Ribosomal RNA on the Surfaces of Nonleukemic Mouse Ascites Tumor Cells

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ABSTRACT. RNAs on the cell surfaces of two nonleukemic and two leukemic strains of mouse ascites tumor cells were studied by fractionating the RNAs released from the cell surface by gentle pronase treatment after sucrose density gradient centrifugation, by indirect membrane immunofluorescence that used anti-RNA antibody and by cell electrophoresis.

RNA was extracted from the cell supernatants of Ehrlich ascites tumor and sarcoma 180 cells (nonleukemic) that had been treated or not treated with pronase (1 µg/ml, 37°C, 20 min) followed by sucrose density gradient centrifugation. It was clearly demonstrated that the amounts of ribosomal RNA (18S and 28S) released after pronase treatment were approximately 80% greater than that of nonpronase-treated cells. Ehrlich ascites tumor cells that had been treated with actinomycin D (100 µg/kg body weight of mouse, 16 h) in vivo released an amount of ribosomal RNA after pronase treatment only 20% greater than the value for untreated cells. Actinomycin D treatment greatly reduced both the cell surface negative charge and the cell surface immunofluorescence when rabbit anti-RNA antibody was used. Under the same experimental conditions with actinomycin D, only ribosomal RNA synthesis showed preferential inhibition, not the syntheses of poly A-containing messenger RNA, 4S or other small-size RNAs.

In contrast, L1210 and C1498 cells (leukemic) showed no change in the amounts of ribosomal RNA released after pronase treatment. L1210 cells also showed no change in the surface negative charge after being treated with actinomycin D. These results suggest that the RNA on the surfaces of nonleukemic ascites tumor cells is composed predominantly of 18S and 28S ribosomal RNA and that these RNAs might be responsible, in part if not completely, for the surface negative charges on nonleukemic tumor cells.

Electrophoretical studies have shown that the negative surface charges of Ehrlich ascites tumor cells (7, 18) and osteogenic sarcoma cells (8, 17) decrease after treatment with RNase. Recent biochemical and immunological studies have shown that these RNase-susceptible, negatively charged groups on the surfaces of such nonleukemic ascites tumor cells as Ehrlich ascites tumor, sarcoma 180 and ascites hepatoma AH66F

Abbreviations used: TBS, tris-buffered saline (10 mM Tris-HCl buffer, 137 mM NaCl, 3 mM KCl, 1 mM CaCl2, and 0.5 mM MgCl2, pH 7.0); FITC, fluorescein isothiocyanate; AMD, actinomycin D; PBS, phosphate-buffered saline (10 mM phosphate buffer, 137 mM NaCl, 3 mM KCl, 1 mM CaCl2, and 0.5 mM MgCl2, pH 7.0).
are RNAs (15) that increase with the suppression of oxygen uptake when glucose is added to the cell suspension (9, 14), a phenomenon known as the Crabtree effect (4). Normal mouse thymic cells and mouse leukemic ascites cells such as L1210, C1498, and T-3-C1-2, which show no Crabtree effect, have no RNA on their surfaces and do not change their surface negative charges on the addition of glucose (9, 14). Presumably, the RNAs on cell surfaces have a marked effect on the surface negative charge in certain types of tumor cells (9). RNA on the surface of Ehrlich ascites tumor cells has been shown to be a component of the cell surface membrane and to be released when the cells are treated with pronase (16).

To determine the type of RNA on the surfaces of nonleukemic ascites tumor cells, we used two types of nonleukemic ascites tumor cells and two types of leukemic ascites cells that had been treated or not treated with pronase. We then extracted the RNAs from the cell supernatants and put them through sucrose density gradient centrifugation.

**MATERIALS AND METHODS**

**Cells.** Two types of ascites tumor cells were used; leukemic and nonleukemic. The leukemic strains were L1210 lymphoid (3) and C1498 myeloid cells (2). Both strains were grown in male BDF1 mice. The nonleukemic tumor strains were Ehrlich ascites tumor and sarcoma 180 cells, both of which were grown in female ddY mice. All the ascites tumor strains were maintained by weekly i.p. inoculation of the mice with 2 × 10⁶ cells.

**Cell preparation.** Ascites tumor cells were removed from mice 5 days after an i.p. inoculation of 2 × 10⁶ cells in saline and were washed 3 times with TBS at 4°C by centrifugation for 5 min at 80 × g, after which they were resuspended in cold TBS. Cells were used within 40 min of removal. The viability of each ascites tumor strain was more than 99%, as determined by the nigrosine exclusion test.

