STIMULATED LYMPHOCYTE CULTURES

Responder Recruitment and Cell Cycle Kinetics

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The proliferative response of cultured lymphocytes following stimulation with either mitogens, or allogeneic cells is used widely to assess the immunological reactivity of an individual, or histocompatibility of two individuals (1–4). However, little is known about cell cycle kinetics, and quantitative aspects of the cellular proliferative response upon stimulation. When cultures are harvested (at the appropriate time after stimulation), the uptake of [^{3}H]thymidine ([^{3}H]TdR)[1] by responding, dividing lymphocytes varies with the concentration of the stimulant (mitogen [5–7], or allogeneic cells [8, 9]). The exact cellular mechanism responsible for this variable response remains unexplored. It might be theorized that among the potentially responsive clone, the number of responding lymphocytes varies with the concentration of the stimulating agent (trigger theory [10]). However, it is alternatively conceivable that all cells of the responsive clone are triggered into proliferation, and that the velocity of their subsequent transit through the cell cycle depends upon the concentration of the stimulant (push theory [10]). Furthermore, responsive cells may enter the division cycle earlier with higher doses of stimulant. All three mechanisms would explain why [^{3}H]TdR incorporation into DNA varies with the strength of lymphocyte stimulation.

In our first approach to investigate this problem, we synchronized in vitro stimulated lymphocyte cultures with hydroxyurea, to study their cell cycle kinetics. If in such a setting the kinetics of the cell proliferation were independent of the strength of the lymphocyte stimulation, one could conclude that the number of responding lymphocytes varies with the strength of stimulation. On the other hand, if one found the duration of the cell cycle related to the strength of stimulation, a faster or slower exponential growth of responding cells accounted for different stimulation ratios.

To study the problem more directly, stimulated lymphocytes were cultured in the presence of colchicine to prevent clonal proliferation of responding cells. In this setting, thymidine uptake is a measure for the number of cells triggered into proliferative response. This allows one to compare the relative number of responding cells with different strengths of stimulation, and to determine when newly responding cells enter the division cycle for the first time.

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[1] *Abbreviations used in this paper:* [^{3}H]TdR, [^{3}H]thymidine; MLC, mixed leukocyte cultures; PHA, phytohemagglutinin; TCA, trichloroacetic acid.

In this paper, we report data on cell cycle kinetics in dog lymphocyte cultures stimulated with phytohemagglutinin (PHA), and allogeneic cells. We present indirect and direct evidence that in stimulated lymphocyte cultures the number of responding lymphocytes varies with the strength of stimulation, and that relatively more responding cells enter the division cycle earlier with greater stimulation.

Materials and Methods

Animals.—Venous blood from normal foxhounds was used as the source of responding lymphocytes. Lymphocytes used as stimulators in the mixed leukocyte cultures (MLC) were prepared from venous blood of normal beagles.

Lymphocyte Culture Techniques.—Four parts heparinized dog blood (50 U heparin/ml whole blood) and one part dextran (mol wt 115,000, Pharmacy Department, Clinical Center, NIH) were mixed, and the red cells allowed to sediment spontaneously. The lymphocyte concentration of the supernate was adjusted with culture medium to 2×10^5/ml for responding cells, and 2×10^6/ml for cells stimulating in MLC. Culture medium consisted of 400 ml Eagle's minimal essential medium (MEM) supplemented with 75 ml decomplemented heparinized pooled dog plasma, 10 ml L-glutamine (200 mM), 10 ml streptomycin-penicillin (5,000 U/ml each), and 5 ml nonessential amino acids (all obtained from Flow Laboratories, Inc., Rockville, Md.). The content of one vial of PHA-P (Difco Laboratories, Detroit, Mich.) was diluted in 5 ml MEM, and 0.1 ml of this stock solution diluted further with MEM as indicated. 1-ml cultures of responding cells were stimulated by addition of different concentrations of PHA in 0.1 ml MEM, or of 0.1 ml of the suspension of stimulating cells. Stimulating allogeneic lymphocytes were irradiated immediately before mixing them with the responding cells, with 2,500 rads at a dose rate of 1,666 rad/min from a cesium source (Kewaunee Scientific Equipment Corp., Adrian, Mich.). The cultures were maintained in a humidified 5% CO_2 air atmosphere at 37°C.

