Biochemical Characterization of Putative Subviral Particulates from Human Malignant Breast Tumors

R. Michalides, S. Spiegelman, and J. Schlom

SUMMARY

Particulates with the properties of cores and/or ribonucleoproteins of RNA tumor viruses have been isolated from Sterox-SL-treated fractions of murine and human mammary adenocarcinomas. These particulates have an RNA-directed DNA polymerase, a 60 to 70 S RNA, and a density of 1.26 g/ml or greater in sucrose equilibrium density gradients. Their uniquely higher densities lead to banding in regions comparatively free of cellular contaminants. These circumstances minimize some of the technical complications of performing the simultaneous detection assay in the presence of cell debris.

INTRODUCTION

Biochemical studies have indicated the presence of a putative oncornavirus in certain human milks (3, 4, 8, 10, 12, 15, 18, 19). Recently, a technique using phospholipase C was used to isolate and characterize particulates from human milk that have the biochemical properties characteristic of cores and ribonucleoproteins of RNA tumor viruses (7). These particulates have a density of 1.23 g/ml or greater in sucrose and contain a reverse transcriptase in association with a 60 to 70 S RNA. The phospholipase C treatment provided a more sensitive method for direction of particulates by minimizing the difficulties generated by the presence of cell-associated debris often found in the “viral” region of density equilibrium gradients.

We report here a modification of the method of Bolognesi et al. (2), using the nonionic detergent Sterox-SL, to isolate putative subviral particulates from murine and human malignant mammary adenocarcinomas. The particulates have the biochemical properties diagnostic of cores and ribonucleoproteins of RNA tumor viruses, i.e., a density of 1.26 g/ml or more in sucrose, and an RNA-directed DNA polymerase in association with a 60 to 70 S RNA. Because of their uniquely higher densities, subviral particulates band at regions comparatively free of cellular contaminants, thus minimizing some of the technical difficulties of the enzyme assays. In addition, the data add further information relevant to characterizing the particles previously identified in human breast cancers.

MATERIALS AND METHODS

Tissues. Murine mammary tumors were obtained from Paris RIII mice [Meloy Laboratories (Springfield, Va.) and Columbia University colonies]. Lactating mammary glands were obtained from normal NIH Swiss mice (Life Sciences, Inc., St. Petersburg, Fla.). Human malignant and benign breast tumors were obtained from HEM Research, Inc., Rockville, Md., via the Office of Resources and Logistics of the Virus Cancer Program, National Cancer Institute. All tissues were stored at -70°C.

Tissue Fractionation and Particulate Isolation. Approximately 8 g of mouse mammary tumor tissue (from 3 to 8 RIII tumors) or 25 g human breast tumor tissue (pools of 3 tumors) were thawed and finely minced at 4°C. Cells were dispersed in a Silverson homogenizer (maximum speed) at 4°C in 2 volumes 5% sucrose (w/w) in TNE2 buffer. The suspension was centrifuged at 4,000 x g for 10 min at 4°C, and the resulting supernatant was centrifuged at 10,000 x g for 10 min at 4°C. Trypsin (Worthington Biochemical Corp., Freehold, N. J.) was added to the resultant supernatant at 1 mg/ml final concentration and incubated at 37°C for 30 min, after which lima bean trypsin inhibitor (Worthington) was added to a final concentration of 1 mg/ml. The mixture was then layered on a 10-mL column of 20% glycerol in TNE and centrifuged at 90,000 x g for 1 hr. This and all subsequent centrifugations were at 4°C in a Spinco SW 27 rotor, unless otherwise indicated. The resulting pellet was resuspended in 3-mL TNE, layered on a linear gradient of 20–50% sucrose (w/w) in TNE buffer containing 0.1 mM DTT and centrifuged at 90,000 x g for 3 hr. The gradient was dripped from below, and 20 equal fractions were collected. Fractions corresponding to densities of 1.15 to 1.20 g/ml were pooled, diluted with 0.01 M Tris-HCl (pH 8.3), and centrifuged at 90,000 x g for 1 hr. The resulting pellet was resuspended in 0.01 M Tris-HCl (pH 8.3) to a final protein concentration of 10 mg/ml (determined using the absorbance ratio of 280 and 260 nm of a sample, which was made to 0.1% sodium dodecyl sulfate). One-half of the sample volume was assayed by the simultaneous detection test (17) for the presence of a 60 to 70 S RNA-directed DNA polymerase. The rest of the sample was brought to 0.1 M DTT and 0.1

