VINBLASTINE-INDUCED RIBOSOMAL COMPLEXES

Effect of Some Metabolic Inhibitors on Their Formation and Structure

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Vinca alkaloids, vinblastine and vincristine sulfate, have a variety of effects on cells in vivo and in vitro (1–10). In low sublethal doses, vinblastine and vincristine arrest cells in mitosis (1–3) and prevent the formation and organization of the spindle microtubules (2, 3). Accumulation of large proteinaceous crystals in platelets (7), in tissue culture cells (8, 10), and in cells of the nervous system (9) by exposure to high doses of vinblastine and vincristine has been reported. The presence of colchicine-binding microtubular proteins in the vincristine- and

vinblastine-induced crystals is suggested by the radioautographic labeling of the crystals in cultured cells by colchicine-^{3}H (11).

The association of a large number of ribosomes and helical polyribosomes with the vincristine- and vinblastine-induced crystals and with complexes of electron-opaque granular material has been recently reported (10, 12). In thin sections a faint linearity is recognizable in the granular material of the complexes, while in other planes sections reveal tubular structures, 280 A in diameter (similar to those seen in cross-sections of the vinblastine-induced crystals), surrounded by the granular material and the ribosomes. These observations suggest that the ribosome-granular material complexes are the probable sites for the formation of the proteins of the crystals and that the associated ribosomes and polyribosomes are involved in their synthesis.

In human lymphoblasts exposed to high doses of vinblastine and vincristine, the granular material-ribosomal complexes (henceforth referred to as ribosomal complexes) are usually large in size and frequency and often show large rows of associated helical polyribosomes (12). In the present investigation we have tried to explore further the nature of these ribosomal complexes by exposing human lymphoblasts and Earle's L-929 fibroblasts in culture to some of the well-known inhibitors of protein synthesis before and concurrent with their exposure to high doses of vinblastine and vincristine.

MATERIALS AND METHODS

Earle's L-929 mouse fibroblasts in monolayer cultures and human lymphoblasts initially isolated from the peripheral blood of a leukemic patient (CCRF-CEM) and maintained in continuous suspension cultures were used for the present study. Cell cultures were exposed to vinblastine sulfate (Velban) or vincristine (Oncovin) (5–10 μg/ml) for 15 min–24 hr.

The following metabolic inhibitors were used before and during the exposure of cells to crystal-inducing concentrations (5–10 μg/ml) of vinblastine and vincristine. (*a*) Puromycin dihydrochloride (Nutritonal Biochemicals Corporation, Cleveland, Ohio) 0.25-10 μg/ml, 15 min–3 hr before the addition of vinblastine or vincristine (10 μg/ml) to the culture media. (*b*) Cycloheximide (Acti-dione, Upjohn Co., Kalamazoo, Mich.) 1.5–10 μg/ml, 30 min–2 hr before the addition of vinblastine. (*c*) p-fluorophenylalanine (Nutritional Biochemicals Corporation) 5 μg/ml, 30 min–3 hr before the addition of vinblastine or vincristine. (*d*) Actinomycin D (Lyovac Cosmegen, Merck, Sharp, and Dohme, West Point, Pa.) 0.0625–1 μg/ml, 15 min–3 hr before the addition of vinblastine or vincristine to the culture media.

Leighton tube cover slips were washed in balanced salt solution, fixed in acetic acid:alcohol (1:3), and stained with carbol-fuchsin and 1% aqueous fast green FCF.

For electron microscope examination, cell buttons were collected by light centrifugation and processed by procedures described elsewhere (10).

OBSERVATIONS

In both the L cell fibroblasts and the CCRF-CEM lymphoblasts exposed to vinblastine or vincristine, a gradual increase in the number and the size of the crystals was seen on continued incubation in the drug-containing medium. Thus, in cells exposed to vinblastine for 15 min no crystals could be recognized, while in cells examined after 30–45 min a large number of small crystals were present in the cytoplasm. In cells incubated for as long as 24 hr with vinblastine, unusually long crystals (some of them approximately 40 μm long) were seen in the cytoplasm. In electron micrographs crystals from cells exposed to vinblastine for 24 hr were seen as large arrays of parallel filaments occupying a major part of the cytoplasm.

