Oncornavirus-like particles released from arginine-deprived human lymphoblastoid cell lines

(Burkitt lymphoblastoid cell lines/induction of oncornavirus/arginine deprivation/Epstein-Barr virus-negative human lymphoblasts)

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ABSTRACT Type-C RNA tumor virus particles were released from three different human lymphoblastoid cell lines after incubation in arginine-deficient medium. The released virus-like particles were characterized by (a) their ability to band in sucrose gradients at a density of 1.16–1.18 g/ml; (b) the presence of an RNA-directed DNA polymerase activity resembling that of the oncornaviruses; and (c) isolation of cores that band at a density of 1.26–1.27 g/ml in sucrose gradients. Examination of the arginine-deprived human lymphoblastoid cell line strain P3HR-1 by electron microscopy revealed the presence of C-type particles in the intracellular spaces.

The search for oncornaviruses associated with different forms of human cancer depends in most instances on the induction in vitro of the latent virus genomes present in the cancer cells. It has been demonstrated that arginine deprivation leads to the induction of oncornavirus synthesis in transformed cells (1–3). Rat cells transformed by the B77 strain of Rous sarcoma virus (RSV) released C-type particles into the medium when deprived of arginine (1). These particles were capable of transforming chick and rat cell cultures (M. Kotler, D. Boettiger, and R. Weiss, to be published). Incubation of leukocytes from patients with lymphatic leukemia in an arginine-deficient medium also resulted in the synthesis and release of C-type virus particles (2, 3). The spontaneous production of similar oncornavirus particles was demonstrated after short-term cultivation of leukemic bone-marrow cells (4, 5) in three myeloblastic cell lines from the peripheral blood of a patient with acute myelogenous leukemia (6) and from a human adenocarcinoma cell line (7).

It was therefore of interest to determine the effect of arginine deficiency on lymphoblastoid cell lines. For this study, we chose: (a) the P3HR-1 line of B-type lymphoblastoid cells derived from Burkitt’s lymphoma, that contain Epstein-Barr virus (EBV) DNA genomes and produce EBV particles (8); (b) nonproducing Raji cells, derived from Burkitt’s lymphoma and consisting of B-type lymphoblastoid cells containing 60 EBV DNA genomes per cell (9); and (c) cell line 1301 consisting of T-type lymphoblasts, originating from a human leukemia (10), that do not contain EBV DNA or EB nuclear antigens. The present study provided evidence that particles resembling oncornaviruses were released from the three human lymphoblastoid cell lines.

MATERIALS AND METHODS

Cells. The P3HR-1, Raji, and 1301 cell lines (kindly supplied by Dr. B. Hampar, National Cancer Institute, NIH) were grown in suspension in Roux bottles containing 100 ml of RPMI 1640 medium (Grand Island Biochemical Co.) supplemented with 10% heat-inactivated fetal calf serum, at a concentration of 10⁶ cells per ml. Arginine deprivation was carried out by incubating the cell cultures at 37° for 24 hr in RPMI medium lacking arginine and containing 2% dialyzed fetal calf serum. The cells were labeled with [³H]uridine (1 μCi/ml; specific activity 18 Ci/mmol, Nuclear Research Center, Negev) for 24 hr.

Virus Purification. Cell debris was removed from the medium by centrifugation for 10 min at 1000 × g in the Sorvall R-5 centrifuge. The supernatant fluids were then centrifuged for 60 min at 25,000 rpm in the Beckman no. 30 or 42 Ti rotor. The pellets were resuspended in RPMI medium to approximately 1/200 of the starting volume. The concentrated preparations were centrifuged in 15% (wt/vol) sucrose layered onto a 65% (wt/vol) sucrose cushion prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) for 60 min at 45,000 rpm in the Beckman SW 50.1 rotor. The particles banding at the top of the 65% sucrose cushion were diluted in buffer and centrifuged in a 15–65% (wt/vol) linear sucrose gradient for 180 min using the same rotor. The gradients were collected dropwise, and the density of selected fractions was determined.

Endogenous DNA Polymerase Assay. Particles banding at a density of 1.16 g/ml were treated with Nonidet P-40 (NP-40) at a final concentration of 0.02% (vol/vol) and 0.25 M dithiothreitol (Sigma, St Louis, Mo.) and kept for 10 min in ice. All the reaction components are the same as those described by Kotler et al. (1).

Preparation of Cells for Electron Microscopy. The cells were washed with Tyrode’s solution, scraped, centrifuged gently into a pellet, fixed for 60 min in 1% glutaraldehyde, washed with buffer, fixed for 30 min in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin (Epon 812). The prepared sections were stained with uranyl acetate and lead citrate before examination in a Philips 300 electron microscope, with 40 μm foil objective aperture and 60 kV accelerating voltage.