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2 The abbreviations used are: TNE, 0.01 M Tris-HCl, pH 8.3, 0.15 M NaCl, and 0.01 M EDTA; DTT, dithiothreitol.
oligo-dT(12) has been shown to increase the efficiency of equal 50-μl aliquots (A, B, and C). Each aliquot was Simultaneous Detection Test. Pellets obtained after equilibrium density gradient centrifugations of tumor extracts were resuspended in 0.01 M Tris-HCl, pH 8.3, and divided into 3 equal 50-μl aliquots (A, B, and C). Each aliquot was incubated for 10 min at 4° in 0.01 M Tris-HCl, pH 8.3, and centrifuged at 98,000 × g for 16 hr. The gradient was dripped from below into 20 equal fractions. For certain indicated experiments, samples were subjected to equilibrium density gradient analysis, using a continuous 10 to 40% CsCl gradient (w/v in 0.01 M Tris-HCl, pH 8.3, containing 0.1 M DTT). In this case the gradient was centrifuged for 2 hr at 40,000 rpm in a Spinc0 SW 41 rotor. Fractions corresponding to densities indicated in “Results” were pooled, diluted with 0.01 M Tris-HCl (pH 8.3), and centrifuged at 98,000 × g for 1 hr. The resultant pellets were resuspended in 150 μl of 0.01 M Tris-HCl (pH 8.3) and assayed by the simultaneous detection test for the presence of a 60 to 70 S RNA-directed DNA polymerase.

Assay of 60 to 70 S RNA-directed DNA Polymerase: Simultaneous Detection Test. Pellets obtained after equilibrium density gradient centrifugations of tumor extracts were resuspended in 0.01 M Tris-HCl, pH 8.3, and divided into 3 equal 50-μl aliquots (A, B, and C). Each aliquot was incubated for 10 min at 4° in 0.33% NP-40 detergent (Shell Chemical Co., New York, N. Y.), 0.1 M DTT, and 0.2 mg actinomycin D per ml. Aliquots A and B were then added to a standard endogenous DNA polymerase reaction mixture (100-μl final volume) containing 5.0 μmoles Tris-HCl (pH 8.3), 0.8 μmole of MgCl2, 1 μmole of NaCl, 0.16 μmole each of dGTP, dCTP, dATP, 0.3 μCi of [3H]TTP (50 Ci/m mole), and 20 μg of oligo-dT10-12. Addition of the primer oligo-dT10-12 has been shown to increase the efficiency of the RNA-directed DNA polymerase of known animal tumor viruses (6). RNase A (Sigma Chemical Co., St. Louis, Mo.), preheated at 80° for 10 min in 0.9 M NaCl, 0.09 M sodium citrate, pH 7.0, to destroy DNase activity, was also added to Aliquot B to a final concentration of 150 μg/ml. Aliquot C was added to the standard reaction mixture, with the exception that dGTP was omitted.

Pellets, obtained after equilibrium centrifugation of Sterox-SL-treated samples, were subjected to the same assays, with the exception that no NP-40 detergent was added in the preincubation mixture.

All polymerase reactions were incubated at 37° for 15 min and were terminated by the addition of NaCl and sodium dodecyl sulfate to final concentrations of 0.4 m and 1%, respectively. After the addition of an equal volume of a phenol:chloroform mixture (7:1) containing hydroxyquinoline (0.185 g/100 ml mix), the mixture was shaken at 25° for 5 min and centrifuged at 5000 × g for 5 min at 15°. The aqueous phase was then layered over a preformed 10 to 30% linear glycerol gradient in TNE and centrifuged at 40,000 rpm for 3 hr at 4° in a SW 41 Spinco rotor. External markers used were [3H]-labeled 70 S avian myeloblastosis virus RNA and tritium-labeled 28 S and 18 S RNA from NC-37 cells. Fractions were collected from below and assayed for acid-precipitable radioactivity as previously described (20).

