Cell Population Heterogeneity in the Inducibility of DNA Synthesis in Human Diploid Fibroblasts

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ABSTRACT The initiation of nuclear DNA synthesis has been studied in cytochalasin B (CB)-induced binucleate human diploid fibroblasts (WI-38 cells). Mitotic cells from different passage levels were rendered binucleate by a brief pulse of CB. The cells were then washed free of the drug, and DNA synthesis was studied by [^{3}H]thymidine labeling. The results showed that, in a small percentage of binucleate cells, one nucleus was labeled (S phase) and the other nucleus was unlabeled (G_1 phase). There was no significant difference in the percentage of these cells with increasing passage levels. The results of this study suggest that some WI-38 cells retire from the cell cycle at different passage levels, and thereby become refractory to inducers of nuclear DNA synthesis generated by sister cells in S phase.

Normal human diploid fibroblasts like WI-38 cells have a finite in vitro lifespan of 50 ± 10 passages (6, 7). The cultures pass through a period of rapid multiplication which is referred to as phase II. This is followed by declining growth and ultimate loss of the cultures which is referred to as phase III. The changes occurring in phase III were interpreted as evidence for the manifestation of aging at the cellular level. Cultures of WI-38 cells are heterogeneous with respect to their ability to divide and form clones (9, 14) and to synthesize nuclear DNA (2). When the cells were labeled with [^{3}H]thymidine ([^{3}H]TdR) for 24 h, the number of cells able to synthesize DNA decreased exponentially with the age of the cultures. DNA cytophotometric studies and autoradiographic analysis showed that cells that are not incorporating [^{3}H]TdR are in G_1 phase of the cell cycle (15). These cells are referred to as nondividing or noncycling cells and are presumed to have retired from the cell cycle. When the cells were labeled with [^{3}H]TdR for prolonged periods (i.e., 5 d), very few unlabeled cells were detected (8). Mitotic cells plated in culture medium containing [^{3}H]TdR followed by autoradiography showed the loss of ability to synthesize DNA by one but not always by both of the daughter cells (4). Cinemicrophotographic observations of cell division patterns showed that cells from late passage cultures divide but that sister cells have unusually different generation times (1). Some of the sister cells did not further divide at all during the period of observation. This suggests, as one possibility, that sister cells are accumulating inducers of nuclear DNA synthesis at different rates (12). The object of the present investigation was to determine whether sister cell nuclei respond to inducers generated by one another and initiate DNA synthesis synchronously when enclosed in a common cell cytoplasm. This was accomplished by rendering WI-38 cells binucleate by a brief exposure to cytochalasin B (CB) followed by [^{3}H]TdR labeling and autoradiography.

MATERIALS AND METHODS

Culture Methods

The culture methods for growing WI-38 cells were described in a previous communication (10). At confluence (5–6 × 10^6 cells/T-75 flask), the cells were split 1:4. At early passage, cells reached confluence in 5–7 d and at late passage in ~10–12 d. All the experimental manipulations were carried out with cells plated onto cover glasses at the densities equivalent to growth in culture flasks.

Production of Binucleates

WI-38 cells from confluent cultures at different passage levels were plated on cover glasses at densities equivalent to a 1:4 split and were allowed to recover for 48 h. The cells were then exposed to 1.0 μg/ml of cytochalasin B for 4 h to produce binucleate cells. At the end of this period, the cells were washed free of the drug and transferred into fresh culture medium for labeling studies.

Cell Fusion

The technique of cell fusion for producing heterokaryons of WI-38 cells × chick erythrocytes was described in detail previously (10). Chick erythrocytes were obtained from 13- to 15-d-old chick embryos. For cell fusion, WI-38 cells were mixed 1:4 with chick erythrocytes in the presence of 100 μl/ml of UV inactivated Sendai virus. After fusion, the cells were distributed into petri dishes containing cover glasses for subsequent labeling studies.

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Labeling Procedures and Autoradiography

The cells were labeled with 0.1 μCi/ml of [^{3}H]TdR (17.1 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 24 h. The unincorporated radioactivity was removed by extracting fixed air-dried cells with 5% TCA for 5 min at 5°C.

Autoradiographs were prepared with Kodak NTB-2 liquid emulsion. The exposure time was 2 or 10 d. The 2-d exposures were required to permit visualization of nuclear morphology at low grain count, while the longer exposures were used to establish more reliably the differences in the labeling between sister cell nuclei. The autoradiographs were stained with toluidine blue (1% wt/vol) through the emulsion.