RESULTS

Isolation of particles from the medium of arginine-deprived human lymphoblastoid cell lines

The human lymphoblastoid cell lines were incubated in arginine-deficient medium in the presence of [³H]uridine for 24 or 48 hr. After removal of the particulate material in each medium preparation by centrifugation, the virus pellets from the supernatant fluids were resuspended and analyzed by centrifugation in sucrose gradients. [³H]Uridine-labeled particles, banding in sucrose gradients at a density of 1.16–1.17 g/ml, were isolated from the medium of arginine-deprived P3HR-1 and 1301 lymphoid cells (Fig. 1A and C). Such particles were not obtained from 1301 lymphoblasts in-

Abbreviations: EBV, Epstein-Barr virus; RSV, Rous sarcoma virus; NP-40, Nonidet P-40.
Particles and density virions after treatments were removed by centrifugation for 60 min at 25,000 rpm to remove cell debris. The supernatant fluids were centrifuged for 10 min at 10,000 rpm to remove cell debris. The supernatant fluids were centrifuged for 60 min at 25,000 rpm in the Beckman no. 10 rotor. The pellets were resuspended in RPMI medium to approximately 1/200 of the starting volume. These pellets were used to obtain RNA-DNA hybrids after treatment with ribonuclease.

Electron microscopy
To determine the morphology of the virus particles released by the human lymphoblastoid cells, after 24 hr of incubation in arginine-free medium, thin sections of P3HR-1 and Raji cells were examined by electron microscopy. Numerous particles resembling type C viruses, like those shown in Fig. 2, were observed in sections of P3HR-1 cells. Similar type C virus particles were seen in Raji cells (not shown). These particles were not found in P3HR-1 and Raji cells that were incubated in an arginine-containing medium. The virus particles of P3HR-1 and Raji cells have a diameter of 85–90 nm and a core diameter of 40 nm. Virus particles were seen budding from the membranes of P3HR-1 cells.

Biochemical and physical properties of induced virus-like particles
Isolation of Cores from Virus-Like Particles. The particles isolated from P3HR-1 cells that banded at 1.17 g/ml in sucrose gradients and particles isolated from the 1301 cell line as cores banded at 1.26 g/ml (Fig. 3) were treated with 0.02% (vol/vol) NP-40, and the kinetics of RNA synthesis was studied in the presence of all four deoxyribonucleoside triphosphates (Fig. 4). Synthesis of DNA was found to occur with the endogenous enzymes of particles obtained from both cell lines (Fig. 4A and B). The DNA polymerase activity was inhibited after treatment with ribonuclease.

Simultaneous Detection of RNA-DNA Hybrids. The particles obtained from the arginine-deprived lymphoblastoid cell lines (P3HR-1, Raji, and 1301) contain virus-like RNA that directs the RNA-dependent DNA polymerase activity, as determined by the simultaneous detection test (12) (Fig. 5). The particles obtained from the P3HR-1, Raji, and 1301 lymphoid cell lines, having a density of 1.16–1.18 g/ml, were recentrifuged in sucrose gradients after treatment with NP-40, and the cores that banded at a density of 1.26 g/ml were collected. Reactions of DNA synthesis by the endogenous RNA-directed DNA polymerases were carried out for 15 min, and the nucleic acid synthesized in vitro was extracted and centrifuged in sucrose gradient under conditions suitable for the isolation of 70S RNA-DNA hybrids. The external marker was 70S RNA extracted from the B77 Rous sarcoma virus strain. DNA was synthesized in vitro on the 70S RNA molecules by the endogenous enzymatic activity present in the particles isolated from all three cell lines, and 70S RNA-DNA hybrid molecules were detected (Fig. 5A–C). Treatment with RNase (not shown) resulted in the
removal of the labeled DNA from the 70S region in the gradient. The results indicate that all three cell lines release particles with the properties of oncornaviruses. Particles labeled with $[^3H]$uridine from the culture medium of Raji cells were used for the isolation of RNA, using Rous sarcoma virus 70S as a marker. Labeled high-molecular-weight RNA was not isolated from Raji particles in the sucrose gradients. The labeled RNA banded as low-molecular-weight fragments. It is possible that the particles are contaminated with nucleases. Further studies are in progress to isolate high-molecular-weight RNA, since the simultaneous detection tests (Fig. 5) demonstrate the presence of 70S viral RNA in the isolated particles.

**DISCUSSION**

The particles released from P3HR-1, Raji, and 1301 lymphoblastoid cell lines by arginine deprivation were characterized by several criteria. These included the morphology of the isolated particles, the density of the particles and the cores, the RNA-directed polymerase, and the synthesis of RNA-DNA hybrids on the 70S RNA present in the isolated particles. The particles isolated from the three cell lines were similar in their properties to type-C virus particles from other mammalian cells. Our results are in agreement with the finding of Hehlmann et al. (13) and Kufe et al. (14, 15) that Burkitt lymphoblastoid cells contain unique RNA sequences and complexes of 70S RNA-instructed DNA poly-

**FIG. 2.** Electron microscopy of virus particles from arginine-deprived P3HR-1 cells. Particles resembling C-type virus particles are present in the extracellular spaces. Inset: higher magnification of the virus particles. Bar represents 100 nm.
centrations of density of isolated as cells incubated lymphoid prived present in polymerase gradients were were for marker particle of type-C layering cell lines. FIG. 3. Isolation and density were described in the legend to Fig. 1. The fractions with a density of 1.16-1.18 g/ml were treated with NP-40 at a final dilution of 1% (v/v), with 0.25 M dithiothreitol (Sigma), and were held for 10 min in ice. The treated preparations were layered onto linear gradients of 15-65% sucrose and centrifuged for 16 hr at 38,000 rpm in the Beckman SW 41 rotor. The sucrose gradients were collected dropwise, and the [3H]uridine radioactivity in each fraction was determined.

