THE EFFECT OF THYMIDINE ON THE

DURATION OF G_1 IN CHINESE HAMSTER CELLS

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ABSTRACT

The generation time of a Chinese hamster cell line was varied by the use of different lots of sera in the culture media. Analysis of the division waves following thymidine synchronization showed that lengthening of the generation time was a result of an increase in duration of the G_1 phase and that thymidine treatment reduced the duration of G_1 back to its minimum value.

INTRODUCTION

Variations in generation time of cultured cells arise not only from diversity among individuals, but also from changes in the biochemical environment. Time-lapse studies of environmental effects on human amnion and kitten lung cells (1) and radioautographic analysis of tritiated thymidine incorporation by HeLa S-3 cells (2) have indicated that increases in generation time arise primarily from a lengthening of the G_1 period. In this report, confirmatory evidence for G_1 expansion is provided for Chinese hamster ovary (CHO) cells, based on measurements of duration of the major intervals in the life cycle following thymidine synchronization. Evidence is further presented suggesting that thymidine synchronization brings about a contraction of the expanded G_1 phase of slowly growing cultures.

MATERIALS AND METHODS

Suspension cultures of CHO cells were set up at 3–5-wk intervals from stock-bottle cultures. At intervals of approximately 90 days, new stock-bottle cultures were started from a large pool of frozen cells. In this way, variations arising from long-term continuous cultivation were minimized. Cells were routinely examined for PPLO contamination with the agar described by Chanock et al. (3). No PPLO were observed.

Cells were grown in suspension culture in F-10 medium (4) without calcium, supplemented with 10% calf and 5% fetal calf sera, and 100 µg/ml each of penicillin and streptomycin. A series of cultures of different generation times was obtained from the stock CHO cell line by the employment of a number of different lots of calf sera in the nutrient medium. As long as a given lot of serum was employed, the generation time of the culture remained constant. When the lot of serum was changed, slowly growing cultures could be made to grow more rapidly, and the reverse was also true.

Our methods of synchrony induction have been described previously (5, 6). Thymidine was added to cultures with generation times in the range of 13–24 hr to a final concentration of 10 mM. After 9 hr in blockade, thymidine was removed by the method of washing the cells and resuspending in fresh medium containing the normal amount of thymidine (10^{-6} M). Cell concentrations were determined to a statistical precision of 1% or better with an electronic particle counter.

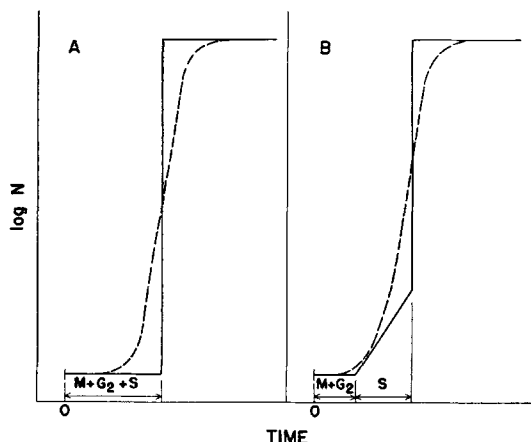Cycloheximide (Acti-dione) was purchased from the Upjohn Company, Kalamazoo, Mich.

FIGURE 1 Effect of dispersion on idealized synchrony waves. Log N (cell concentration) is plotted against time after release from thymidine blockade. In Fig. 1 A, it is assumed that the entire population was collected at and released from the G_1/S boundary. The solid line in A represents the expected pattern of division, assuming no dispersion; the broken line represents the division pattern, assuming symmetrical dispersion. In Fig. 1 B, it is assumed that the S cells were stopped in situ, while the remainder of the population was collected at the G_1/S boundary. The solid line in B represents the expected pattern of division, assuming no dispersion; the broken line represents the division pattern, assuming symmetrical dispersion. The method of determination of duration of the combined M, G_2, and S phases is also shown in both A and B, illustrating the rationale for utilizing the midpoint of the synchrony wave.

DESCRIPTION OF DIVISION

PATTERNS

Temporal mapping of the location of specific events within the life cycle of the cell (7, 8) has been based on the observed time lag between initiation of an action (e.g., application of inhibitor) and appearance of an effect on the mitotic index or on cell division, the advantage in the latter case being the ease and precision with which total cell number can be determined electronically.

