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Definition of the Cell Cycle Segment with Special Sensitivity to Vinblastine[1] (40022)

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Vinblastine sulfate, an alkaloid of the *Vinca rosea* group which is a potent inhibitor of cell division, has been used for many years as a cancer chemotherapeutic agent. It acts by inhibiting the assembly of microtubular proteins into spindle microtubules (1–3), producing a metaphase arrest similar to that caused by colchicine, that has been observed *in vitro* and *in vivo* (1, 2). In spite of the similarity of their effects on cells, the mechanism of action of vinblastine (Vb) and colchicine is probably different (3–5). Any drug or agent capable of prolonging the G2 phase or mitosis, permitting premature centriole replication and maturation of daughter centrioles, may induce multipolar spindles, leading frequently to cellular death, defective cytokinesis, and multinucleated tetraploid cells (6, 7). Irradiated cultured mouse cells (7) and Earle's L cells exposed to Vb (4) have been noted to have more than one pair of centrioles, and to undergo multipolar mitosis with abnormal chromosomal grouping (7) and multinucleation (4). We have found that exposure of HeLa cells to low concentrations of Vb produces similar effects, with high yields of multinucleated cells (8).

The purpose of this study was to define a discrete period in the cell cycle during which events take place that are especially sensitive to vinblastine, in the hopes of uncovering at least one cell cycle region which is critical for the formation of an intact mitotic spindle. This was approached by determining the period of the cell cycle during which Vb causes multinucleation.

Materials and methods. Cell culture. HeLa S 3 cells were grown in monolayers in 950-ml glass bottles in Eagle's minimal essential medium, supplemented with 10% heat-inactivated fetal calf serum and 1% glutamine.

Cell volume and number were obtained with an electronic particle counter (Coulter Electronics Inc.) equipped with a 25-channel particle size distribution analyzer, Model B.

Vb dose and time of exposure for optimal multinucleation. Exponentially growing monolayers of HeLa cells were treated with different concentrations of Vb (0.001 to 1.0 μg/ml of culture medium) for variable periods of time (30 min to 4 hr). After removal of Vb, the cell monolayers were washed with warm phosphate-buffered saline and placed at 37° with fresh medium. At 2-hr intervals, for a period of 24 hr, mitotic cells were removed from culture flasks by gentle rocking of the medium over the monolayers (9, 10) and seeded onto sterile glass coverslips attached to metal rings, which were placed in petri dishes (11). The concentration of cells on each coverslip ranged from 2 to 5 $\times$ 10^4/ml.

The cells were allowed to attach to and grow on the glass for 20 to 24 hr, while incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Then, the coverslips were fixed in Carnoy fixative (3:1 ethanol–acetic acid) and stained with 0.5% toluidine blue (TB) for light microscopy observation. The percentage of multinucleated cells was calculated by counting 600 to 1200 cells at different times and variable Vb concentrations.

HeLa cell cycle analysis. The phases of the cell cycle (G1, S, G2) were timed by scoring the proportion of labeled metaphases (12–14). The cells were grown on coverslips in petri dishes for 24 hr before undergoing a 30-min exposure to medium containing 0.5 μCi/ml (sp act, 7 Ci/mmole) of tritiated thymidine. After exposure, the radioactive medium was removed, and the cells were placed at 37° in fresh medium with an atmosphere of 5% CO_2 in air. At

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hourly intervals, for a period of 24 hr, coverslips with cell monolayers were removed and prepared for autoradiography. The duration of phases of the cell cycle was determined from the curve depicting the rate of appearance and disappearance of labeled mitoses (13) (Fig. 1). Mitotic figures (metaphases) with four or more grains were considered labeled. Background grains were zero to one grain per cell cytoplasm. For each coverslip, a minimum of 250 mitotic figures was scored.