**Pronase treatment of cells.** Cells were treated with pronase (RNase free, Calbiochem-Behring Corp., La Jolla, autodigested at 37°C for 2 h) at a concentration of 1 µg/ml 5 × 10⁷ cells in silicone-coated, 10 ml glass tubes for 10 min at 37°C with shaking to release the cell surface materials. They then were cooled quickly at 4°C and centrifuged at 160 x g for 5 min.

**Extraction of RNA from the supernatant released by pronase treatment.** Extraction of the RNA from the supernatant was based on the procedures of Izawa et al. (5). The supernatant from the centrifugation was centrifuged at 1,500 x g for 10 min, then its supernatant was centrifuged at 12,000 x g for 60 min. Sodium dodecysulfate (Nakarai Chemical Corp., Kyoto, Japan) was added to the final supernatant to make a final concentration of 0.1%, and the mixture was shaken vigorously for 10 min at room temperature. The same volume of 80% phenol containing 0.1% 8-hydroxyquinolin then was added and the mixture shaken for 10 min, after which it was centrifuged at 9,500 x g for 10 min. The same volume of 80% phenol containing 0.1% 8-hydroxyquinolin was added to the aqueous upper layer, after which shaking and centrifugation were performed as above. Two volumes of cold ethanol, were added to the final aqueous layer which then was stored at −20°C overnight.

The RNA precipitate was sedimented at 4,300 x g for 10 min then washed by centrifugation with 80% ethanol containing 0.1 M NaCl. The resulting precipitate was dissolved in a small amount of 0.1 M NaCl. After adding two volumes of ethanol, the solution was stored at −20°C for several hours. The RNA was precipitated again by centrifugation and dissolved in a small amount of 0.1 M NaCl. For RNA extraction from leukemic ascites cells,
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the RNA was treated with 1 M NaCl to remove small size RNAs (which do not precipitate in 1 M NaCl) before sucrose density gradient centrifugation. It has been shown experimentally that the RNAs were extracted from the control, pronase-treated and AMD-treated cell surfaces at the same rate.

Sucrose density gradient centrifugation. The RNA obtained was layered on a 5 to 20% linear sucrose density gradient in a centrifuge tube. This RNA was centrifuged at 46,500 × g for 16 h at 4°C in a Hitachi RPS-55 centrifuge. After centrifugation, 0.25 ml fractions were collected, and the absorption at 260 nm was measured for each tube.

AMD treatment. Five days after cell transplantation, mice received 100 µg/kg of body weight of AMD (Sigma Chemical Co., St. Louis, Mo.) i.p. Cells were removed from the mice 16 h after the AMD administration. Control mice were injected with the same volume of saline.

[3H] Uridine labeling of ascites tumor cells. Eight hours after the AMD injection, mice were labeled in vivo with 100 µCi of [3H]uridine (specific activity, 25.3 Ci/mmol, Amersham Corp., England). Ascites tumor cells were collected from these mice 0.5 or 2 h after labeling.

Isolation of RNA from cytoplasm. Cells were suspended in a hypotonic solution (2 mM MgCl₂) for 10 min at 4°C then homogenized in a Chaikoff homogenizer with an equal volume of solution containing 0.5 M sucrose and 2 mM MgCl₂, followed by centrifugation at 12,000 × g for 30 min. RNA was extracted from the supernatant formed as described above.

Fractionation of RNA into poly A- and nonpoly A-containing RNAs. Poly A-containing RNA was separated from nonpoly A-containing RNA by poly U Sepharose 4B affinity column chromatography and the methods of Lindberg (6). Poly U Sepharose 4B was prepared from poly U (Sigma) and cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method of Obchinnikov (11). The nonpoly A-containing RNA was separated into the 18S plus 28S RNA and the 4S plus other small size RNAs by 1 M NaCl precipitation.

Treatment of cells with RNase. Cells (2 × 10⁷) were incubated in 2 ml PBS pH 7 at 37°C for 30 min together with 100 µg/ml of RNase A from bovine pancreas (type 1-A, 5 times crystallized, protease free, Sigma) with continuous shaking. The enzyme-treated cells were cooled quickly to 4°C, washed once with cold PBS then resuspended in PBS for electrophoresis.

Cell electrophoresis. As described previously (14), cell mobility was measured in PBS at 4 ± 0.2°C in a microelectrophoresis apparatus (Sugiuura Lab., Inc., Tokyo, Japan) of the type originally described by Bangham et al. (1). The time taken to move a distance 35 μm in both directions in a cylindrical tube containing a 5.5 V/cm gradient was measured for 20 cells per sample.

Preparation of anti-RNA antibody. Anti-RNA antibody was prepared by immunizing rabbits with yeast total RNA-methylated bovine serum albumin as described elsewhere (15) by the method of Plescia et al. (13). Rabbit serum containing anti-RNA antibody was purified by affinity column chromatography with an RNA-Sepharose 4B column as described previously (15).