When DNA synthesis is inhibited by adequate quantities of thymidine (the minimum amount of thymidine required to bring about an immediate cessation of DNA synthesis), cells in the S phase (the DNA synthetic period) of the life cycle are unable to continue and appear to suspend progress toward division, whereas cells in all other parts of the life cycle are unaffected (5, 9, 10). When the latter cells reach S, they also stop and accumulate

at the G_1/S boundary. All cells initially in G_2, M, and G_1 eventually accumulate, and these phases are devoid of cells. Upon removal of thymidine block, progress around the life cycle resumes, and a characteristic pattern of cell division is observed (5, 10) which reflects the pattern in which the cells were immobilized by blockade. Specifically, there is a period equal to the duration of G_2 + M during which the cell number remains constant, followed by a period equal to the duration of S during which cells trapped in the S phase during block reach division and during which the cell number rises exponentially at a rate equal to that of the original, random culture (since the S cells were immobilized during block). The pattern of division produced by these cells has been called the "S division-wave" or simply the "S wave." When cells accumulated at the G_1/S boundary reach division, there is a rapid increase in cell number, the "synchronous division wave" or "synchronous wave." Finally, division will cease and cell number will remain constant until the S wave returns again a generation later.

The steepness of the synchronous wave will be limited by two factors: the degree of "compaction" attained by thymidine treatment and the amount of subsequent dispersion resulting from nonuniform rates of traverse of the life cycle. Experimental results on rate of decay of synchrony (11) indicate the latter to be the determining factor. For accurate timing, account must be taken of the effect of dispersion on division pattern.

If initial synchronization were perfect and there were no dispersion, the division pattern would be that given by the solid line of Fig. 1 A, a step function in which all cells divide simultaneously. However, if dispersion occurs and if it is symmetrical (i.e., if there are equal numbers of cells moving faster and more slowly than the average), then the pattern will resemble the broken line of Fig. 1 A. A finite time is required for the population to divide, the curve becoming sigmoid, but the central portion of the curve approximates a straight line and, most important, the midpoint corresponds in time to the undispersed wave.

For a single-thymidine block in which S cells are not synchronized, the situation is as illustrated in Fig. 1 B. The solid line is the idealized case in which M + G_2 is the time to the first resumption of growth and S is the additional time to the synchronous wave. Symmetrical dispersion results in the broken line in Fig. 1 B. Again the midpoints
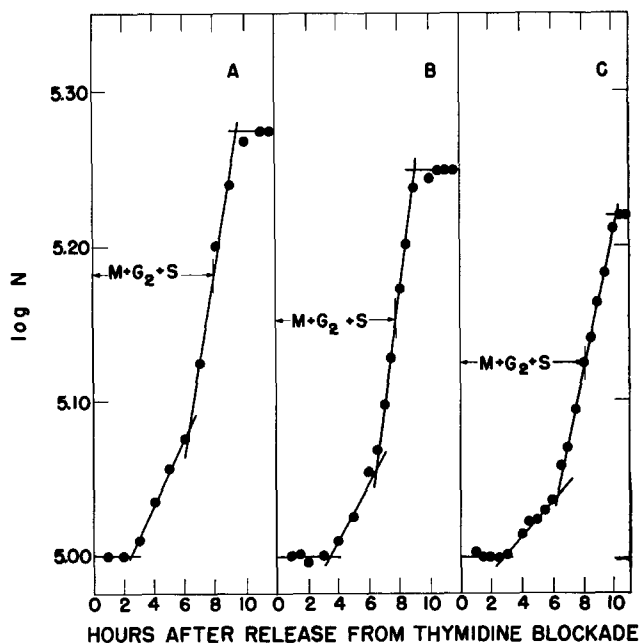
FIGURE 2 Patterns of division for three CHO cultures treated once with 10 mM thymidine for 9 hr and then resuspended in normal medium at $t = 0$. Generation times for the three cultures prior to thymidine addition were 13.3 hr (A), 16.5 hr (B), and 24.1 hr (C). The variations in generation time could be ascribed to the lot of serum employed in the growth medium. The duration of the $M + G_2 + S$ phases is taken to be the time from release to the midpoint of the linear approximation to the synchronous division wave. The synchronous division wave is the pattern of division following reversal of thymidine block which represents the cells trapped at G_1/S during block.

correspond in time, and straight-line approximations to the data do not introduce significant error, provided the midpoint of the synchronous wave is used for identification of the G_1/S boundary.[1] Similarly, the fraction of cells in S (amplitude of the S wave) should be well approximated by intersection of the lines, but the slope of the S wave will appear too steep.

