. of samples strongly positive for MTV virion</th>
<th>No. of samples weakly positive for MTV virion</th>
<th>Percent of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>33</td>
<td>4</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Second</td>
<td>32</td>
<td>9</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>Third</td>
<td>18</td>
<td>11</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Fourth</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>Fifth</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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Detection of mammary tumor virus (MTV) in fresh milk samples by reaction in immunodiffusion with rabbit antiserum containing antibodies against MTV virion antigen(s).
no MTV is present in the milk. Previous studies utilizing MTV prepared from various extracts of normal and neoplastic mammary tissues have clearly shown that the concentration of MTV within a tissue may be crucial in the successful use of that preparation as antigen in the immunodiffusion plates (5). Thus, in the present experiments, the immunodiffusion procedure was used to detect relatively large quantities of MTV but not to detect minimal amounts of MTV.

Since the relative number of MTV-positive milk samples in the breeding population increased with each lactation, one might predict that, once MTV was detected in a milk sample, it would be detected in later milk samples from that female. Contrary to this expectation, 5 of the 6 females that produced detectable MTV during their first lactation did not do so during later lactations. Further data are limited, since most of the remaining females produced detectable MTV only in the last sample tested. However, as predicted 5 MTV-positive females did continue to produce MTV-positive milk in succeeding lactations.

It is of interest that the milk of many of the BALB/cfC3H females contained sufficient MTV virions to react in immunodiffusion without the use of concentration procedures. This is in contrast to our previous reports which indicated that MTV obtained from extracts of milk-filled lactating mammary gland tissue and partially purified by precipitation in the ultracentrifuge was not effective as antigen in immunodiffusion. One explanation for this is that the precipitation of the virus in the ultracentrifuge was not effective as antigen in immunodiffusion. One explanation for this is that the precipitation of the virus in the ultracentrifuge created clumps of virus which could not be resuspended, thus effectively reducing the concentration of virions in the extract. Evidence for this has been obtained from more recent experiments utilizing preparative procedures which do not include such a step. If MTV is centrifuged onto a cushion of high density material, such as 65% sucrose (w/v), MTV preparations reactive in immunodiffusion can readily be obtained from a number of tissue extracts which yielded negative results in the previous studies.

MTV-induced mammary tumors began to develop in the breeding females after most of the females had completed their second pregnancy and lactation (Table 2). Since the females were removed from the experiment when the tumors were detected, the number of females available for testing decreased during the period when samples were obtained from the third and fourth lactations. When the number of breeding animals remaining became too small for the collection of meaningful data, the experiment was terminated. At this time the females were 11 months old. Of the original 43 females, 28 had developed mammary tumors within the 11-month period, 3 had died without developing tumors, and 12 remained alive without tumor development. Thus, 65% of the females had developed mammary tumors by 11 months of age as a result of their infection with MTV during neonatal life.

The incidence of MTV-positive milk samples during the first and second lactations was low, and no correlation between the presence of detectable amounts of MTV in the milk and the early development of a mammary tumor was observed.

However, the presence of detectable MTV in the later lactations was correlated with the development of a mammary tumor during the experimental period (Table 2). Of the samples tested during the third and fourth lactations, 13 of the 15 samples obtained from females which later developed mammary tumors were positive for MTV, whereas only 2 of the 12 samples obtained from females that were tumor-free at the end of the experiment were positive for MTV.

Thus, although the milk of the early lactations contains relatively little virus, even in those females which rapidly develop tumors, the presence of virus in the milk of the later lactations is predictive of eventual tumor development. Although this relationship between virus production and tumor development may be coincidental, it is more likely causal in nature. The production of large quantities of virus in the mammary parenchyma may directly increase the probability of neoplastic transformation in this tissue. On the other hand, the correlation between virus production and tumor development may result from heterogeneity of some other physiologic characteristic in the mouse population. Since endocrinologic and immunologic mechanisms influence not only the development of mammary tumors in MTV-infected mice but also the replication of MTV both in vivo and in vitro (reviewed in 6), diversity in one of these mechanisms may be involved.

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