Cells lying close to one another can be mistaken for binucleate cells. In the autoradiographs, binucleate cells were distinguished from two cells merely lying close to one another by the following criteria. Only cells that retained the fibroblast morphology and in which the nuclei were close to one another were scored. Fibroblasts are usually fusiform with an ellipsoidal nucleus. The long axis of the nucleus is oriented parallel to the long axis of the fibroblast. This morphological orientation is usually retained in the binucleate cells (Fig. 1). On rare occasions, the long axis of the nuclei was oriented at right angles to the long axis of the cell. In this case the morphology of the cell was considerably altered because of this unusual orientation of the nuclei. In both the classes of the binucleate cells the sister nuclei are roughly of identical size and morphology.

RESULTS AND DISCUSSION

Effect of CB on Nuclear DNA Synthesis

Naturally occurring binucleate cells in populations of WI-38 cell cultures are extremely rare. Binucleate cells were produced by exposing cells to CB. The effect of CB on the production of binucleate cells and the initiation of DNA synthesis were studied by exposing WI-38 cells from early and late passage cells to 1, 2, 5, and 10 μg/ml of CB for different lengths of time. The exposure of cells to 5 and 10 μg/ml for 4 h resulted in abnormal cell morphology. The exposure of cells to 1 and 2 μg/ml for 12 h or more did not result in any changes in the morphology of the fibroblasts. There was no significant difference in the percentage of binucleates produced at these two concentrations of CB. In all further experiments the cells were exposed to 1.0 μg/ml of CB for 4 h.

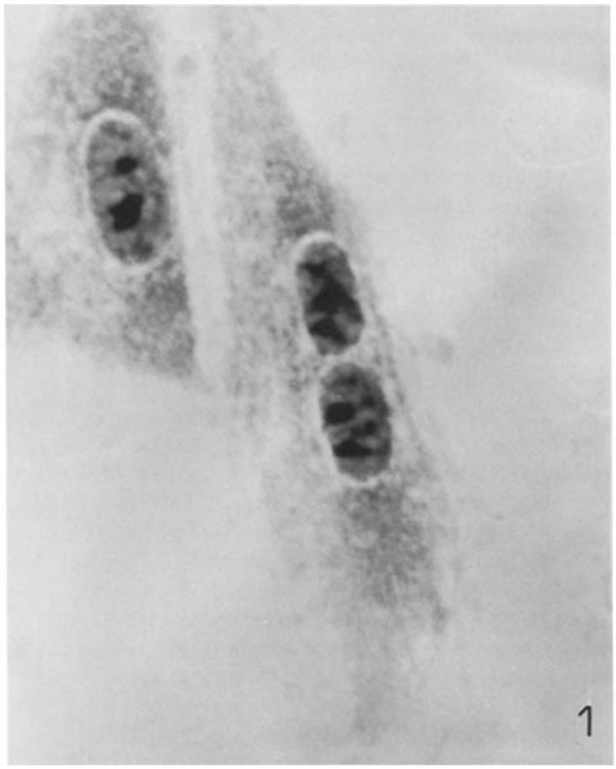

FIGURE 1 Photomicrograph of CB-induced binucleate cell from passage 40 showing identical morphology of the sister nuclei. The cells were plated on cover glasses at 1:4 split and allowed to recover for 48 h and then exposed to 1.0 μg/ml of CB for 4 h. The cells were then washed free of CB and returned to normal medium. This preparation was made after 24 h of growth in normal medium.

TABLE I

Results of [^{3}H]TdR Labeling of WI-38 Cells and Heterokaryons of WI-38 Cells × Chick Erythrocytes Exposed to CB

	Percentage of labeled mononucleate cells
A. Cells	
WI-38 cells—no CB (controls)	61
WI-38 cells + CB	59
	Percentage of chick nuclei labeled in the heterokaryons
B. Heterokaryons*	
No CB (controls)	44
CB prefusion	44
CB postfusion	44

* In this experiment WI-38 cells at passage 35 were exposed to CB for 4 h before or after cell fusion and then labeled with 0.1 μCi/ml of [^{3}H]TdR for 24 h. The percentage of labeled mononucleate cells was determined from 1,600 to 2,000 cells from the same cover glasses that were scanned for heterokaryons.

TABLE II

Results of [^{3}H]TdR Labeling in CB-induced Binucleate Cells from Different Passages of WI-38 Cells

Passage No.*	Both nuclei labeled	Both nuclei unlabeled	One labeled and one unlabeled nucleus	Percentage of labeled mononucleate cells
19	77 (69)	27 (24)	8 (7)	95
33	54 (61)	28 (32)	6 (7)	65
42	67 (70)	23 (24)	6 (6)	50
	201 (70)	65 (23)	20 (7)	

WI-38 cells plated on cover glasses were exposed to 1.0 μg/ml of CB for 4 h. The cells were then washed free of CB and labeled with 0.1 μCi/ml. of [^{3}H] TdR for 24 h. The numbers in parentheses indicate the percentage of cells in the population of binucleate cells. The percentage of labeled mononucleate cells was determined from a sample of 1,600 to 2,000 cells from the same cover glasses that were scanned for binucleate cells.

