Particles with Characteristics of Leukoviruses in Cultures of Marrow Cells from Leukemic Patients in Remission and Relapse
(human leukemic cells in culture/electron microscopy/reverse transcriptase)

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Communicated by Leon O. Jacobson, May 28, 1974

ABSTRACT An enzyme activity with the characteristics of RNA-directed DNA polymerase (reverse transcriptase) was detected in marrow from patients with leukemia in relapse and in firm hematological remission. Material having the enzyme activity, when analyzed in sucrose gradients, appeared as two distinct homogeneous bands of particles with densities of about 1.17 and 1.23 g/ml. The enzyme activity was stimulated by exogenous template poly(C)-(dG)2,3-8 but not by (dT)2,3-8. The enzyme activities in these bands also increased (1.7- to 24-fold) after culture, and both bands with enzyme activity were obtained from the cultured cells and from the supernatant medium. Electron microscopic studies showed that the two bands contained particles resembling leukoviruses or their cores.

Leukocytes from patients with leukemia contain a DNA polymerase with the characteristics of an RNA-directed DNA polymerase (reverse transcriptase) (1-4). This enzyme activity is associated with a cytoplasmic subcellular fraction having a density characteristic of leukoviruses (5, 6). Further, Gallo et al. (7) have demonstrated homology between DNA synthesized by human reverse transcriptase from an endogenous template and RNA of leukemia and sarcoma viruses of simian and murine origin.

Recently, we reported that reverse transcriptase activity increases after culture of marrow cells from leukemic patients in relapse and in remission (4). In the present communication we show that this reverse transcriptase activity is associated with two discrete bands of particles with densities of about 1.17 and 1.23 g/ml, respectively. The amount of reverse transcriptase activity found at these densities increases after culture. Particles with reverse transcriptase activity can be identified not only in the cells but also in the external supernatant fluid. The biochemical and morphological properties of the particles are consistent with those of leukoviruses.

MATERIALS AND METHODS

Patient Material. Fourteen specimens of bone marrow were obtained from 13 patients with leukemia and two specimens from nonleukemic patients during the course of hematological investigation. Morphological diagnoses of acute myeloblastic leukemia (AML) and acute lymphoblastic leukemia (ALL) were made by described criteria (8). Patients were considered to be in marrow remission when their marrow preparations contained less than 5% blast cells in association with normal erythropoiesis and platelet formation, with normal blood levels of granulocytes and platelets in excess of 100,000/mm3.

Abbreviations: AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelocytic leukemia.

Suspension Culture Procedure. Nucleated marrow cells, obtained from buffy coat preparations, were cultured in suspension as described (4). The cultures consisted of 5 × 10⁶ nucleated cells in 5 ml of medium (Flow Laboratories) with 20% (v/v) fetal calf serum, all contained in 10-ml plastic test tubes. In some cultures, [14C]Juridine was added at a concentration of 1 μCi/ml. The tubes were incubated for 7 days at 37° in a moist atmosphere of 5% CO₂ in air. Ten tubes, containing a total of 5 × 10⁶ nucleated cells, were routinely cultured for each sample. For electron microscopy cells were cultured either in the absence of serum or with 1% pooled human serum.

Preparation of Subcellular Material for Measurement of DNA Polymerase Activity. Cultures prepared as described above were pooled after 7 days. The cells were separated from the supernatant culture medium by centrifugation at 500 × g for 10 min. The cells were resuspended in 1 ml of solution A [0.4 M sucrose–50 mM Tris (pH 8.0)–2 mM dithiothreitol] and were mechanically disrupted in a Teflon fitted homogenizer (5 strokes). The homogenate was centrifuged at 500 × g for 10 min and the pellet was discarded. Both this supernatant and the culture medium were centrifuged separately at 12,000 × g for 15 min. The resultant pellets were discarded and the supernatants were centrifuged at 100,000 × g for 60 min. The resulting sediments were resuspended in 1 ml of phosphate-buffered saline (pH 7.2) and were centrifuged in a 20-70% sucrose gradient for 16-18 hr in an SW 41 rotor at 150,000 × g. Twenty fractions were collected, and each fraction was assayed for endogenous DNA polymerase activity as described (4), with the exception that 6 mM Mg acetate was used instead of 1 mM Mn acetate. In addition, where [14C]Juridine had been added to the cultures, 14C counts were also measured. Densities of the fractions were calculated from refractive index measurements.