RESULTS

Typical division patterns of cultures grown in media containing different serum lots and synchronized by a single thymidine blockade are shown in Fig. 2. The generation times prior to thymidine addition were 13.3 hr (Fig. 2 A), 16.5 hr (Fig. 2 B), and 24.1 hr (Fig. 2 C). The duration of the combined $M + G_2 + S$ period following thymidine removal was determined graphically as indicated. Application of the same method to similar data from other cultures (grown in different lots of serum) gave the results summarized in Fig.

[1] Estimating the midpoint of the synchrony wave by linear interpolation on a semilogarithmic plot corresponds to choosing the time at which the population has risen by a factor $\sqrt{k}$, rather than by the rigorously correct factor $(1 + k)/2$, where k is the factor by which cell concentration increases during the wave. For the data at hand, the difference amounts to only 0.1 hr in timing.
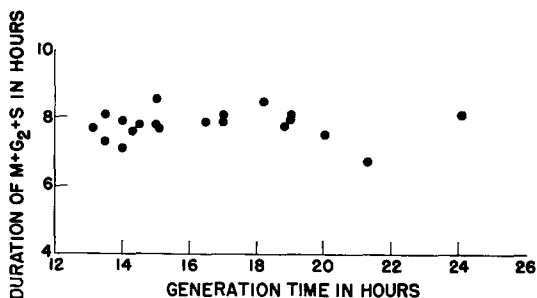

FIGURE 3 Duration of the $M + G_2 + S$ period in a series of synchronized CHO cultures with different generation times. Differences in generation time of the cultures were due to the different lots of sera employed in the growth medium. Conditions of synchronization were those described in Fig. 2.

3, in which the duration of $M + G_2 + S$ is plotted against generation time. In all cases, the duration of thymidine blockade was 9 hr. The calculated mean duration of $M + G_2 + S$ following block removal for the 20 cultures in Fig. 3 was 7.8 $\pm$ 0.1 hr, in excellent agreement with the value of 7.75 $\pm$ 0.5 hr obtained from life cycle analysis of Colcemid-treated random cultures of CHO cells (12).

It is apparent that the measured duration of the $M + G_2 + S$ period following reversal of a single-thymidine block is independent of generation time of the culture. The simplest interpretation of this
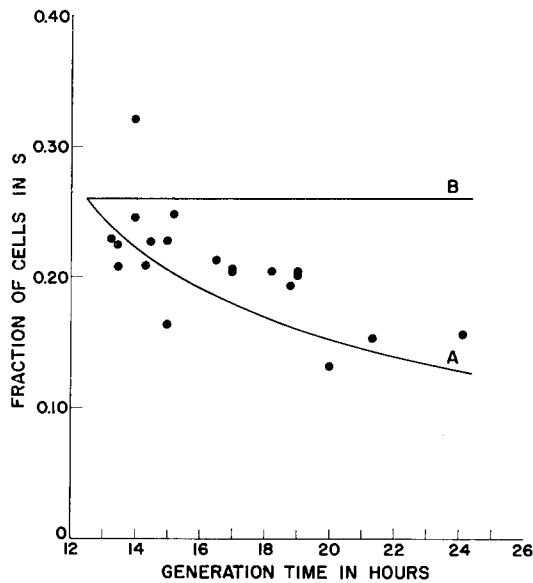
FIGURE 4 Measured fraction of cells trapped in the S phase during thymidine block (data points) for the cultures with different generation times described in Fig. 3. Curve A represents the theoretically expected fraction of cells dividing if only G_1 expanded in the slowly growing cultures. Curve B represents the theoretically expected fraction of cells dividing if all phases of the life cycle had expanded in slowly growing cultures and all phases were reduced proportionally following thymidine treatment.

observation is that in slowly growing cultures the lengthening of generation time can be accounted for by an expansion in duration of the G_1 phase (which is the difference between generation time and duration of the $M + G_2 + S$ phases). The data in Fig. 3 do not exclude the alternate possibility that all phases of the life cycle were expanded proportionally in slowly growing cultures and that thymidine treatment merely reduced the durations of all phases back to the values of rapidly growing cultures.