* The passages were not successively derived from a single passage for this study. DNA synthesis in binucleate cells from 12 different passages were studied, but results from early, mid, and late passage levels were tabulated here.

The effect of CB on nuclear DNA synthesis was studied in two separate experiments. In the first experiment the cells were exposed to 1 μg/ml of CB for 4 h. The cells were then washed free of CB and then labeled for 24 h with [^{3}H]TdR. As shown in Table I, there was no significant difference in the percentage of labeled nuclei between cells exposed to CB and controls that were not exposed to CB. In the second experiment the effect of CB on the initiation of DNA synthesis was studied in WI-38 cell × chick erythrocyte heterokaryons. WI-38 cells from passage 35 were exposed to CB for 4 h and then washed free of CB and fused with chick erythrocytes. In a separate experiment the cells, including heterokaryons, were exposed to CB for 4 h immediately after cell fusion. They were then washed free of

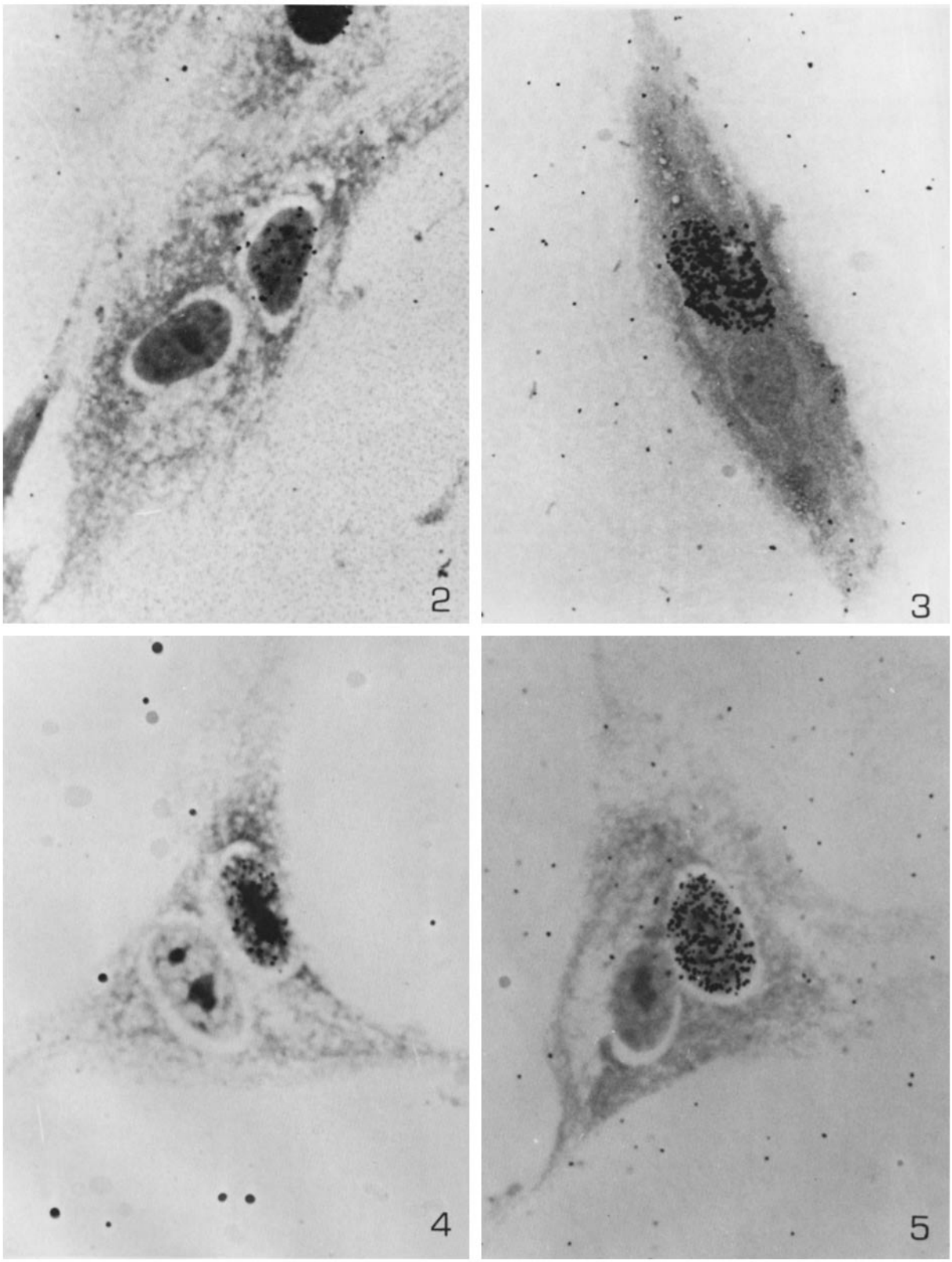

FIGURE 2 Radioautograph of a binucleate cell from passage 33 showing a labeled nucleus and an unlabeled nucleus. This is the most usual morphology of the binucleate cell. This radioautograph was exposed for 2 d to show the identical morphology of the sister nuclei. There are ~25 silver grains over the labeled nucleus.