Electron Microscopy. Fractions corresponding to peaks of reverse transcriptase activity were pooled, diluted with phosphate-buffered saline, and centrifuged at 100,000 × g for 60 min. The supernatant fluid was removed and the sediment (not usually visible) was resuspended in a minimal amount (0.1-0.2 ml) of phosphate-buffered saline. A small quantity of the suspension was applied with a platinum wire loop to a carbon-formvar coated electron microscope grid and allowed to adsorb for 1 min. The specimen was then washed briefly in phosphate-buffered saline, fixed for 3 min in 1.5% glutaraldehyde, washed in distilled water, and dehydrated by immersing successively in 50%, 75%, and 95% ethanol containing 1% uranyl acetate and finally in absolute ethanol. After
the specimen was washed in ethanol, excess fluid was removed from the grid with a piece of filter paper and the remaining thin film of liquid was allowed to dry. For negative staining the grids were removed and dried from the 95% ethanol–uranyl acetate solution. Specimens were examined in a Siemens Elmiskop 1A at 80 kV and magnifications of 20,000× or 30,000×.

**RESULTS**

In our previous study (4) reverse transcriptase activity in cells from marrow of patients with leukemia increased after 7 days in suspension culture. We asked whether this reverse transcriptase activity was associated with particles of densities similar to those described by Kufe et al. (6). Fig. 1 contains representative reverse transcriptase activity profiles obtained by sucrose density gradient centrifugation of subcellular particles before and after culture; the top and middle panels of the figure contain data for cells, while the bottom panel contains the similar information for the culture supernatant. For most preparations two distinct peaks of activity are observed; one at a density of 1.17 g/ml, the other at a density of 1.23 g/ml. It is evident from Fig. 1 and Table 1 that, in agreement with our previous observations, reverse transcriptase activity increased after 7 days in culture. In the experiment shown in Fig. 1, [14C]Uridine was added to the cultures. Peaks of [14C]radioactivity and reverse transcriptase activity are found at identical positions on the gradient.

Detailed results of similar studies on nine marrow specimens obtained from eight of the 13 patients with leukemia are summarized in Table 1. Endogenous reverse transcriptase activity banding in two peaks, one with densities in the range 1.20–1.25 g/ml (peak I) and the other in the range 1.16–1.19 g/ml (peak II), was identified in all specimens regardless of the clinical status. After culture, reverse transcriptase activity associated with particles of densities characteristic of either or both of peaks I and II was found in cells and supernatants. In each case, the total activity was greater after culture than before, although the extent of the increase varied greatly from patient to patient. In one patient, G.A.R., reverse transcriptase activity was lower in remission than in relapse, but increased almost to the relapse level after the cells were cultured. Since cell number decreased during culture, reverse transcriptase activity after culture is expressed in terms of cpm per culture.
rather than cpm per cell. This method of data expression minimizes the increase, but avoids possible confusion resulting from nonspecific cell lysis of cells containing reverse transcriptase. In two instances the cultures tested were found to be free of mycoplasma by the uridine phosphorylase assay (9).

**Characterization of the DNA Polymerase Activity.** The characteristics of the DNA polymerase activity were determined, and are presented in Fig. 2. The figure shows sucrose density gradient profiles of the supernatant of a culture of marrow cells from a patient with AML. The fractions from the gradient were assayed for both endogenous and template-stimulated DNA polymerase activity; for the latter, two templates were used, poly(rC)·(dG)12-18 and (dT)12-18. It is evident that the enzyme activity was stimulated by the artificial template poly(rC)·(dG)12-18 but not by (dT)12-18. These properties are consistent with those of leukovirus reverse transcriptase and not those of a terminal transferase (10). Treatment with Nonidet had only a small effect on the endogenous reverse transcriptase reaction, suggesting that the particles may be unstable.