PHA-stimulated cultures were harvested on day 4, and MLCs on day 7: [^{3}H]TdR (2 μCi in 0.1 ml; obtained from New England Nuclear, Chicago, Ill.) was added for 4 h. The cultures were then centrifuged at 4°C for 10 min at 250 *g*; the pellet was washed once with 4 ml phosphate-buffered saline (pH = 7.4), and resuspended in 4 ml 5% trichloroacetic acid (TCA). After a 30 min incubation at 4°C, the acid-insoluble material was washed again, first with 4 ml 5% TCA, then with 4 ml absolute methanol. The pellet was finally dissolved at 60°C for 15 min in 0.5 ml of hydroxide of hyamine (New England Nuclear), and this transferred into 12 ml of scintillation cocktail consisting of a 0.6% solution of 2, 5-dyphenyloxazole (New England Nuclear) in toluene (Fischer Scientific Co., Fair Lawn, N. J.). The radioactivity of the TCA-precipitable material was determined in a liquid scintillation counter (Tri-Carb model 3390, Packard Instrument Co., Inc., Downers Grove, Ill.). Corrections for quenching were made by internal standard. The strength of lymphocyte stimulation in a particular experiment (stimulation ratio) was expressed as the ratio: mean of four stimulated cultures/mean of four unstimulated cultures.

Hydroxyurea (Ben Venue Laboratories, Inc., Bedford, Ohio) was dissolved, and stored frozen in 1-ml aliquots at −20°C until use. It was then further diluted with MEM to 12 times the concentration needed for the cultures, and added in 0.1-ml vols.

Dose-Related Suppression of DNA Synthesis by Hydroxyurea.—In order to determine what

[2] In this context, the term "synchrony" is used to describe the phenomenon that all cells of a dividing cell population pass through the same stages of the division cycle simultaneously (as a cohort). "Homogeneity" and "heterogeneity," respectively, denote the degree of variability in the duration of cell cycle phases among cells of a synchronized cell cohort.

concentration of hydroxyurea suppresses DNA synthesis, hydroxyurea was added to PHA-stimulated cultures on day 4, and to MLCs on day 7 to yield final concentrations between 0.125 and 4.0 mM. To the controls 0.1 ml MEM was added. 15 min later, 2 μCi [^{3}H]TdR was added to all cultures, which were harvested 4 h later. The ratio of [^{3}H]TdR incorporation between hydroxyurea-treated, and hydroxyurea-untreated cultures was calculated from the means of quadruplicate samples.

Synchronization of Stimulated Lymphocyte Cultures.—We assumed that a concentration of hydroxyurea that suppressed DNA synthesis was equally effective in inhibiting cells from proceeding from G_1 into S phase of the cell cycle. After 4 mM hydroxyurea was found to suppress DNA synthesis in stimulated lymphocyte cultures (see details under Results), this concentration was used throughout the following studies in an effort to synchronize stimulated lymphocyte cultures. Hydroxyurea was added to PHA-stimulated cultures on day 3, and to MLCs on day 6. In the initial studies, the cells were incubated with hydroxyurea for 20 hr; after cell cycle kinetic data of these experiments were available, the incubation time with hydroxyurea was shortened to 14 h. After incubation with hydroxyurea, the cultures were centrifuged at 250 *g* for 10 min, the supernate discarded, and the cells forming the pellet washed once with 2 ml of culture medium; they were then resuspended in 1 ml culture medium. The time when the 2-ml wash vol was added was considered to be the time of release from the hydroxyurea block ($t = 0$) in all experiments.

Rate of DNA Synthesis in Synchronized Cultures.—The rate of DNA synthesis was determined hourly after cultures were released from the hydroxyurea block: quadruplicate cultures were pulse labeled ([^{3}H]TdR added for 30 min), then harvested after addition of 0.1 ml of 20 mM cold thymidine in MEM. Using this method, a relative measure for the mean rate of DNA synthesis of all cells incorporating [^{3}H]TdR was obtained.

Metaphase Arrest of Dividing Cells.—3 h after lymphocyte cultures were released from a hydroxyurea block, 0.55 μg of colchicine (Eli Lilly & Co., Indianapolis, Ind.) in 0.1 ml MEM was added to all cultures. Quadruplicate cultures were removed hourly thereafter; the cells were hypotonically swollen, smeared, stained, and inspected for the presence of arrested mitoses (11). Accurate determination of the percentages of cells arrested in metaphase, or cell counts in the cultures after the release from the hydroxyurea block were impossible because of cell clumping.

