Correlation between the Detection of Specific Mouse Mammary Tumor Proviral Sequences and the Presence of Pulmonary Metastases in Mice Bearing Spontaneous Mammary Tumors

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ABSTRACT

Pulmonary metastases in C3H/He mice bearing spontaneous mammary tumors were detected and characterized by histological criteria and immunocytochemical staining for mouse mammary tumor virus antigens. The same lungs containing metastases were also positive when assayed for a specific subset of mouse mammary tumor virus proviral DNA sequences. These sequences, termed tumor-associated sequences, have previously been shown to be present in the DNA of spontaneous mammary tumors that arise before 1 yr of age in C3H/He mice but are absent in DNA's of apparently normal tissues of C3H/He mice. Reconstruction experiments demonstrated that the nucleic acid hybridization method will detect at least one mammary tumor cell/250 lung cells. While DNA from 13 lungs of apparently normal C3H/He mice did not contain sequences homologous to mouse mammary tumor virus tumor-associated-sequence RNA, DNA from lungs of 9 of 12 C3H/He mice bearing spontaneous mammary tumors did contain these sequences. Since the entire DNA content of the lung can be assayed as one sample, the hybridization method minimizes false negatives resulting from histological analysis of random biopsy sampling. The hybridization procedure described here thus represents a sensitive and quantitative element as an adjunct for the detection of micrometastatic lesions in mice bearing viral-mediated spontaneous mammary carcinomas.

INTRODUCTION

The mouse system has proved to be an excellent model for investigating the multiple etiological factors involved in mammary tumors. The roles of viruses, hormones, chemical and physical carcinogens, genetics, diet, and the immune system have all been extensively investigated. The murine model also represents an excellent system for the study of metastatic dissemination of mammary tumor cells. Pulmonary metastases have been detected and characterized by numerous investigators in such mouse strains as C3H, BALB/cfC3H, BALB/cRIII, R111, (BALB/c X DBA) F1, and (C57BL X A/He) F1. Incidences of pulmonary metastases that range from 2 to 95% of tumor-bearing animals have been reported; the primary factor in this wide range has been reported to be the size of the primary mammary tumor (1). Several methods have been used to score for pulmonary metastases; these include gross and microscopic examination of lung tissues and bioassays by trocar implants of lung tissues into syngeneic mice.

The C3H/He mouse has been extensively used in studies of both the etiology and metastatic spread of mammary carcinoma. Recent nucleic acid hybridization results have demonstrated that sequences homologous to a part of the RNA genome of the highly oncogenic MuMTV of C3H/He mice, i.e., MuMTV(C3H), can be detected in the DNA's of mammary tumors that occur early in the life span of C3H/He mice (before 1 yr of age); these sequences are absent in the DNA's of apparently normal livers from these same tumor-bearing animals (5). We report here the use of a radioactive probe, which contains sequences homologous to these "TA" sequences, that can detect specific MuMTV proviral sequences in lungs that contain micrometastatic lesions of C3H/He mice bearing spontaneous mammary tumors. Pulmonary metastases have also been characterized by histochemical staining for MuMTV antigens. The sensitivity and specificity of the nucleic acid hybridization technique is also delineated.

MATERIALS AND METHODS

Animals and Tissues. Spontaneous mammary tumors and tissues were obtained from C3H/HeCrGl or C3H/HeJ retired breeder females. Some tumor-bearing C3H/HeJ mice were kindly supplied by Dr. B. Diwan, Meloy Laboratories, Inc., Springfield, Va. Mice were monitored for mammary tumors by palpation. Tumor-bearing mice were held 3 to 4 weeks before sacrifice. Autopsy of all tumor-bearing animals revealed that the internal organs, including the lung, appeared normal with no gross evidence of metastases.