RESULTS

Subviral Particulates from Mouse Mammary Tumors. RIII mouse mammary tumors, previously shown to contain mouse mammary tumor virus RNA (11, 16), were minced, homogenized, and subjected to velocity and sucrose equilibrium gradient centrifugation as described in “Materials and Methods.” Resulting gradients were divided into 5 distinct density regions (between densities 1.15 and 1.30 g/ml). Material from each region was diluted and centrifuged at 90,000 × g for 1 hr, and the resulting pellets were assayed for endogenous RNA-directed DNA polymerase and 60 to 70 S RNA by the simultaneous detection test (17). As seen in Chart 1, there were several density regions with evidence of de novo DNA synthesis. Although some of the DNA synthesized is in the 60 to 70 S region (Chart 1, C, D, and E), the results are not at all conclusive. This may be due to the presence of RNase and other degradative enzymes in these density regions of these tumor preparations. Treatment of the 1.15 to 1.20 g/ml density region with phospholipase C at concentrations of 0.01 to 0.1 mg/ml, and recentrifugation as described (7), proved unsuccessful in increasing the sensitivity of the assay.

The biochemical demonstration of virus in these tumor preparations can be made more certain, however, by the use of Sterox-SL for the generation of subviral particulates, i.e., cores, ribonucleoproteins, or transitional forms with some of the core membrane removed. One-half of the original material banding between densities of 1.15 and 1.20 g/ml (density of mouse mammary tumor virus in sucrose is approximately 1.18 g/ml) was pelleted, treated with Sterox-SL, as described in “Materials and Methods,” and subjected to sucrose equilibrium gradient centrifugation. Resultant fractions were pooled into the same 5 distinct density regions as described above, and each fraction was diluted, pelleted at 90,000 × g, and assayed by simultaneous detection test. As can be seen in Chart 1F, there is no longer any DNA synthesis on a high-molecular-weight template in the “viral” (1.15 to 1.20 g/ml) region of the gradient; this activity has now shifted to the 1.28 to 1.30 g/ml subviral particulate density region of the gradient (Chart 1F).

To demonstrate that the distinct DNA peak observed in the 60 to 70 S region (Chart 1F) is due to a DNA synthesis using heteropolymeric 60 to 70 S RNA as a template, parallel reactions were carried out in the absence of deoxyribonucleoside triphosphate (dGTP) and with the addition of RNase; both of these reactions were negative (Table 1).

A titration of the NP-40 detergent, used in the subviral particulate endogenous DNA polymerase reaction mixture, revealed that no NP-40 is required. Final NP-40 concentrations of 0.02 and 0.2% in the preincubation mixture led to DNA synthesis on a 60 to 70 S RNA template of 50 and 60% less cpm, respectively.

Fractions from lactating mammary glands of NIH Swiss mice, a strain whose milk is believed to be free of mouse mammary tumor virus (5, 13), were prepared and tested as described above for the RIII tumors. The 1.15 to 1.20 g/ml viral regions of gradients were negative for particulates with

1004 CANCER RESEARCH VOL. 35
Characterization of Putative Subviral Particulates

Table 1

<table>
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<tr>
<th>Density Range</th>
<th>% of Total Reaction</th>
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<td>60-70 S region</td>
<td>100</td>
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revert transcriptase and 60 to 70 S RNA. Furthermore, when the 1.15 to 1.20 g/ml density regions were treated with Sterox-SL and then subjected to equilibrium density gradient centrifugation, the resulting subviral particulate regions (1.23 g/ml and greater) were also negative.

To demonstrate that the shift of the simultaneous detection activity observed in the RIII tumor fractions after Sterox-SL treatment was indeed due to the conversion of mouse mammary tumor virions to subviral particulates of mouse mammary tumor virus, immunological tests were performed. Antisera to purified, disrupted mouse mammary tumor virus from RIII milk, adsorbed against BALB/c milk, were tested in micro Ouchterlony plates against sucrose equilibrium density gradient-purified fractions of RIII tumors. The 1.15 to 1.20 g/ml density regions before Sterox-SL treatment and the 1.23 g/ml or greater density regions after Sterox-SL treatment were both positive and displayed a line of identity with cores prepared from RIII mouse milk (R. Michalides, S. Spiegelman, and J. Schlam, unpublished data).