These alternatives can be distinguished on the basis of the number of cells which are immobilized in the S phase during thymidine block. In a random population with generation time T_G, the fraction of cells $N(\Delta T)$ present in the interval between T_1 and T_2 is given by the equation (8),

$$N(\Delta T) = 2^{T_2/T_G} - 2^{T_1/T_G} .$$

We have used this equation to calculate the fraction of cells in the S phase of the life cycle as a

a function of generation time on the assumptions that (a) lengthening of the life cycle is due solely to a lengthening of G_1, or (b) all phases lengthen proportionally. In the first case, the S fraction declines with increasing T_G; in the second, it remains constant.

Before comparison with the experimental data, a small correction is necessary owing to the fact that, in the process of thymidine synchronization, $M + G_2$ cells divide before they are collected in the synchronized population. Their increased number has the effect of reducing slightly the fraction of S cells as determined from the division pattern following release from block. The theoretical curves (representing the expected fraction of S phase cells in cultures of different generation time) for the two models are shown in Fig. 4.

Although dispersive forces tend to blur the boundaries of the S wave, the number of cells comprising the S wave can be determined directly from straight-line approximations to the data as in Fig. 2. One determines the cell concentration at the point at which the S and synchronous division waves intersect. The difference between this cell concentration and the initial concentration of cells determined shortly after thymidine removal represents the number of S phase cells which divided in the culture. The ratio of S phase cells to the total number of cells which divided in the combined S and synchronous division waves[2] is then

[2] The mean increase in cell number for the 20 experiments in Fig. 3 was 71%, with extremes of 64 and 88% rather than the 100% expected if all cells divide. Similar reduced yields have been observed in different lines when cells were synchronized with amethopterin (13) or by temperature shock (14, 15), indicating that the reduced yield is not peculiar to the thymidine synchronization technique employed. Approximately 70% of the total CHO cell population divides in each succeeding division wave for several subsequent generations, and a second thymidine block does not further reduce the fraction dividing (unpublished observations). Furthermore, thymidine-synchronized CHO cultures and nonblocked control cultures yield similar results for the trypan blue permeability test. The reduced number of dividing cells is, therefore, not attributable to a selective killing of part of the population by thymidine. Experiments in progress suggest that the reduced fraction may be due to delayed separation of daughter cells, and a few clones of CHO cells have recently been obtained in which the cell number (determined with a particle counter) doubles following the thymidine blockade procedure.

equivalent to the fraction of S phase cells in the culture.

Measured fractions of S phase cells (data points) are given in Fig. 4, along with theoretical curves for the expected fraction of S phase cells for the G_1 expansion model (Fig. 4 A) and for the model of proportional expansion and contraction of all phases of the life cycle (Fig. 4 B). The data are consistent with expansions in the G_1 phase only.

One further line of evidence argues against a proportional expansion model. The time of action of the inhibitor of protein synthesis, cycloheximide, is defined as the latest time in the life cycle preceding division that the drug can prevent the cells from dividing (5); cells closer in time to division will divide in the presence of the drug. This time point, located in late G_2, should change if all phases of the life cycle expand proportionally as the generation time increases. In experiments with randomly growing cultures with generation times of 13.0, 16.5, 19.0, and 21.0 hr, the corresponding times of action measurement (2 μg/ml cycloheximide) were 60, 61, 62, and 60 min, respectively.