Immunocytochemistry. The entire lung and mammary tumors were removed from 3 animals and were fixed and submitted for immunocytochemistry. Random biopsies, representing approximately one-fifth of the total tissue volume, were taken from lungs and tumors of 4 animals for immunocytochemistry. The remaining tissue was frozen in liquid nitrogen for biochemical analysis. Random cu mm samples of mammary tumor and lung of each animal were fixed in Tellyezniski's solution, embedded in paraffin, sectioned at 4 μm, and mounted on precleaned glass slides. Following incubation at 56°C overnight, the slides were deparaffinized in xylene. The sections were stained by the peroxidase-antiperoxidase technique (13, 16). Briefly, sections were preincubated in 10% normal goat

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serum (Antibodies, Inc., Davis, Calif.) in 0.1 M PBS (pH 7.4) for 30 min at ambient temperature. Rabbit anti-MuMTV, characterized previously (4, 13), was absorbed with BALB/c lactating serum (Antibodies, Inc., Davis, Calif.) in 0.1 M PBS (pH 7.4) for wash in PBS. Rabbit antiperoxidase-peroxidase-soluble complex (Litton Bionetics, Kensington, Md.) that had been diluted 1:50 in PBS was placed on sections for 45 min followed by a 15-min wash in PBS. Rabbit antiperoxidase-peroxidase-soluble complex was placed on the sections for 2 to 5 min. The slides were rinsed with PBS with hematoxylin. Substitution and deletion controls were routinely done (3). The slides were evaluated and photographed with a Zeiss photomicroscope using a Kodak BG-12 filter and Panatomic-X filter.

Viruses. MuMTV(C3H) was obtained from supernatant fluids of the C3H mouse mammary tumor cell line Mm5mt/c1 (10). Propagation of cells and purification of MuMTV from supernatant fluids was as previously described (5). No type C virus was detected by electron microscopy, by radioimmunoassays, or by divalent cation preference of DNA polymerase (Mg2+:Mn2+ ratio of 23:1) using oligodeoxyguanylate:polyribocytidylate as template. Competition hybridization experiments were used to demonstrate that the virus produced in Mm5mt cells was also free of contaminating endogenous type C virus. These studies have been detailed previously (5).

Purification of Viral RNA. The procedure for the isolation and iodination of MuMTV 60 to 70 S RNA was as described previously (5). Iodinated RNA had a specific activity of approximately 2 x 107 cpm/µg and was 99% acid precipitable and greater than 95% RNase sensitive.

Purification of Cellular DNA. DNA was extracted from tissue using a procedure previously described (5). DNA prepared in this way had a 260:280 nm ratio of at least 1.70 and was usually above 1.80. After sonication, the sedimentation coefficient of DNA as determined by alkaline sucrose sedimentation was between 8 and 9S.

Molecular Hybridization. Hybridization conditions used have been described previously (5). Cellular DNA was annealed to MuMTV(C3H) 125I or 3H 60 to 70 S RNA at 68° under the following conditions. Cellular DNA (first boiled in 0.01 M NaPB, pH 6.8, for 1 min in a bath of ethylene glycol), at a concentration of 1.5 mg/ml in 0.4 M NaPB (pH 6.8) and 0.05% sodium dodecyl sulfate, was mixed with labeled MuMTV RNA and incubated for various times to obtain the desired C0f. C0f values are corrected to 0.12 M NaPB. Aliquots of 0.067 ml (100 µg of DNA and 1000 cpm of MuMTV RNA) were diluted with 2.0 ml of 2 M NaCl and 0.015 M sodium citrate to obtain a final DNA concentration of 50 µg/ml. RNase’s A and T1 were added to one-half of the sample at final concentrations of 15 µg/ml and 3 units/ml, respectively. Both tubes were incubated at 37° for 30 min. The samples were chilled for 10 min, and trichloroacetic acid was added to a final concentration of 10%. After being kept on ice for 30 min, the acid-insoluble material was collected on 0.45-µm nitrocellulose filters (Gelman, Inc.). A zero time control, which was boiled for 1 min in a bath of ethylene glycol prior to the addition of RNase, was included in all experiments. The cpm of radioactivity present in the RNase-treated portion of this sample was deducted from the amount obtained in all other samples. The percentage of hybridization was determined by dividing the cpm present in the RNase-treated sample by the cpm present in the untreated sample.

Isolation of TA Sequences. The method used is a modification of that of Shoyab et al. (14) and has been detailed previously using MuMTV (5). Briefly, 300,000 cpm of MuMTV(C3H)-labeled 60 to 70S RNA were hybridized to 30 mg of apparently normal C3H/He liver DNA to a C0f of 15,000 using the hybridization conditions described above. Liver was used since it has been reported to be free of many MuMTV markers (9). The sample was then diluted to 150 µg of DNA per ml, heated to 60°, and applied to a column of 150 ml of packed hydroxyapatite at 60°. The sample that eluted was reloaded, and the column was rinsed with 0.01 M NaPB (pH 6.8) containing 0.01% SLS until no material eluted which absorbed at 260 nm. Single-stranded RNA was then eluted with a solution of 0.14 M NaPB (pH 6.8) in 0.01% SLS and was then dialyzed against 3 changes of 6 liters of water containing 0.01% SLS. After dialysis, 1 to 2 mg of yeast carrier RNA were added, and the "TA" RNA was precipitated with 2 volumes of alcohol. The RNA was then pelleted, dissolved in 1 ml of 0.001 M NaPB, and stored at −20° until use.