Putative Subviral Particulates from Human Breast Tumors. The technology developed for the preparation of subviral particulates of mouse mammary tumor virus from murine mammary tumors was applied to detect similar particulates in human malignant adenocarcinoma tissue. Human breast tumors were treated in an identical manner, as were the murine mammary tumors. Following mincing, homogenization, and velocity sucrose equilibrium gradient centrifugation, individual density regions from 1.15 to 1.30 g/ml were tested by the simultaneous-detection assay. None of 9 human tumor samples prepared in this manner yielded a distinct peak of DNA in the 60 to 70 S region of glycerol velocity gradients in any of the density fractions tested. However, positive results were obtained when the material banding between the densities of 1.15 to
1.20 g/ml in the sucrose equilibrium gradient was treated with Sterox-SL and centrifuged to equilibrium in a 20 to 50% sucrose gradient. The resulting density regions were concentrated and assayed by the simultaneous detection test. Using this Sterox-SL treatment method, particulates with densities of 1.26 g/ml or greater that contain a reverse transcriptase in association with a 60 to 70 S RNA were observed. Chart 2 illustrates the results obtained from such tumor preparation. The trichloroacetic acid-precipitable [3H]TTP in the 60 to 70 S region of these glycerol gradients was eliminated when RNase was added to reaction mixtures (Table 2). The omission of one of the deoxynucleoside triphosphates (dGTP) also greatly reduced these peaks.

When RNase-treated reaction products were analyzed by glycerol velocity sedimentation, acid-precipitable [3H]TTP was observed only in the low-molecular-weight regions (approximately 4 to 20 S) and not in the 60 to 70 S region as observed in the reactions not treated with RNase.

In one case, the 60 to 70 S RNA-directed DNA polymerase activities after Sterox-SL treatment were observed at densities less than 1.26 g/ml. This is shown in Chart 3D where the resulting activity is at 1.20 to 1.24 g/ml. This may be due to the partial removal of membranes from putative viral particles. Material banding at densities 1.24 to 1.30 g/ml of this tumor preparation was also concentrated and was negative for 60 to 70 S RNA-directed DNA polymerase activity.

<table>
<thead>
<tr>
<th>Tumor Sample A</th>
<th>6H cpmin in 60-70 S region %</th>
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<tbody>
<tr>
<td>Endogenous reaction</td>
<td>205</td>
</tr>
<tr>
<td>Plus RNase A (150 µg/ml)</td>
<td>1</td>
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<tr>
<td>Minus dGTP</td>
<td>52</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumor Sample B</th>
<th>6H cpmin in 60-70 S region %</th>
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<tbody>
<tr>
<td>Endogenous reaction</td>
<td>585</td>
</tr>
<tr>
<td>Plus RNase (150 µg/ml)</td>
<td>10</td>
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Chart 2. Simultaneous detection activity in Sterox-SL-treated fractions of human malignant breast tumors. Twenty-five g of human breast adenocarcinoma tissue were minced, homogenized, clarified, and treated with trypsin, as described in “Materials and Methods.” All density regions tested before Sterox-SL treatment by the simultaneous detection assay were negative. One-half of the material banding between densities of 1.15 and 1.20 g/ml was concentrated, treated with Sterox-SL, and centrifuged at 90,000 x g for 3 hr, the gradient was dripped from below and 20 equal fractions were collected. Fractions within regions of the indicated densities (A to C) were pooled and one-half of each region was concentrated and assayed by the simultaneous-detection test as described in “Materials and Methods.”

Chart 3. Conversion of simultaneous detection activity in fractions of human malignant breast tumors from viral to subviral particulate density regions. Twenty-five g of human malignant breast tumors (a pool of 3 tumors) were minced, homogenized, and clarified, and the resulting 10,000 x g supernatant was trypsin treated, as described in “Materials and Methods.” Following centrifugation at 90,000 x g, the resulting pellet was resuspended and layered over a 20 to 50% linear sucrose gradient. After centrifugation at 90,000 x g for 16 hr in a linear 20 to 70% sucrose gradient. Results are given as acid-precipitable [3H]TTP in the 60 to 70 S region of resulting 10 to 30% glycerol velocity gradients.