Labeled cells in metaphase first appeared between 2 and 3 hr (minimal G2 + 1/2 mitosis) after exposure to tritiated thymidine. One-half of the time for mitosis was added because mitoses were scored in metaphase, which is about the midpoint of mitosis. The frequency of labeled metaphase cells rose to 98% at 10 hr after point zero (maximal G2 + 1/2 mitosis). The average G2 + 1/2 mitosis was 4.5 hr as determined by the time between exposure and that at which 50% of metaphase cells were labeled. At 19 to 20 hr the proportion of labeled mitoses declined 2.5%.

The duration of S phase was obtained from the difference between the two times at which 50% labeling occurred, and was found to be 10 hr (Fig. 1).

Labeling of multinucleated cells. Exponentially growing monolayers of HeLa cells were treated simultaneously with 0.01 μg/ml of Vb and 0.5 μCi/ml of tritiated thymidine for a period of 30 min. After removal of these compounds by washing several times with warm phosphate-buffered saline, the cultures were shaken at 2-hr intervals, for a period of 24 hr, to obtain mitotic cells, which were plated out on coverslips by the method described above for optimal multinucleation. One-half of the coverslips were processed for light microscopy and the other half for autoradiography.

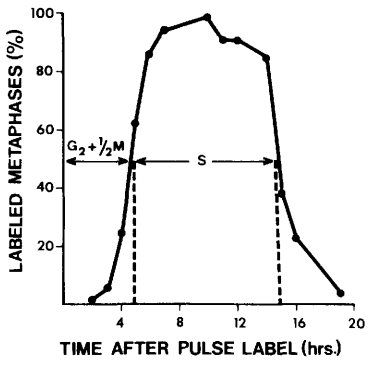

FIG. 1. HeLa *S* phase of the cell cycle determined by scoring at hourly intervals the proportion of labeled mitotic cells (metaphases) after 30-min exposure to 0.5 μCi/ml of [^{3}H]thymidine.

Autoradiography. Coverslips were fixed in 3:1 ethanol–acetic acid at 0°, rinsed in ethanol, mounted cell-side-up on a microscopic slide and dipped in Kodak NTB 3 liquid emulsion (diluted 1:1 with distilled water at 43°). The slides were then exposed in a dry, dark atmosphere (sealed box containing desiccant) at 4° for 2 days. After exposure, the coverslips were developed in Kodak D-19 developer for 2 min at 20°, fixed in Kodak acid fixer, washed for 5 min in running water, dried, and stained through the emulsion with 0.5% TB.

Effect of inhibition of DNA synthesis on Vb-produced multinucleation. Randomly growing monolayers of HeLa cells were exposed to inhibitors of DNA synthesis (17, 18) [hydroxyurea (HU), 2 m*M*) and cytosine arabinoside (Ara C, 10 μg/ml)] for a period of 1 hr. During the second half-hour of exposure, 0.01 μg/ml of Vb was added. Simultaneous controls were exposed only to Vb in all experiments. Afterward, the cells were washed and incubated with fresh medium. At 2-hr intervals, until 6 hr had elapsed from removal of the inhibitors, mitotic cells were collected by shaking the cultures, seeded, incubated for 20–24 more hr, and then fixed.

Results. The ability of Vb to produce multinucleation in HeLa cells was used to define a period of the cell cycle which determines the successful outcome of subsequent mitosis. This was accomplished by determining the period of the cell cycle of maximal sensitivity to Vb.

Maximal multinucleation [16 ± 8%(SD)] was found when cells were exposed to 0.01 μg/ml of Vb for 30 min, starting at 5 hr 26 min before mitosis (Table I), a period which is in late S, but close to the S-G2 boundary of most cells in these cultures. The majority of the multinucleated cells had 2 to 6 nuclei (Fig. 2), but cells with 10 to 18 were not infrequent. Simultaneous exposure to Vb and tritiated thymidine showed that DNA

