A Method for the Selection of a Synchronized Population of Cloning HeLa Cells[1] (37216)

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A number of methods have been described for the synchronization of mammalian cells in tissue culture. These generally fall into three categories: (a) the removal of cells from a full sheet by gentle agitation which will dislodge those cells in the process of mitosis (8, 10); (b) the use of metabolic blocking agents or the removal of an essential nutrient which arrest the cells at a particular point in the cell cycle (3, 6, 9, 11, 12); (c) the use of cold shock treatment which arrests the cells at the beginning of the S phase (5, 7). These methods have been used most commonly for the synchronization of large numbers of cells either in a cell sheet or in suspension culture. Synchronization methods which utilize blocking agents or cold shock are deleterious to the cell function of small populations and it is also unlikely that the cells resume their normal metabolic function immediately following removal of the block (2, 9). In addition, there is no certainty that the cells are not permanently damaged by cold shock or the chemical blocking agents. Cells dislodged in mitosis frequently are in suspension in various sized clumps, which even when filtered still contain 2 and 3 cell groups which must be further treated to obtain single cells. More important, these procedures are not amenable to the study of small cell populations in clonal growth. Assays that examine the clonal growth of a small number of cells require very stringent techniques and optimum conditions for growth, and the techniques described above are unsatisfactory. In order to examine cloned cells in synchrony, we devised a technique whereby cells could be selected immediately after division, and their clonal growth observed in synchrony for 72 hr.

Methods and Materials. HeLa cells (obtained from Flow Laboratories, Rockville, MD) were maintained as a continuous cell line by accepted techniques (4). Cells were grown in minimal essential medium with Earle's salts (MEM-Earle) with 10% heat inactivated (56° for 30 min) fetal calf serum (FCS) and 50 units of penicillin and 50 units of streptomycin/ml. A barely confluent sheet of HeLa cells was used to prepare the cell suspension. The growth medium was decanted from the cell sheet and the surface was rinsed once with warm saline. A volume of 0.25% trypsin or trypsin-EDTA (Gibco) just necessary to cover the cells was then added (trypsin-EDTA is more likely to assure a single cell suspension). The bottle was then incubated at 37° for 5–10 min. As soon as the cells were released from the surface of the bottle, the trypsin activity was halted by adding MEM-Earle with 20% FCS, and the cells were pipetted up and down several times using a 5 ml pipette to assure a single cell suspension. The suspension was then centrifuged for 5 min at 22*g*, the supernatant medium was discarded and the cells were suspended in MEM with 10% FCS. After mixing thoroughly by pipetting, the suspension was examined microscopically in a hemocytometer chamber; an adequate suspension was one that contained less than 0.5% of the cells in clumps of 2, 3 or more. If the suspension contained too many clumps of cells, it was discarded. Two thousand cells in 0.2 ml

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of medium were pipetted into milk dilution bottles (12 × 4 × 4 cm) and the final volume was brought to 10 ml with MEM with 10% FCS. For experimentation purposes, this final volume was adjusted to 9 ml so that additives in volumes of not more than 1 ml could be added after the synchronized cells were selected. Bottles were then placed in a CO_2 incubator (5%) at 37° immediately, and allowed to incubate for 1–1.5 hr. At the end of this time the bottles were removed from the incubator and examined by inverting them and scanning the surface with a dissection microscope at 30–80×. Any cells that could be found that were double, were assumed to have divided after the essentially single-celled suspension had been added to the bottle. The double cells were marked for future identification by circling them with a felt-tipped black marking pen. It is also advisable to prepare a chart and mark the position of the double cell in the circle so that there will not be any confusion with cells which may have been included in the circle. The bottles were then replaced in the incubator and examined at various time intervals for cell growth. It is advisable to treat the bottles gently at all times, since cells in mitosis tend to detach from the glass more easily than nondividing cells (1).