**Rebanding Experiments.** Leukoviruses usually have densities of about 1.17, while their cores are denser. It was possible, therefore, that the two peaks observed in our experiments represented complete virions and their cores. We examined this possibility by rebanding material from each peak. The data from one of two similar experiments are given in Fig. 3. The top panel of the figure is the original sucrose density profile;
Fig. 4. Electron micrographs of material from peaks I and II. Particles from band of density 1.17 were positively stained with uranyl acetate and dried from ethanol (a and b). Particles from a band of density 1.23 are shown in c and d, negatively stained with uranyl acetate. Bar represents 100 nm.

the middle panel shows that obtained when peak I was rebanded. Some activity was recovered at the density characteristic of peak I, while the rest was distributed throughout the gradient. In contrast, when material from peak II was rebanded (bottom panel of Fig. 3), activity was recovered at densities characteristic of both peaks. This result is compatible with a breakdown of particles with density characteristic of peak II to others of density characteristic of peak I.

Electron Microscopy. Marrow from four patients with leukemia was cultured to obtain specimens for electron microscopy. Materials banding at the densities of peak I and peak II were examined after positive and negative staining with uranyl acetate. Fig. 4a and b shows particles typical of those observed after positive staining of material of peak II. The particles are about 100 nm in diameter, have densely stained peripheries and in some instances, central dense regions with angular outlines. Particles of smaller diameter were also present. Specimens prepared from material of peak I and negatively stained consisted almost entirely of smaller particles, as shown in Fig. 4c and d. The mean diameter was 71 nm, and 90% of the particles had diameters in the range 55–80 nm. Peripheral projections were visible around many of the particles. Their shape varied according to the plane of view, but in some instances they appeared as rings of diameter 8–10 nm. Similar structures were apparent on the surface of some particles (Fig. 4c and d).

DISCUSSION
In agreement with Sarin et al. (5) and Kufe et al. (6), we have found that the reverse transcriptase activity in cells from patients with leukemia is associated with particles of densities characteristic of leukoviruses. We report here that the enzyme activity associated with such particles increases in cultures of leukemic marrow cells, and that particles are recovered in the supernatants of such cultures. Material with reverse transcriptase activity was detected in two regions of the sucrose gradients, one at density range 1.20–1.25 g/ml (peak I), the other at density range 1.16–1.19 g/ml (peak II).

As seen in the electron microscope, material from peak I consisted almost entirely of particles having a limited size range, 55–80 nm, and having common structural features, in-
including surface projections or subunits. It has been shown by Nermut et al. (11) that a murine leukemia virus (Friend) possesses a core covered with regular subunits, and recently it has been reported that cores from pig leukovirus show a similar structure (12). The particles that we observed in specimens made from the denser peak of human leukemic material are of about the same size and density as murine leukemia virus cores [65-70 nm and 1.24-1.26 g/ml, respectively (13)], and possess surface subunits. Although the arrangement of the subunits lacks regularity, probably due to damage during the preparative procedure used, they bear some resemblance to the morphological subunits or capsomeres of which viral capsids are composed.

Recently, Benz and Moses (14) have reported particles with a virus-like morphology in fetal calf serum. To avoid the possibility of confusion with such particles, we prepared cultures for electron microscopy in the absence of serum or with 1% pooled human serum.

The present study confirms our previous findings (4) that reverse transcriptase activity is present in cells of patients with leukemia both in relapse and in remission, and this activity increases in suspension culture. The present studies provide evidence that the increase is associated with new synthesis, since $[^{14}C]$uridine was incorporated into material that banded in sucrose with reverse transcriptase activity (Fig. 1).

We have examined marrow specimens from two patients without malignant disease and failed to find discrete peaks or reverse transcriptase activity or any other evidence of leukovirus-like particles either before or after culture. These controls are inadequate to allow us to conclude that there is a relationship between the leukovirus-like particles and leukemia in man; hematopoietic cells derived from fetal liver or from marrow recovering after injury would be more appropriate control materials. Nonetheless, the particles obtained from patients with leukemia have similarities in their physical, biochemical, and morphological characteristics to C-type virus particles known to cause leukemia in other animal species (15). These similarities are the major basis for considering that the particles reported in these experiments may be significant in human leukemia.

We thank L. Schachtschabel, M. Bates, and J. Mathers for their capable technical assistance. This work was supported by grants from the Ontario Cancer Treatment and Research Foundation, The Medical Research Council of Canada, and the National Cancer Institute of Canada.