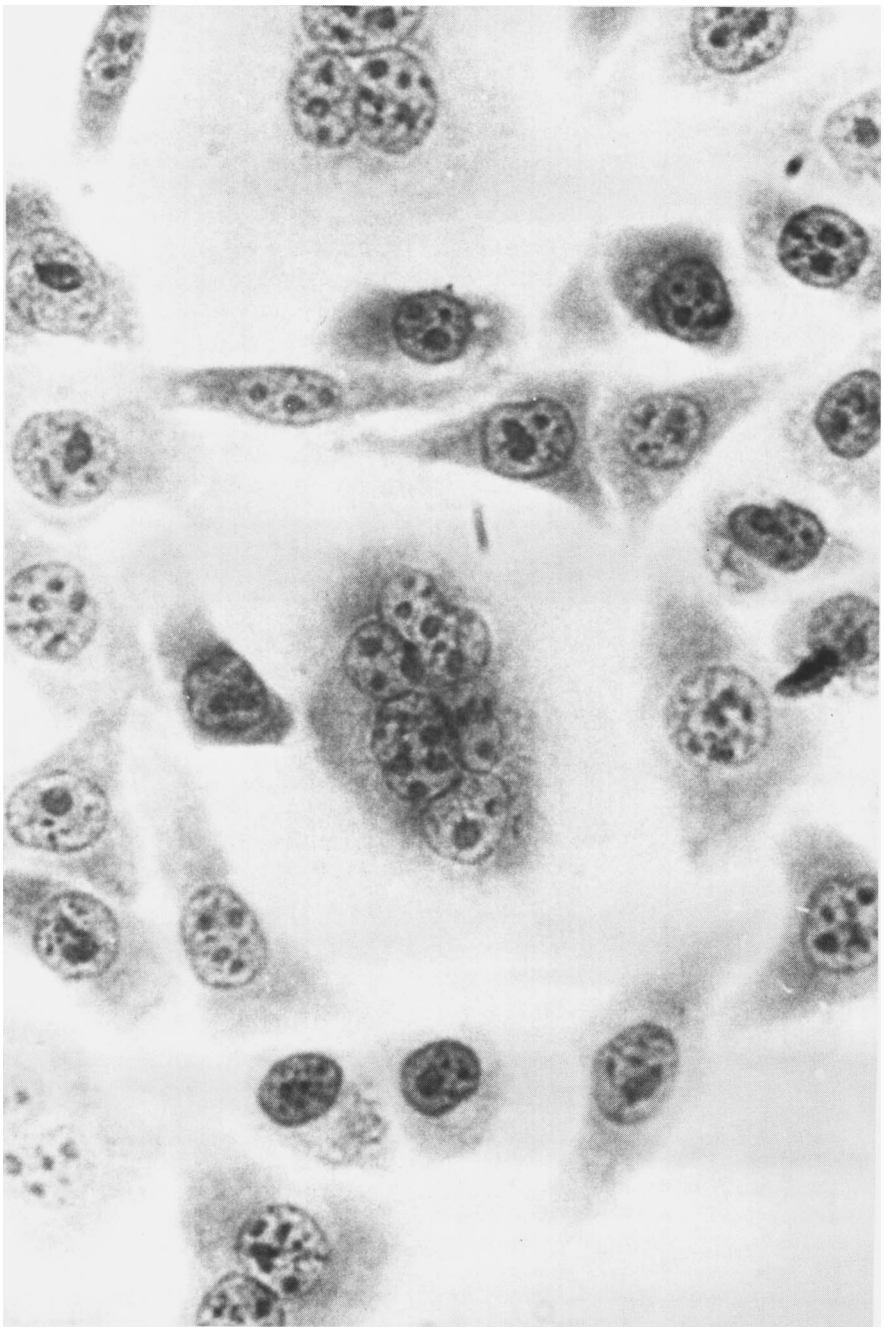

FIG. 2. Multinucleated cell obtained after 30-min exposure to 0.01 μg/ml of vinblastine, 6 hr prior to mitosis, and fixed 20 hr later. Toluidine blue, × 400.

synthesis was taking place during the period in which Vb produced multinucleation. It was observed that 100% of the multinucleated cells incorporated the radioisotope, but the intensity of autoradiographic labeling of multinucleated cells was less than that on mononuclear cells. This might have occurred because Vb-affected cells were those members of the culture which were traversing the end of the DNA synthetic phase of

TABLE I. DOSE-RELATED VINBLASTINE-INDUCED MULTINUCLEATION (0.5-hr EXPOSURE).