FIGURE 3 Radioautograph of a binucleate cell from passage 35 showing a labeled nucleus and an unlabeled nucleus. This radioautograph was exposed for 10 d. There are numerous silver grains over the labeled nucleus and none over the sister nucleus. The morphology of the labeled nucleus could not be discerned because of the numerous silver grains over the labeled nucleus.

FIGURE 4 Radioautograph of a binucleate cell from passage 31 showing a labeled nucleus and an unlabeled nucleus. The long axes of the nuclei are oriented at right angles to the long axis of the cell. This radioautograph was exposed for 2 d. This brief exposure was intended to show the identical morphology of the sister nuclei. There are ~25 silver grains over the labeled nucleus.

FIGURE 5 Radioautograph of a binucleate cell from passage 21 showing a labeled nucleus and an unlabeled nucleus. The long axes of the nuclei are oriented at right angles to the long axis of the cell. This radioautograph was exposed for 10 d. There are numerous silver grains over the labeled nucleus but none over the unlabeled nucleus.

CB and labeled with [^{3}H]TdR for 24 h. The results are shown in Table I. There was no significant difference in the percentage of labeled erythrocyte nuclei in heterokaryons as a function of whether or when they were exposed to CB. There were no heterokaryons containing a labeled WI-38 nucleus and an unlabeled chick nucleus. These control experiments show that initiation of DNA synthesis is independent of CB. This interpretation is consistent with the previous reports of Fournier and Pardee (3) and Rao and Smith (11).

DNA Synthesis in CB-induced Binucleate WI-38 Cells

The potential ability of a daughter cell in S phase to induce DNA synthesis in a sister cell in G_1 was studied in CB-induced binucleate cells. The results of [^{3}H]TdR labeling of the CB-induced binucleate cells are shown in Table II. There are three subpopulations of binucleate cells with respect to [^{3}H]TdR incorporation in the nuclei: (*a*) in 70% of the binucleate cells both of the nuclei were labeled, (*b*) in 23% of the binucleate cells both of the nuclei were unlabeled, and (*c*) in 7% of the binucleate cells one nucleus was labeled and one nucleus was unlabeled (Figs. 2–5). This latter unique class of binucleate cells containing a labeled and an unlabeled nucleus was observed at all population doubling levels. There was no significant difference in the proportion of these binucleate cells between the cells from early and late passages.

The important observation is that in a fraction of CB-induced binucleate WI-38 cells, one of the nuclei incorporated [^{3}H]TdR while the other did not. Fournier and Pardee previously reported that a very small number of binucleate cells produced by exposing baby hamster kidney (BHK) cell cultures to CB included a labeled nucleus and an unlabeled nucleus (3). These authors further reported that there are naturally occurring binucleate cells in BHK cell cultures. The timing of the formation of these naturally occurring binucleate BHK cells in the previous study is not precisely known, however (3). It is likely that differential labeling in these binucleate cells is caused by asynchronous completion of DNA synthesis in sister nuclei rather than by difference in the actual initiation of DNA synthesis. This interpretation is consistent with the studies of DNA synthesis in naturally occurring binucleate cells in myeloblastic leukemia cells (13). The control experiments in Table I further argue against the possibility that the present results are caused by artifacts of exposing cell cultures to CB.

Naturally occurring binucleate cells are very rare in WI-38 cell cultures. The brief exposure of cell cultures of CB followed by [^{3}H]TdR labeling monitors the initiation of DNA synthesis in sister cell nuclei included in a common cell cytoplasm. The results of this study sharply differ from those on the initiation of DNA synthesis in the heterokaryons resulting from the fusion of HeLa × mouse and HeLa × hamster cells (5). These cells have different durations of cell cycle phases including G_1. Initiation of nuclear DNA synthesis occurred synchronously in the nuclei of these heterokaryons at a time corresponding to the shorter G_1 of the mouse or hamster parent, and several hours before the mononucleate HeLa cells entered the S phase (5).

In the present study, the labeled nucleus (S phase) in the binucleate cells containing a labeled nucleus (S) and an unlabeled nucleus (G_1) traversed G_1 and reached S independent of the sister nucleus residing in the same cell cytoplasm. The G_1 nucleus in these binucleate cells is therefore presumed to have "retired" from the cell cycle. The results of the present study suggest that WI-38 cells retiring from the cell cycle at different passage levels become refractory to inducers of DNA synthesis generated by the sister cells in S phase.

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