Thermal Stability of DNA-RNA Hybrids. The technique for assaying the thermal stability of the DNA-RNA hybrids is as described previously (5). The hybridization mixture contained 1 mg of cellular DNA and 6000 cpm of radioactive MuMTV RNA. After hybridization to a C0f of 20,000, the DNA was diluted to 50 µg/ml, and the solution was applied to a 1.0-ml column of packed hydroxyapatite maintained at 60° in 0.006 M NaPB (pH 6.8). These are the correct conditions established by Martinson and Wagenaar (8) for monitoring thermal stability of nucleic acids on hydroxyapatite. Using these conditions, approximately 95% of the cellular DNA remains bound to the column. While at 60°, the column was washed twice with 10-ml samples of 0.12 M NaPB (pH 6.8) containing 0.01% SLS. This procedure was repeated at 5° increments until a temperature of 100° was attained. The thermal dissociation of the DNA-RNA hybrids was measured by monitoring the absorbance at 260 nm in the effluent fractions, and that of the radioactivity-labeled RNA-DNA hybrids was measured by determining the trichloroacetic acid-precipitable cpm in the effluent fractions.

RESULTS

Immunocytochemistry. Lungs and spontaneous mammary tumors from C3H/He mice were examined by conventional histological techniques and by immunoperoxidase staining for MuMTV antigens. The 8 mammary tumors from 7 animals available for examination. Metastases were detected, however, in only 1 of 4 lungs sampled by random biopsy; 3 of these 4 identical lung samples, on the other hand, were positive when assayed by the nucleic acid hybridization procedure described below. Pulmonary metastases appeared as masses within the pulmonary arteries or as expansile masses within the pulmo.
nary parenchyma (Figs. 1 and 2). The metastatic cells frequently extended through the wall of the blood vessel (Fig. 1). There was no evidence of individual tumor cells lodged in blood vessels. No cells outside the tumor mass were positive for MuMTV antigens. Multiple metastatic foci were present in most positive sections. The tumor masses, when present, occupied as much as 10 to 20% of the cross-sectional area of the section. The histological pattern of the metastases closely resembled that of the primary tumor. MuMTV antigens were identified by the immunoperoxidase technique in each of the metastases observed.

**Hybridization of MuMTV(C3H) Radioactive 60 to 70S RNA to Murine DNA's.** We have previously shown that MuMTV(C3H) radioactive 60 to 70S RNA hybridized approximately 55% to the DNA's of spontaneous C3H/He mammary tumors and 49% to the DNA's of apparently normal livers of these same animals or other C3H/He mice free of mammary tumors (5). In an extension of these studies, the DNA's from the livers and lungs of 4 apparently normal C3H/He mice and 4 C3H/He mice bearing mammary tumors were hybridized to $^{125}$I-labeled 60 to 70S RNA to MuMTV(C3H). Using this probe (Table 1), one cannot distinguish between DNA's obtained from livers of tumor-bearing and apparently normal animals or between livers and lungs from tumor-bearing animals.

**Hybridization of Radioactive MuMTV(C3H) TA-Sequence RNA to Murine DNA's.** Previous studies have shown that radioactive MuMTV(C3H) TA-sequence RNA hybridizes approximately 55% to the DNA's of spontaneous mammary tumors of C3H/He mice but does not hybridize above the 6% background level to livers of C3H/He mice bearing mammary tumors or to livers of apparently normal C3H/He mice (5). In an extension of these studies, it is observed that $^{125}$I-labeled TA-sequence RNA also failed to hybridize to hearts, lungs, kidneys, brains, and livers of each of 5 apparently normal C3H/He mice (Table 2). DNA's of Animals 1, 2, 3, and 4 were the same as those shown in Table 1, thus ruling out the possibility that the lack of hybridization was due to the quality of the DNA's. As a further control, this same $^{125}$I-labeled TA-sequence RNA was annealed to DNA's of various internal organs of C3H/He mice bearing spontaneous mammary tumors, 6 of 8 lungs gave a positive hybridization (Table 3), whereas all hearts, kidneys, brains, and livers scored negative. The lack of detection of TA sequences in the DNA's of other highly vascularized organs, such as liver, provides evidence that the hybridization to lung DNA is not simply detecting circulating tumor cells.