FIG. 3. Isolation of RNA-containing cores released from the 1301 (A) and Raji (B) cell lines. B77 virus (a strain of RSV) was used as a marker for type-C particle density (C) and as a marker for core density (D). The particles banding at a density of 1.16 g/ml (see Fig. 1) were treated with NP-40 at a final dilution of 1% (v/v), with 0.25 M dithiothreitol (Sigma), and were held for 10 min in ice. The treated preparations were layered onto linear gradients of 15-65% sucrose and centrifuged for 16 hr at 38,000 rpm in the Beckman SW 41 rotor. The sucrose gradients were collected dropwise, and the [3H]uridine radioactivity in each fraction was determined.

FIG. 4. Kinetics of DNA synthesis by the RNA-directed DNA polymerase present in the particles released from arginine-deprived lymphoid cell lines. (A) The particles released by P3HR-1 cells incubated in an arginine-deficient unlabeled medium, were isolated as described in the legend to Fig. 1. The fractions with a density of 1.16-1.18 g/ml were treated with NP-40 at a final concentration of 0.02% (v/v) and used as the enzyme source. Disrupted merase and 70S RNA. Similar complexes of 70S RNA and reverse transcriptase were found by Baxt et al. (16) and Gallo and his collaborators (6, 17), in human leukemic cells.

In our previous report we demonstrated that arginine deprivation induced the release of type-C particles from leukocytes taken from patients with lymphatic leukemia (2, 3). In the present study we extend our findings to three human lymphoblastoid cell lines, demonstrating that EBV DNA positive and negative cell lines contain an oncornavirus that particles (100 µl) were distributed to each of four tubes. The first tube then received 10 µl of TE buffer; the second, 10 µl of 250 µl/mil of ribonuclease A (Sigma); the third, 10 µl with 0.01 unit (A 280 nm) of poly(rA)-oligo(dT), and the fourth, 10 µl with 0.01 unit (A 280 nm) of poly(dA)-oligo(dT) (purchased from Collaborative Research, Waltham, Mass.). The tubes were incubated for 45 min at 4° and for 10 min at 37°. To each tube 150 µl of the polymerase reaction mixture containing the four deoxynucleoside triphosphates and 1 mM MnCl₂, final concentration, instead of MgCl₂ (see refs. 1 and 6) was then added and the mixtures were incubated at 37°. Duplicate samples of 25 µl each were removed at the indicated time intervals. The samples were transferred into a cold trichloroacetic acid solution, and the trichloroacetic acid-precipitable radioactivity was determined. (B) The cores of unlabeled particles from arginine-deprived 1301 cells were isolated, after centrifugation in sucrose gradients, from the band with a density of 1.26 g/ml (as described in the legend of Fig. 3). DNA synthesis in the presence and absence of RNase A was determined as described in part A.
can be induced by arginine deprivation. However, the possibility of a laboratory contamination with other oncornaviruses always exists since the cells were propagated for many years in different laboratories. Nevertheless, there are several indications against external contamination. First, all three human cell lines were grown in our laboratory in a special room in which only human cells were grown and no work with oncornaviruses was done there. Second, no monkey viruses have been grown in our laboratory. Third, the virus particles were found in the culture medium only when the cells were deprived of arginine and not in underpriviledged cells.

At present we do not know whether the particles isolated from the lymphoid cells are associated with the transformed state of these cells. From previous studies we know that arginine deprivation of nonproducer B77 virus transformed rat cells results in the induction and release of Rous sarcoma virus capable of transforming both chick and rat cells in tissue culture (ref. 1 and M. Kotler, D. Boettiger, and R. Weiss, to be published). It would be of special interest to compare the virus particles released by arginine deprivation from human lymphoid cell lines to those spontaneously released from cultured human leukemic cells (4–6), both by radioimmunooassay and nucleic acid sequence homology.

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![Graph](image-url)

**FIG. 5.** Characterization of the DNA synthesized on the RNA template present in particles released from (A) P3 HR-1, (B) Raji, and (C) 1301 cells. The reactions were carried out for 15 min at 37°C as described in the legend of Fig. 4A except that 10 mM MgCl2 was used. The reactions were then stopped by adding Baycovin (Bayer, Leverkusen, Germany) (5 μl/1.25 ml), 5% (w/v) N-lauroyl sarcosine (Sigma, St. Louis, Mo.), and 50 mg of yeast RNA to act as a carrier. The RNA-DNA hybrids were centrifuged at 15–30% (w/w) sucrose gradients for 100 min at 45,000 rpm in the SW 50.1 Beckman rotor. [3H]uridine-labeled RNA of B77 virus was used as an external marker (RSV-RNA). The sucrose gradients were collected dropwise, and the radioactivity in each fraction was determined after precipitation with trichloroacetic acid.