Indirect membrane immunofluorescence. As reported elsewhere (15), a membrane immunofluorescence technique, after Möller(10), was used to identify the anti-RNA, antibody-binding sites on the surfaces of the tumor cells.

RESULTS

Extraction of RNA from the surfaces of ascites tumor cells. To obtain RNA from
their cell surfaces, two nonleukemic ascites tumor (Ehrlich and sarcoma 180) cells and two leukemic ascites (L1210 and C1498) cells were treated with pronase. After cell sedimentation, the RNA in the supernatant was fractionated by sucrose density gradient centrifugation. The absorptions at 260 nm of the RNA fractions released from nonleukemic Ehrlich ascites tumor cell surfaces is shown in Fig. 1A. Peaks containing 18S and 28S RNA were 80% higher in the RNA extracted from L1210 cells was precipitated in 1 M NaCl before sedimentation.

Fig. 1. Sucrose zone sedimentation pattern of the RNA obtained from surfaces of Ehrlich ascites (A) and L1210 (B) tumor cells. RNA was extracted from the supernatants of ascites tumor cells that had been treated with pronase or from untreated cells. The RNA extracted from L1210 cells was precipitated in 1 M NaCl before sedimentation.

Fig. 2. Indirect membrane immunofluorescence of Ehrlich ascites tumor cells using rabbit anti-RNA antibody and FITC-conjugated anti-rabbit IgG. (A): control, (B): AMD-treated cells.
shown). The absorptions at 260 nm of the RNA fractions from leukemic L1210 cells is shown in Fig. 1B. There was no difference in the 18S and 28S RNA contents of the supernatants of pronase-treated and non-treated cells. C1498 cells gave the same results as L1210 cells (data not shown).

**Effects of AMD on the amounts of RNA on the surfaces of Ehrlich ascites tumor cells.** To analyze the RNA on the surfaces of nonleukemic ascites tumor cells, we investigated the effect of AMD. Immunofluorescence on the surfaces of Ehrlich ascites tumor cells that was produced by rabbit anti-RNA antibody and FITC-conjugated anti-rabbit IgG in nonAMD-treated (2A) and AMD-treated (2B) cells is shown in Fig. 2. AMD-treated cells showed markedly weaker surface fluorescence than nonAMD-treated ones.

The effects of AMD on the electrophoretic mobility of Ehrlich ascites tumor cells are presented in Fig. 3. Ehrlich ascites tumor cells that had been treated with AMD had lower surface negative charges than the nonAMD-treated controls. RNase treatment of nonAMD-treated Ehrlich ascites tumor cells produced a marked decrease in mobility, and RNase treatment of the AMD-treated Ehrlich ascites tumor cells decreased the mobility to that of the nonAMD-treated RNase treated cells. In contrast, L1210 cells showed no difference in the cell surface negative charges of AMD-treated cells and the nonAMD-treated controls (data not shown). Ehrlich ascites tumor cells that had been treated with AMD were, or were not, treated with pronase. RNA was extracted from their supernatants and put through sucrose density gradient ultracentrifugation.

The U.V. absorption spectra of the extracted RNAs is shown in Fig. 4. The amount of the RNA extracted by pronase treatment of AMD-treated cells was only 20% more than the values for the nonpronase-treated AMD-treated control cells; but, the RNA extracted by pronase treatment of the nonAMD-treated cells was 80% more than that from the nonAMD- and nonpronase-treated cells. These results show

![Graph showing electrophoretic mobility of Ehrlich ascites tumor cells](image)

**Fig. 3.** Changes in the electrophoretic mobility of Ehrlich ascites tumor cells due to AMD treatment. Bars indicate the S.E.
that AMD decreases the amount of RNA on the surfaces of Ehrlich ascites tumor cells. The absorption at 260 nm of the RNA fractions released from AMD-treated Ehrlich ascites tumor cells is shown in Fig. 5. Pronase treatment slightly increased the 18S and 28S RNAs over the value obtained for nonpronase-treated cells. This suggests that the amounts of 18S and 28S RNA on the surfaces of Ehrlich ascites tumor cells decreased because of AMD treatment.

Inhibition of RNA synthesis in Ehrlich ascites tumor cells by AMD. To determine what kind of RNA synthesis was inhibited in Ehrlich ascites tumor cells by AMD treatment, we labeled RNA with \(^{3}H\)uridine in vivo then extracted it from the cytoplasm of Ehrlich ascites tumor cells that had been treated, or not treated, with AMD.

Fig. 4. Ultraviolet light absorption spectra of the RNAs extracted from Ehrlich ascites tumor cells that had been treated with AMD then with RNase, and from untreated cells.