Assessment of Synchronization and Homogeneity.[2]—The degree of synchronization in a hydroxyurea-incubated cell population can be assessed from the rate of DNA synthesis after the wave of synchronized cells has proceeded through S: If the rate of DNA synthesis falls to near zero levels after the S peak, one can conclude that the cell cohort is highly synchronized. A continuing high rate of DNA synthesis after an initial S peak would indicate that a considerable part of the dividing cells is not synchronized.

By pulse-labeling synchronized cultures for longer than one expected cell cycle, one may obtain two or more peaks of DNA synthesis, depending on the degree of desynchronization that occurs with the time. Desynchronization, however, is a function of the degree of heterogeneity of a cell population. By definition, the greater the degree of homogeneity, the less likely one is to observe desynchronization. If one can detect two or more peaks of DNA synthesis, it can be concluded that a high degree of homogeneity exists in the synchronized cell population under study.

Determination of the Duration of Cell Cycle Phases.—The initial increase in DNA synthesis after release from a G_1-S block can be attributed to [^{3}H]TdR uptake by those cells which begin DNA synthesis first; the last uptake occurring in those cells of the cohort completing S phase last. The time interval between these two points is therefore the longest possible duration of S. Intercellular variations in the durations of cell cycle phases can be expected to be small in a well synchronized, homogeneous cell cohort. Hydroxyurea incubation of stimulated lymphocytes resulted in such a cell population (see details under Results). Therefore, the mean duration of S (TS) of a synchronized lymphocyte cohort was considered to be the time

interval between $t = 0$, and the time when the rate of DNA synthesis failed to decrease further after the S peak. The time interval between identical points of two subsequent peaks of the DNA synthesis rate was considered to be the mean generation time (duration of the cell cycle, TC).

The interval between termination of the hydroxyurea block, and the first appearance of colchicine-arrested metaphases marks the shortest possible summation of S + G_2. Having obtained data for the duration of S, a mean duration of G_2 (TG_2) can be calculated. The duration of the mitosis (TM) in mammalian cells has been found to range between 0.5 and 2.5 h (12–15). For the present study, TM was therefore assumed to be 1 h for lymphocyte cultures. The duration of G_1 (TG_1) was calculated according to the formula:

$$TG_1 = TC \text{ (measured)} - TS \text{ (measured)} - TG_2 \text{ (calculated)} - TM \text{ (estimated)}.$$

Direct Assessment of the Number of Responding Cells.—Lymphocyte cultures were established as described, and stimulated with different doses of PHA. For 6 days, [^{3}H]TdR was added to quadruplicate cultures at 12-h intervals, and cultures were harvested 12 h later. To the first three sets of quadruplicate samples, colchicine (0.55 μg in 0.1 ml) was added simultaneously with PHA; to the remaining cultures, colchicine was added 36 h before [^{3}H]TdR, and left until they were harvested. Colchicine was added in these experiments in order to produce mitotic arrest of all dividing cells. When [^{3}H]TdR is added 36 h later, cells which have previously gone through division will all have been arrested in M. Therefore, any cell incorporating [^{3}H]TdR during the 12-h labeling period will be a cell newly triggered into proliferation (first generation responder). Since identical cells contain the same amount of chromosomal DNA (16), any cell has to reduplicate the same amount of DNA during S. [^{3}H]TdR incorporation into DNA in the presence of colchicine is then a measure for the number of first generation responders passing through S during the labeling period. The cumulative DNA-bound [^{3}H]TdR activity of successive labeling periods during such an experiment reflects the total number of cells responding to the stimulating agent.

RESULTS

Suppression of DNA Synthesis by Hydroxyurea.—Fig. 1 demonstrates the extent of suppression of DNA synthesis in PHA-stimulated cultures, and in mixed lymphocyte cultures with varying concentrations of hydroxyurea. 2-4 mM hydroxyurea almost completely suppresses DNA synthesis, whereas a concentration of 0.125 mM hydroxyurea has minimal effect. Further experiments, in which the time interval between addition of hydroxyurea, and [^{3}H]T dR was shortened, showed that DNA synthesis is suppressed within minutes after addition of 4 mM hydroxyurea.