In addition to the crystals, cells exposed to vinblastine or vincristine contained large cytoplasmic ribosomal complexes. In the CCRF-CEM lymphoblasts exposed to vinblastine or vincristine, unusually large ribosomal complexes occurred more frequently than in L cells similarly treated. Although ribosomal complexes were absent in cells from cultures incubated for 15 min with vinblastine or vincristine, large numbers of ribosomal complexes could be recognized in cells examined after 30 min of exposure. Fig. 1 shows a large ribosomal complex in the cytoplasm of a CCRF-CEM cell exposed to vinblastine for 3 hr (arrows point to helical polyribosomes). Although a faint linearity can be recognized in the substructure of the granular material, it lacks the prominent linear structure seen in the crystals.

In some earlier studies it was reported that puromycin, a strong inhibitor of protein synthesis, did not prevent the formation of the crystals in Earle's L cells exposed to vinblastine or vincristine (8, 10). In CCRF-CEM lympho-

blasts incubated with puromycin (10 μg/ml) for 30 min before the addition of vinblastine (10 μg/ml) for 3 hr to the medium, ribosomal aggregates and helical polyribosomes were rarely seen in the cytoplasm. These cells also had a reduced number of ribosomal complexes compared to cells exposed to vinblastine alone. Fig. 2 shows the fine structure of an unusually large ribosomal complex in the cytoplasm of a CCRF-CEM cells exposed to puromycin (10 μg/ml) for 30 min before the addition of vinblastine (10 μg/ml) for 3 hr. As seen in this electron micrograph, no large aggregates of ribosomes or helical polyribosomes (as seen in cells exposed to vinblastine alone, Fig. 1) are seen in association with the ribosomal complexes or in the cytoplasm of cells exposed to puromycin.

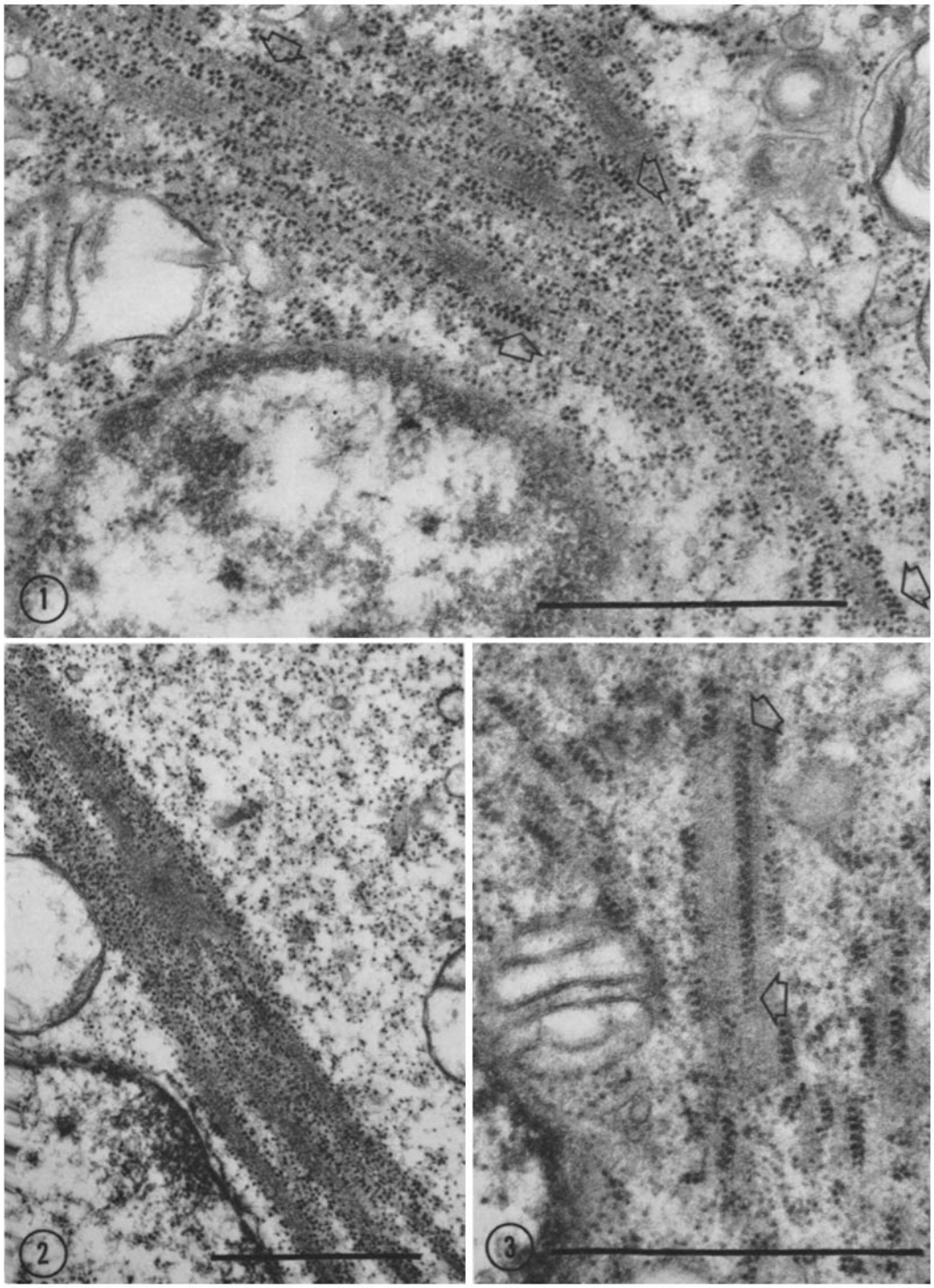
Cycloheximide (like puromycin) does not inhibit the initial formation of the crystals in Earle's L cells. CCRF-CEM lymphoblasts exposed to cycloheximide (5 μg/ml) and leucine-^{3}H before exposure to vinblastine (10 μg/ml) show a marked inhibition in the uptake of the radioactive label (unpublished observations). Like puromycin, cycloheximide did not prevent the formation of the ribosomal complexes, but in cells incubated with cycloheximide for 1 hr before the addition of vinblastine for the next 3 hr, there was an apparent reduction in the number of the crystals and the ribosomal complexes compared to cells from cultures exposed to vinblastine alone. Although in cells exposed to cycloheximide and vinblastine the crystals and the ribosomal complexes were morphologically similar to those of cells exposed to vinblastine alone, an apparent reduction in the number of helical ribosomes associated with the complexes was evident in the cycloheximide-treated cells. However, in contrast to cells exposed to puromycin, there was no apparent breakdown of the ribosomal clusters and polyribosomes in the cytoplasm of these cells.