Concentration (μg/ml)	Peak time[a]	Maximal multinucleation (%)
0.001	2 hr	8
0.005	6 hr	10
0.01[b]	5 hr, 26 min	16
0.03[c]	6 hr	13.0
0.05[d]	4 hr, 30 min	14
0.1	4 hr	12
0.3	6 hr	11
1	6 hr	7

[a] Overall mean, 5 hr, 6 min ± 1 hr, 34 min (SD). Peak time and maximal multinucleation represent average values.
[b] Seven experiments.
[c] Two experiments.
[d] Four experiments.

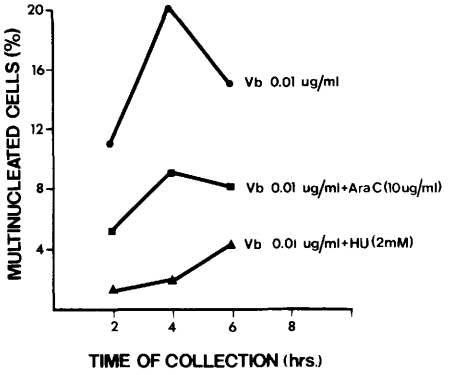

FIG. 3. Exponentially growing monolayers of HeLa cells were exposed to vinblastine (Vb), Vb + cytosine arabinoside (Ara C), and Vb + hydroxyurea (HU) for 1 hr. After removal of the drugs, mitotic cells were collected, seeded on coverslips, and incubated for 20–24 hr. After this period, coverslips were fixed and examined microscopically for determination of percentage of multinucleated cells.

the cell cycle, and the period of exposure to the radioisotope included only a few minutes of the S phase, or because Vb interfered with the uptake of the isotope.

Treatment with inhibitors of DNA synthesis markedly reduced the multinucleation produced by Vb (Fig. 3).

Discussion. The sequential events which result in cell reproduction are poorly understood. The main landmarks are DNA synthesis and mitosis, but it is obvious that a large number of processes must occur in precise order to ensure cell division. It is already well established that histone synthesis is coupled to DNA synthesis (15, 16, 19), and it would also appear that mitotic spindle formation takes place during early stages of mitosis (20, 21). It seems reasonable to assume that events preparatory for the spindle must precede formation of the visible spindle. Work reported here suggests that events crucial for orderly assembly of the spindle microtubules take place shortly before replication of the chromosomes is completed. This would explain the observed maximal disruptive effect near the S–G2 boundary. This conclusion is supported by the inhibitory effect of hydroxyurea and cytosine arabinoside, since some DNA synthesis appears to be necessary for the Vb-sensitive microtubule formative events to take place. It would appear, therefore, that the last period of chromosome replication is accompanied by events which are preparatory for microtubule assembly and are exquisitely sensitive to Vb.

Summary. The ability of Vb to produce multinucleation in HeLa cells was used to define a period of the cell cycle with special sensitivity to vinblastine. Exponentially growing monolayers of HeLa cells were treated with Vb for 30 min. After removal of Vb, mitotic cells were collected, seeded on coverslips, and examined microscopically. Maximal multinucleation was found when the cells were treated with Vb about 5 hr before mitosis, the period of time which corresponds to the late S, close to the S-G2 boundary of these cells. Simultaneous exposure to Vb and [^{3}H]thymidine showed that DNA synthesis was taking place during the period of maximal sensitivity to Vb, and treatment and inhibitors of DNA synthesis abolished this Vb effect. These findings indicate that events take place near the end of the S phase which affect the integrity of the spindle at the subsequent mitosis.

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