**Table 1**

<table>
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<th>Mouse no.</th>
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% of hybridization to individual mice

- **Heart**: 4.4% to 5.2% to 4.3% to 3.8%
- **Lungs**: 38% to 5.4% to 5.4% to 4.1% to 1.5%
- **Kidney**: 5.1% to 5.4% to 5.8% to 4.1% to 1.5%
- **Brain**: 5.2% to 4.6% to 3.8% to 4.4% to 2.9%
- **Liver**: 3.9% to 3.1% to 6.0% to 3.9% to 5.1%

Table 2

| % of hybridization to individual organs of apparently normal C3H/He mice |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| DNA                        | Heart | Lungs | Kidney | Brain | Liver | Tumor |
| 1                          | 4.4   | 5.2   | 4.3   | 3.8   | 4.1   | 3.7   |
| 2                          | 3.8   | 5.1   | 4.1   | 1.5   | 4.4   | 4.6   |
| 3                          | 5.1   | 5.4   | 5.8   | 4.1   | 1.5   | 4.4   |
| 4                          | 5.2   | 4.6   | 3.8   | 4.4   | 2.9   | 3.3   |
| 5                          | 3.9   | 3.1   | 6.0   | 3.9   | 5.1   | 3.8   |

% of hybridization to individual mice

Table 3

| % of hybridization to individual organs of tumor-bearing C3H/He mice |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| DNA                        | Heart | Lungs | Kidney | Brain | Liver |
| 1                          | 4.6   | 3.9   | 5.1   | 5.2   | 5.2   | 5.2   |
| 2                          | 1.3   | 10.6  | 10.6  | 14.7  | 26.2  | 26.2  |
| 3                          | 4.0   | 3.3   | 4.9   | 3.4   | 5.7   | 5.7   |
| 4                          | 4.2   | 5.1   | 5.7   | 3.2   | 2.6   | 5.3   |
| 5                          | 4.6   | 5.5   | 5.2   | 5.7   | 4.6   | 5.3   |
| 6                          | 45.1  | 53.6  | 56.9  | 54.2  | 60.3  | 51.9  |
| 7                          | 49.3  | 46.9  | 46.9  | 49.3  | 46.9  | 46.9  |

% of hybridization to individual mice

- **Heart**: 4.6% to 5.1% to 5.2% to 5.9% to 5.2% to 5.2% to 5.2%
- **Lung**: 1.3% to 10.6% to 10.6% to 14.7% to 26.2% to 26.2% to 28.4%
- **Kidney**: 4.0% to 3.3% to 4.9% to 3.4% to 5.7% to 5.7% to 4.5%
- **Brain**: 4.2% to 5.1% to 5.7% to 3.2% to 2.6% to 5.3% to 3.8%
- **Liver**: 4.6% to 5.5% to 5.2% to 5.7% to 4.6% to 5.3% to 3.2%
- **Tumor**: 45.1% to 53.6% to 56.9% to 54.2% to 60.3% to 51.9% to 49.3% to 46.9%

Table 3

**Hybridization between radioactive MuMTV TA-sequence RNA and individual organs of mammary tumor-bearing C3H/He mice**

Hybridization was performed as described in "Materials and Methods." In an extension of these studies, the DNA's from the livers and lungs of C3H/HeJ mice but does not hybridize above the 6% background level to livers of C3H/He mice bearing mammary tumors or to lungs of apparently normal C3H/He mice (5).