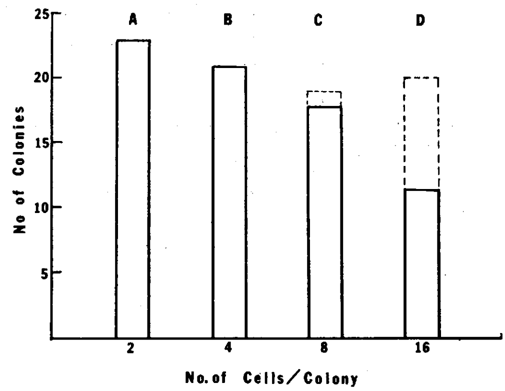

FIG. 2. Longevity of synchrony method. (A) Originally selected 2 cell colonies; (B) 24 hr postselection; 21/23 of the colonies are 4 cells; (C) 48 hr postselection; 18/23 of the colonies are 8 cells; (19/23 are 7 or 8 cells); (D) 72 hr postselection; 12/23 of the colonies are 16 cells; (20/23 are 14, or 16 cell colonies).

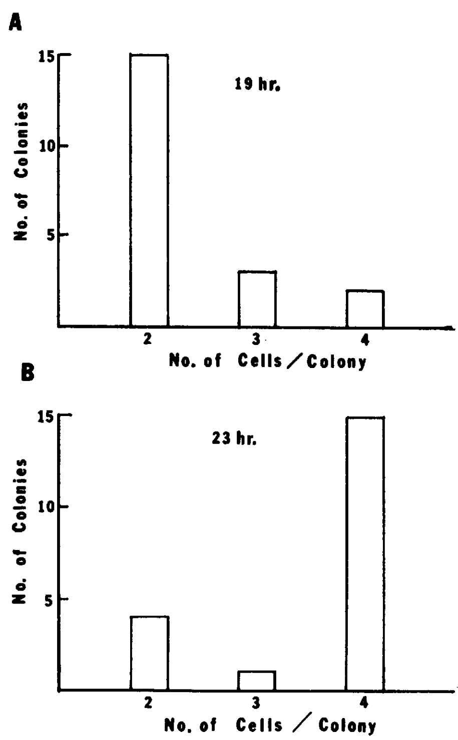

FIG. 1. Examaination of 20 selected 2-cell colonies at 19 hr (A) and 23 hr (B) after attachment to the glass.

Results and Discussion. When 2-cell HeLa colonies are selected in the manner described, 75% or more of them divide synchronously into four cell colonies (over a period of 3–4 hr) approximately 20 hr after their selection. This time period varies depending upon: (a) the length of the cycle of the particular line of cells being synchronized, (b) the general metabolic health of the cells at the beginning of the experiment, (c) the rapidity with which the preparation and selection of cells is carried out. Figure 1 shows the collective data for 20 clones in the same bottle and their distribution as 2-, 3- and 4-cell colonies when the bottle was examined 19 hr (Fig. 1A), and 23 hr (Fig. 1B) after the addition of cells (16 to 20 hr following selection). In this experiment 15 of the 20 colonies were in synchrony and divided within 4 hr of each other. Figure 2 demonstrates the length of time that selected cells remain in synchrony. In these experiments 21/23 of the 2-cell colonies selected were in absolute synchrony at 24 hr. By 48 hr, only 18/23 of the colonies were in absolute synchrony, and by 72 hr this number had dropped to 12/23. If, however, instead of enumerating only the 8- and 16-cell colonies as being in synchrony, we take into account the single cell which may have failed to divide and count the colonies which contained either 7 or 8 cells (at 48 hr) and the 14 or 16 cells (at 72 hr) the number of clones in synchrony then becomes 19/23 and 20/23, respectively. This is shown by the dotted continuation of the Fig. 2 histogram.

This technique then offers almost complete cell synchrony for 72 hr after the cells are selected. In addition, these cells have not been subjected to harsh treatments and their growth rate is completely normal; experimental procedures examining the effects of radiation, growth inhibitors and cytotoxins, etc., on the cell cycle are easily carried out using this method.

Summary. A method is described in which individual clones of cells in synchrony can be selected out of a nonsynchronized population of cells. These cells have progressed no more than 3 hr into the G1 phase of the cell cycle. The clonal growth of these colonies can be observed microscopically for up to 72 hr before they begin to lose synchronous cell division.

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