Properties of the DNA polymerase of putative subviral particulates from human malignant breast tumors

Each tumor sample consisted of 25 g of human malignant breast tissue (from 3 tumors). Tumors were minced, homogenized, and subjected to velocity and sucrose equilibrium gradient centrifugation as described in “Materials and Methods.” Material banding between densities 1.15 and 1.20 g/ml was concentrated, treated with Sterox-SL, and again subjected to sucrose equilibrium density gradient centrifugation. Material at densities of 1.23 g/ml or greater was then concentrated and divided into 3 equal aliquots. Each aliquot was treated as indicated and assayed by the simultaneous-detection test, as described in “Materials and Methods.” Results shown are acid-precipitable [3H]TTP above gradient background (20 cpn) in the 60 to 70 S region of resulting 10 to 30% glycerol velocity gradients.
Human breast adenocarcinoma fractions were also analyzed after density equilibrium gradient centrifugation in cesium chloride. No activity was found (Chart 4) in various density regions before Sterox-SL treatment. After Sterox-SL treatment of material in the 1.20 to 1.24 g/ml density region [the density of mouse mammary tumor virus in CsCl is approximately 1.22 g/ml (14)], and recentrifugation in CsCl to equilibrium, the 60 to 70 S RNA-directed DNA polymerase activity was now observed at densities between 1.40 and 1.44 g/ml (Charts 4 and 5). Subviral particulates of avian myeloblastosis virus (9) and mouse mammary tumor virus also band at approximately this density in CsCl.

Table 3 is a composite of results obtained from Sterox-SL-treated fractions of human malignant breast tumors when tested by the simultaneous detection assay. Given are the [3H]DNA cpm in the 60 to 70 S regions of glycerol velocity gradients, as well as gradient backgrounds. Six of 9 pooled tumor preparations (Table 3, Samples 1 to 3, 6, 8, and 9) clearly showed de novo synthesis of DNA, which subsequently sediments at 60 to 70 S. Pools of 3 tumors were used to provide adequate quantities. Therefore, no statement can be made on the frequency of positive tumors.

No 60 to 70 S RNA-directed DNA polymerase activity was observed in fractions of 12 (4 pools) human benign breast tumors or normal “nonlactating” breast tissue when tested either before or after Sterox-SL treatment. In none of these cases was acid-precipitable [3H]TTP in the 60 to 70 S region of resulting 10 to 30% glycerol velocity gradients.

**Chart 4.** Cesium chloride equilibrium density gradient analysis of the putative subviral particulates from human malignant breast tumors. Twenty-five g of human malignant breast tumor tissue were treated as described in the legend to Chart 4. Material banding between densities of 1.40 to 1.44 g/ml after Sterox treatment and CsCl density gradient analysis was concentrated and assayed by the simultaneous detection test as described in “Materials and Methods.”

**Table 3.** Assay of extracts from human malignant breast tumors for putative subviral particulates by the simultaneous detection test

<table>
<thead>
<tr>
<th>Sample</th>
<th>[3H] cpm in 60-70 S region</th>
<th>Gradient background</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>233</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>4418</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>553</td>
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<td>4</td>
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<td>6</td>
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<td>8</td>
<td>351</td>
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</tr>
<tr>
<td>9</td>
<td>568</td>
<td>19</td>
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</tbody>
</table>
DISCUSSION

The studies reported here provide biochemical evidence for the presence of an RNA tumor virus in purified fractions of human malignant breast tumors. These particles are similar to the subviral particulates of mouse mammary tumor virus isolated from identical fractions of murine mammary tumors. Furthermore, they exhibit several features diagnostic of cores and ribonucleoproteins of oncornaviruses, namely, (a) an RNA-instructed DNA polymerase in association with a 60 to 70 S RNA template and (b) a density of 1.26 g/ml or greater in sucrose, and a density of 1.40 g/ml or greater in CsCl, after treatment with Sterox-SL. No such particulates were observed in similar extracts from human benign breast tumors.

Comparison of Charts 1, 3, and 4 reveals that the simultaneous detection of 60 to 70 S RNA and reverse transcriptase is improved by prior treatment of the material banding at 1.15 to 1.20 g/ml with Sterox followed by another equilibrium density gradient centrifugation. This may be due to the elimination of contaminating cell debris which bands at 1.15 to 1.20 g/ml.

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REFERENCES

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