If, in the slowly growing cultures, the G_1 phase had remained expanded during thymidine treatment, then the thymidine block time of 9 hr would be too short (by a factor of about two) to permit accumulation of all cells outside of S at the G_1/S boundary, since a large fraction would still be traversing G_1 at the time of release. The synchronous division wave, therefore, would be reduced in amplitude and would be followed by another group of cells exhibiting the rate of division of the initial random culture. Dispersion of the population and experimental error might blur the boundary between these two populations, but the effect would be to reduce the rate of division of cells in the synchronous wave in slowly growing cultures. The fraction of cells in the combined S and synchronous waves would also be reduced in slowly growing cultures, since a large number of cells would divide in the third group of cells following the S and synchronous waves. Values for apparent doubling times of the synchronized cell populations (cells trapped at the G_1/S boundary during blockade) for the various cultures are presented in Fig. 5 A, and the total fraction of cells dividing in the combined S and synchronous waves is presented in Fig. 5 B. The patterns of division in all 20 cultures were similar to those in Fig. 2; there was no evidence for a sizable third group of cells following the synchronous wave. The rate of
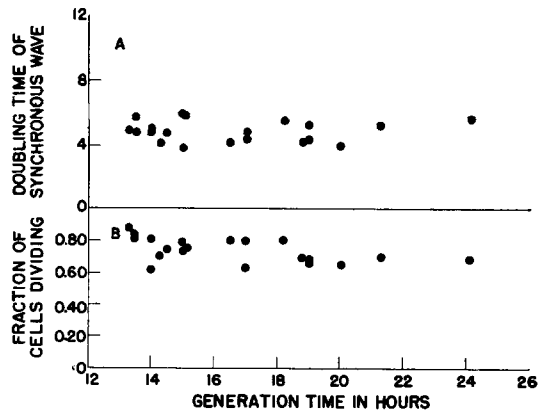

FIGURE 5 Doubling time of the synchronous division waves (A) and fraction of cells dividing in the S and synchronous division waves (B) after a single-thymidine block for the series of cultures with different generation times described in Fig. 3. The S division wave cells are those trapped in S during thymidine block. The synchronous division wave cells are those trapped at the G_1/S boundary during block. The patterns of division from which these data were calculated were biphasic (as in Fig. 2) for all cultures.

division of cells in the synchronous wave (Fig. 5 A) is approximately the same for all 20 cultures. While a very slight trend toward a decreased fraction of dividing cells in slowly growing cultures is observed in Fig. 5 B, the data are clearly inconsistent with a model in which the enlarged G_1 phase in slowly growing cultures remains expanded throughout the period of thymidine block. Rather, the results indicate that essentially all cells outside of the S phase in the various cultures have been collected at the G_1/S boundary during the 9-hr period of thymidine blockade, suggesting that thymidine treatment brings about a contraction of expanded G_1 phases in slowly growing cultures (i.e., those in which the variability in generation time is due to factors in the nutrient medium) to a duration close to the 5-hr value characteristic of rapidly growing cultures.

DISCUSSION

In studying the effects of thymidine on large populations of CHO cells, we have confirmed the findings of Sisken and Kinosita (1) and Terasima and Tolmach (2) that expansion of the G_1 phase is primarily responsible for lengthening the generation time in cultures of mammalian cells in which the variation in generation time can be ascribed to the composition of the growth medium. It is

also apparent that, under the conditions of thymidine treatment described here, the expanded G_1 phase in slowly growing cultures is reduced to a duration approximately equivalent to the minimum duration of G_1 observed in rapidly growing cultures. Galavazi and Bootsma (16) reported an apparent contraction in both the G_1 and G_2 phases in a human kidney cell line following two successive thymidine treatments (i.e., double-thymidine block); the bulk of G_2 reduction occurred apparently only after the second block. Rao and Engelberg (17) also observed a shortening of duration of the G_2 + S phase following release of HeLa cells from a double-thymidine block.

Although we have not attempted to measure the duration of the M + G_2 phase in our experiments because of effects of dispersion in obscuring the end of this period, the uniformity of duration of the combined M + G_2 + S phases for all cultures studied suggests that duration of the G_2 and M phases is not grossly different among the various cultures following a single-thymidine treatment. In 11 double-thymidine block experiments with CHO cells, we have observed a small reduction in duration of the M + G_2 + S phases from the 7.8 hr observed here to 7.4 ± 0.05 hr. It is possible

that our shorter duration of the M + G_2 + S phases obtained after double-block is due to a shortening of the G_2 period, as reported by Galavazi and Bootsma (16). However, we are unable to determine from our double-block data where the shortening occurs. Final resolution of this problem will require additional experiments.

In view of the effect of thymidine on duration of the G_1 phase following a single block, the proper duration of the thymidine block for optimum synchrony appears to be independent of generation time of the culture, but is instead equal to the sum of the durations of the G_2 + M + G_1 phases in a rapidly growing culture (about 9 hr for the CHO cells employed in this study). Since prolonged thymidine blockade has been reported to lead to chromosome abnormalities (18), the block period should be kept as short as possible, consistent with complete collection of the population at the G_1/S boundary.

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