Degree of Synchronization and Homogeneity after Hydroxyurea Incubation.—Fig. 2 shows the results of a representative experiment in which the rate of DNA synthesis in PHA-stimulated, hydroxyurea-synchronized lymphocyte cultures was determined by repeated pulse labeling. DNA synthesis resumes almost immediately after hydroxyurea is removed from the cultures, its rate increasing rapidly to a maximum about 3 h after hydroxyurea was washed out. By 8 h after removal of hydroxyurea, DNA synthesis had fallen almost to zero. When the DNA synthesis rate of cultures was followed by pulse labeling, three peaks could be seen. These results indicate that hydroxyurea can be used to obtain a highly synchronized and homogeneous population of stimulated lymphocytes.

Duration of Cell Cycle Phases.—Nine experiments were performed in which

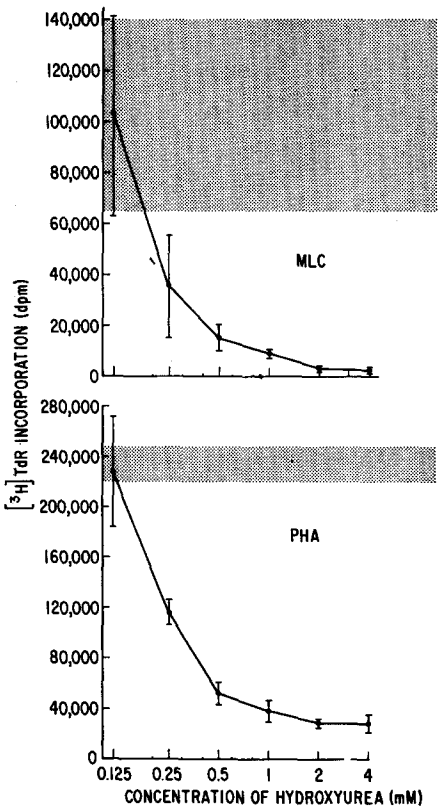

FIG. 1. Suppression of DNA synthesis in dog lymphocytes stimulated with allogeneic lymphocytes (top), and PHA (bottom). Indicated are the means (closed circles) and standard deviations of [^{3}H]TdR uptake in quadruplicate samples. The shaded areas indicate [^{3}H]TdR uptake in control cultures without hydroxyurea.

lymphocyte cultures were stimulated with either PHA, or allogeneic cells; the data are summarized in Table I. The duration of S varied between 7.0 and 9.0 h (mean, 8.3 $\pm$ 0.6 h). There appeared to be no correlation between the duration of S, and the strength of lymphocyte stimulation.

The first colchicine-arrested metaphases were observed 11 h after the release of the stimulated lymphocyte cultures from the hydroxyurea block. From the mean duration of S phase (8.3 h), a mean duration of G_2 of 2.7 h was calculated.

In five experiments, cultures were followed for up to 34 h by hourly pulse labeling, after they were released from a hydroxyurea block. The generation times determined in these experiments are listed in Table I. A typical experiment is shown in Fig. 3. The duration of the cell cycle (TC) ranged between 13.0 and 14.5 h (mean, 14.0 $\pm$ 0.6 h), regardless of the type or strength of the lymphocyte stimulation. From these data, the mean duration of G_1 can be calculated to be about 2 h.

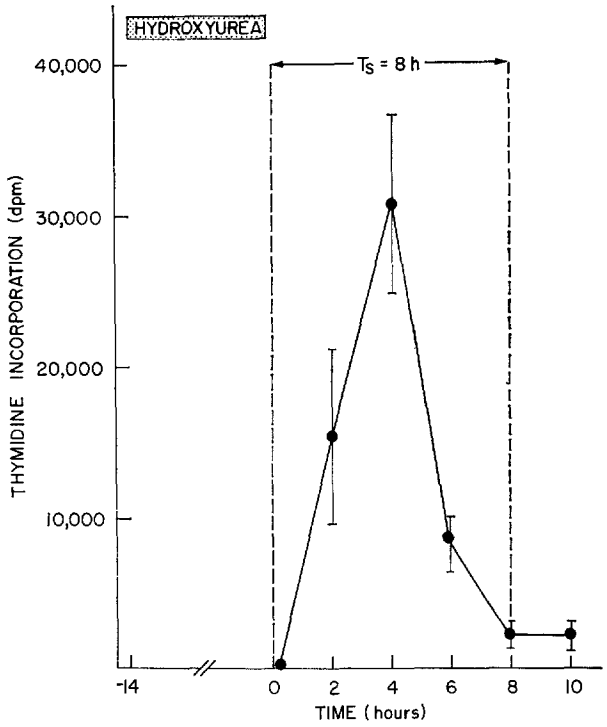

FIG. 2. Rate of DNA synthesis in PHA-stimulated dog lymphocyte cultures after release from a 14-hr hydroxyurea block. Indicated are the means (closed circles), and standard deviations of quadruplicate samples.