Since the proteins forming the crystals and the granular material associated with the ribosomal complexes are supposedly similar to the microtubular protein, and *p*-fluorophenylalanine (a phenylalanine analogue) has been shown to induce the formation of morphologically abnormal cilia (13), we exposed CCRF-CEM cultures to this analogue before the addition of vinblastine to the medium. Compared to the inhibitory effect of puromycin and cycloheximide on the formation of helical polyribosomes and ribosomal complexes, this compound had no morphologically detectable effect on either the crystals, the ribosomal complexes, or the associated helical polyribosomes.

Pretreatment of CCRF-CEM cells and L cells with 0.25–1 μg/ml of actinomycin D for 1 hr before the addition of vinblastine did not prevent the formation of crystals. Similarly exposure to low doses of actinomycin D (0.06 μg/ml) for 30 min before the exposure to vinblastine did not inhibit the formation of the ribosomal complexes. Fig. 3 shows part of a large complex from a CCRF-CEM cell exposed to low doses of actinomycin D for 30 min before the addition of vinblastine for the next 3 hr. Arrows point to an unusually large polyribosome in the middle of the complex. In cells exposed to high doses of actinomycin D (0.1–1 μg/ml), a definite inhibitory effect on the number and size of the ribosomal complexes formed was evident. In cells exposed to 0.1 μg/ml of actinomycin D for 3 hr before the addition of vinblastine, only occasional ribosomal complexes with few polyribosomes were seen. In cells exposed to 1μg/ml for a similar length of time, small ribosomal complexes were rarely seen.

DISCUSSION

As shown in the present study, crystals seen in cells exposed to vinblastine for long periods of time are considerably larger than those in cells exposed to similar concentrations of vinblastine for shorter lengths of time. There are two possible explanations for this observation: either there is continued synthesis of the material for the crystals in the presence of the alkaloid, or the large crystals result from the aggregation and the alignment of smaller crystals. The submicroscopic appearance of the larger crystals suggests more a continuity of structure than the aggregation of smaller crystals. It is probable that newly synthesized material is added on the surface of the growing crystals initially composed of proteins synthesized before the exposure of the cells to the alkaloids. This view is supported by our preliminary electron microscope radioautographic study with leucine-^{3}H showing incorporated label on the edges of the crystals. The effect of cycloheximide in reducing the size as well as the number of the crystals also supports the above observations. Evidence from the present studies and those reported earlier (8, 10, 11) makes



All figures are from CCRF-CEM lymphoblasts incubated with vinblastine (10 μg/ml) for 3 hr unless otherwise indicated. Magnification markers indicate 1 μ.