**Kinetic Analysis of MuMTV TA Sequences in Lung DNA's.**

Kinetics of hybrid formation was analyzed in an attempt to determine the frequency of proviral sequences in DNA's of lungs positive for sequences homologous to MuMTV TA-sequence RNA. One hundred μg of DNA from a positive lung were hybridized with 1000 cpm of $^{125}$I-TA-sequence RNA and were incubated for various time intervals. As is seen in Chart 2, an accurate $C_{0f/2}$ could not be obtained, since the percentage of hybridization was still rising at a $C_{0f}$ of 50,000. For comparison, $^{125}$I-labeled polyadenine-selected normal murine DNA (5) was hybridized to the same DNA under identical conditions; a $C_{0f/2}$ of 3800 was obtained (Chart 2, arrow; Ref. 5). When compared to this value for "unique" sequence RNA, it can be estimated that the DNA of these lung tissues contains less than one copy of TA-sequence proviral DNA per haploid genome. When compared to the $C_{0f/2}$ of 380 obtained between $^{125}$I-TA-sequence RNA and mammary tumor DNA (5), it can be estimated that there is a maximum value of...
Detection of Mammary Cell Metastases

In an attempt to determine the approximate sensitivity of the hybridization method to detect mammary tumor cells in pulmonary tissue, mixing experiments were performed. Lungs from apparently normal C3H/He mice were trypsinized, and individual lung cells were counted and mixed in various ratios with C3H mammary tumor cells. Cells from a mammary tumor cell line were used to eliminate possible errors in numbers of mammary tumor cells due to the existence of normal mammary stroma and infiltrate. DNA's were extracted from the various mixtures of lung and mammary tumor cells, and these were then hybridized to MuMTV(C3H) TA-sequence 125I-RNA. As seen in Chart 3, hybridization above background levels could be detected in ratios of at least 1 mammary tumor cell/250 normal lung cells.

DISCUSSION

Pulmonary metastases are the most frequent form of distal metastases detected in patients with breast cancer, being found in 60 to 65% of autopsies. The murine model presents an excellent system to study metastatic spread of mammary cells to lung tissue in that various studies have reported that between 2 and 95% of animals with spontaneous mammary tumors possess pulmonary metastases. The tumor-bearing C3H/He females studied here had typical MuMTV-positive mammary adenocarcinomas and intrapulmonary masses. Since the masses were intravascular, MuMTV antigen positive, and histologically similar to the mammary cancer, they most probably originated in the mammary tumor. The lesions were expansive and invasive, indicating active growth in situ rather than passive entrapment of circulating tumor cells. Further, individual antigen-positive tumor cells were not found in the lung. The pathological diagnoses of pulmonary metastases from primary mammary tumors were reinforced by the obser-
viation of TA DNA in the lungs of only tumor-bearing mice and by the lack of TA DNA sequences in other heavily vascularized organs such as the liver (Table 3).

The clear evidence of blood-borne metastases found here deserve mention. Although rarely emphasized, blood-borne metastases are a major source of distant metastases in human breast cancer (6). Thus, this aspect of the human disease has a biological counterpart in the murine mammary tumor model.

Three of the random biopsies studied here had no histologically identifiable tumor cells. Since the same lungs contained TA DNA, occult tumor cells were present. These observations point out the sampling error inherent in random biopsies and underline the usefulness of the hybridization technique described here.

The nucleic acid hybridization studies reported here conceptually add a new dimension in the detection of metastatic spread. To our knowledge, it is the first attempt to use the technique of molecular hybridization to such an end. There are, of course, several pros and cons to the hybridization methodology. The disadvantages include: (a) the availability of a specific radioactive probe to detect unique nucleic acid sequences in tumor cells; (b) mammary tumors from different animals may vary in their MuMTV proviral sequence copy number; (c) the need of highly trained personnel and specialized reagents to conduct nucleic acid hybridization experiments; and (d) like any other biochemical detection technique, hybridization only detects the presence of a marker (DNA) associated with cancer; it does not reveal the biological behavior or potential of the cells carrying the DNA. Some of the advantages of this technique include: (a) the high degree of sensitivity; (b) the specificity of the hybridization reaction; and (c) the large amounts of a given tissue that can be tested in one assay. This latter property reduces the possibility of missing metastatic cells in some tissues due to sampling error when only a part of a given tissue is examined histologically.

The time factor involved in the molecular hybridization procedure (approximately 10 days from start to finish due to incubation times; see "Materials and Methods") is of course much slower than histological or immunocytochemical techniques, but it is much more rapid than transplant bioassay techniques. In summary, the results reported here provide an adjunct and predictive element to the study of metastatic spread in a defined animal model; furthermore, these studies conceptually indicate that, with the proper nucleic acid probe, molecular hybridization may be used as a sensitive and specific adjunct for the study of metastatic lesions.

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REFERENCES

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