![Ultraviolet light absorption spectra](image)

Fig. 5. Sucrose zone sedimentation patterns of the RNA obtained from surfaces of AMD-treated Ehrlich ascites tumor cells.

![Sucrose zone sedimentation patterns](image)
The RNA extracted first was separated into poly A-containing messenger RNA and nonpoly A-containing RNA by poly U Sepharose 4B affinity column chromatography. Next, the nonpoly A-containing RNA was separated into the 18S and 28S RNAs and into the 4S and other small size RNAs by 1 M NaCl precipitation.

The tritium counts of the poly A-containing messenger RNA and the nonpoly A-containing RNA from AMD-treated and nonAMD-treated cells are given in Fig. 6A. The radioactivity of the 18S and 28S RNAs and of the 4S and small size RNAs from the nonpoly A-containing RNA are given in Fig. 6B. The results show that AMD treatment remarkably inhibited the syntheses of the 18S and 28S ribosomal RNAs, but that the syntheses of poly A-containing messenger RNA and other small size RNAs were not much inhibited.

**DISCUSSION**

RNAs have been shown to be present on the surfaces of nonleukemic ascites tumor cells but not on the surfaces of leukemic cells (15). Nonleukemic ascites tumor cells that have RNAs on their surfaces have been shown to form caps after incubation with anti-RNA antibody at 4°C followed by the elevation of temperature to 37°C. Pronase treatment of their cell surfaces completely inhibit their ability to react with anti-RNA antibody. It has been suggested that the RNAs on the surfaces of nonleukemic ascites tumor cells are linked to the membrane protein to which cytoskeletons are bound (16).

Immunological and electrophoretical studies have shown that the RNAs on the surfaces of nonleukemic ascites tumor cells are not adsorbed contaminants from lysed cells but are components of the cell surface membrane (15, 16). RNA was extracted from the cell surface of two types of nonleukemic cells by pronase treatment, after which the amount of RNA extracted was compared with that of cells not treated with pronase. After pronase treatment of the nonleukemic ascites tumor cells, 80% more RNA was extracted than from nontreated cells. In contrast, leukemic cells did not release more RNA after pronase treatment.

The RNA was fractionated after sucrose density gradient centrifugation, and the amounts of the 18S and 28S ribosomal RNAs were shown to have increased in the
supernatant from pronase-treated nonleukemic ascites tumor cells in comparison to nontreated cells, whereas the amounts of the 18S and 28S ribosomal RNAs did not increase in pronase-treated leukemic cells when compared with nontreated cells. Therefore, we concluded that the RNA on the surface of nonleukemic ascites tumor cells is ribosomal type RNA.

To validate our assumption, we extracted the RNA from AMD-(an inhibitor of RNA synthesis) treated Ehrlich ascites tumor cells. The results of our electrophoretical and indirect membrane immunofluorescence studies suggest a decrease in the amounts of RNA on the surfaces of AMD-treated Ehrlich ascites tumor cells. The amount of RNA extracted actually decreased in comparison to the amounts found for nonAMD-treated control cells. Decreases in the amounts of 18S and 28S ribosomal RNA on the cell surface were shown after sucrose density gradient centrifugation. Moreover, inhibition of RNA synthesis after AMD treatment was found when we measured the [$^3$H]uridine uptake for each RNA; only ribosomal RNA synthesis was greatly inhibited, messenger RNA and 4S, as well as other small size RNAs, syntheses were not much inhibited. These results suggest that the decreased RNA on the surfaces of Ehrlich ascites tumor cells after treatment with AMD is ribosomal RNA, evidence that the RNA on the surfaces of Ehrlich ascites tumor cells is mainly ribosomal RNA.

Ehrlich ascites tumor cells and L1210 cells released RNAs during incubation in TBS at 37°C; but, during this incubation, they showed no change in mobility (data not shown). Therefore, it is unlikely that the RNAs released from cells not treated with pronase are electrophoretic surface components of the cell. Whether these RNAs initially were located at intracytoplasmic or intramembranous sites has yet to be determined.

Ribosomal RNA synthesis is inhibited preferentially when cells are treated with a low concentration of AMD, whereas messenger- and t-RNA syntheses are not (12).

Our investigations indicate that ribosomal RNAs are present on the surfaces of nonleukemic ascites tumor cells. We now must determine whether ribosomal RNAs on surfaces of nonleukemic ascites tumor cells have ribosomal structure and work as sites of protein synthesis. A biochemical study of the syntheses of cell surface RNAs is now in progress in our laboratory. We also are using electron microscopy to study the surface structure of nonleukemic ascites tumor cells to see whether ribosomal particles are present in Ehrlich ascites tumor cells.

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