FIGURE 1 A large ribosomal complex is shown in this electron micrograph of a lymphoblast exposed to vinblastine for 3 hr. Arrows point to helical polyribosomes seen in association with the complex. × 45,310.

FIGURE 2 An unusually large ribosomal complex in a lymphoblast exposed to puromycin (10 μg/ml) for 30 min before the addition of vinblastine. In contrast to Fig. 1, large ribosomal aggregates or helical polyribosomes are rarely seen in puromycin-treated cells. × 30,720.

FIGURE 3 Part of a large ribosomal complex from a lymphoblast exposed to actinomycin D (0.06 μg/ml) for 30 min before the addition of vinblastine for 5 hr. Arrows point to an unusually large helical ribosomal configuration. × 54,500.

it probable that vinblastine-induced crystals are formed not only from the material synthesized by the cells before their exposure to vinblastine, but also from additional material synthesized in the presence of the alkaloid.

As described earlier (12) and seen in the present study, in the human leukemic lymphoblasts (CCRF-CEM) the ribosomal complexes not only are more frequent but also are very long in size. On the basis of a faint linearity recognizable in the granular material, the presence of occasional tubular cross-sections, and the association of ribosomes and polyribosomes with both the complexes and the crystals, it is probable that the material associated with the ribosomal complexes is similar to that forming the crystals and that the complexes are the sites for the synthesis of the crystalline material. However, in view of the recent demonstration (14) that vinblastine can precipitate structural proteins other than the microtubule proteins, it is probable that some of the complexes are formed from proteins other than those of the microtubular variety.

None of the various protein synthesis inhibitors used in the present study completely prevented the formation of the ribosomal complexes. Puromycin has been shown to inhibit protein synthesis by forming a peptidyl-puromycin complex (15, 16), and disaggregation of polyribosomes in vivo and in vitro by puromycin has been recently demonstrated (see 17 for references). As seen in the present study, cells exposed to puromycin before their exposure to vinblastine show very few ribosomal aggregates and helical polyribosomes in the cytoplasm and in association with the ribosomal complexes. Although the complexes in puromycin-treated cells are smaller in size and fewer in number, this protein synthesis inhibitor does not completely prevent their formation.

Cycloheximide, a strong inhibitor of protein synthesis, acts by preventing the transfer of amino acids from the sRNA to the nascent polypeptide chain (18, 19). In contrast to puromycin, cycloheximide blocks the "read out" process, preventing the breakdown of the polyribosomes (or ergosomes) and inhibiting the amino acid incorporation (18). In the present study, cells exposed to cycloheximide had fewer numbers of ribosomal complexes, but in contrast to puromycin there was no breakdown of the ribosomal masses or helical polyribosomes into individual ribosomes either in the cytoplasm or in association with the ribosomal complexes. Similarly, actinomycin D in lower doses did not prevent the formation of the complexes, while in higher doses there was a distinct reduction in the size and number of the complexes. From these observations, one can probably postulate that in the presence of low doses of vinblastine there is continued synthesis of material, but binding of the alkaloid to the nascent polypeptide chain causes a freezing of this metabolic process as manifested by the shape of the ribosomal complexes. Protein synthesis inhibitors used in the present study can stop this synthesis (as seen in the reduced number of complexes) but do not effect the precipitation by vinblastine of the complexes which were already engaged in the synthesis. Needless to say, further work is needed to assess the validity of this hypothesis.

SUMMARY

A gradual increase in the size and the number of vinblastine-induced crystals is seen in Earle's L cell fibroblasts and human lymphoblasts (CCRF-CEM) exposed to this antitumor alkaloid for 24 hr. Besides the crystals, a large number of ribosomal-granular material complexes are seen in CCRF-CEM cells incubated with vinblastine. In cells incubated with puromycin and cycloheximide before the addition of vinblastine to the medium, a reduction in the number and size of the ribosomal complexes was seen. In puromycin-treated cells disaggregation of ribosomal clusters and helical polyribosomes was evident. No morphologically detectable effect on the crystals or the ribosomal complexes of cells incubated with *p*-fluorophenylalanine was seen. Actinomycin D at high doses had a definite inhibitory effect on the number and size of these ribosomal complexes.

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