TABLE I

Duration of S Phase and Cell Cycle in Stimulated Lymphocyte Cultures

Stimulation with:		Duration of S	Duration of cell cycle	Stimulation ratio
PHA	Allogeneic cells			
		h	*h*	
	+	9.0		40
	+	8.0		44
	+	8.0	14.5	68
1:100		8.5		60
1:240		8.0		280
1:450		8.5	14.5	22
1:150		7.0	13.0	38
1:60		9.0	14.0	140
1:240		8.5	14.0	105
Mean ± SD		8.3 ± 0.6	14.0 ± 0.6	

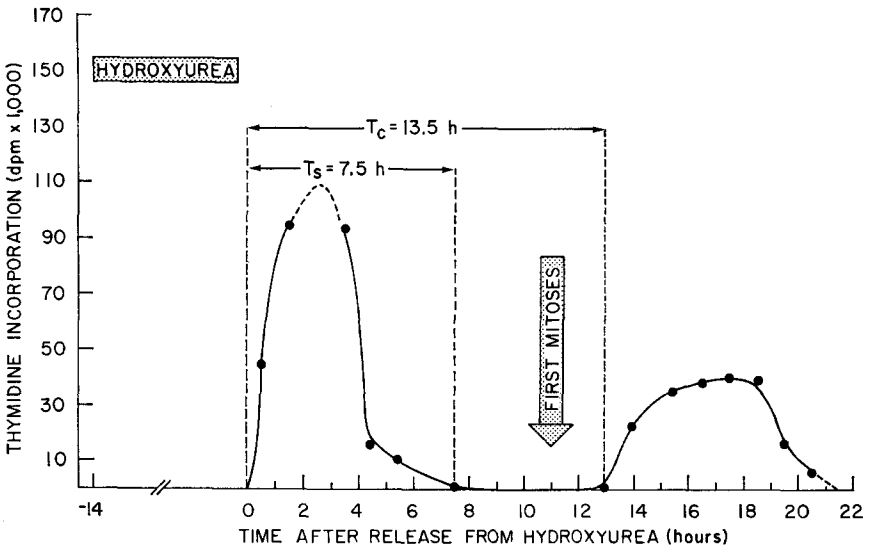

FIG. 3. Determination of the durations of cell cycle phases in a hydroxyurea-synchronized PHA-stimulated dog lymphocyte culture. The rate of DNA synthesis is plotted against the time after termination of the synchronizing block. Closed circles represent the means of quadruplicate cultures.

Direct Assessment of the Number of Responding Cells.—When lymphocyte cultures were grown in the presence of colchicine, the following observations were made (Fig. 4): The cumulative DNA-bound [^{3}H]T dR activity, which represents a measure for the total number of first generation responders, was related to the [^{3}H]T dR uptake in control cultures without colchicine. PHA-stimulated lymphocytes were first triggered into proliferation between 28 and 40 h after stimulation. Of all 12-h labeling periods, the highest number of first generation responders entered the division cycle during these 12 h; in subsequent labeling periods, the number of newly proliferating responders decreased more and more. With increasing PHA stimulation, relatively more of all responding cells were triggered into proliferation during the earlier labeling periods. These experiments provide direct evidence that the number of cells triggered into proliferative response is related to the level of stimulation; and that with greater PHA stimulation, a shift to earlier proliferative response of the responsive cells occurs.

Attempts to perform similar experiments using allogeneic-irradiated lymphocytes for stimulation failed because the quantity of DNA-incorporated [^{3}H]T dR activity during any 12-h interval was not significantly different from the background activity. Experiments designed to overcome this obstacle by growing MLCs for 7 days in the continuous presence of colchicine and [^{3}H]T dR were inconclusive since the label was degraded early.

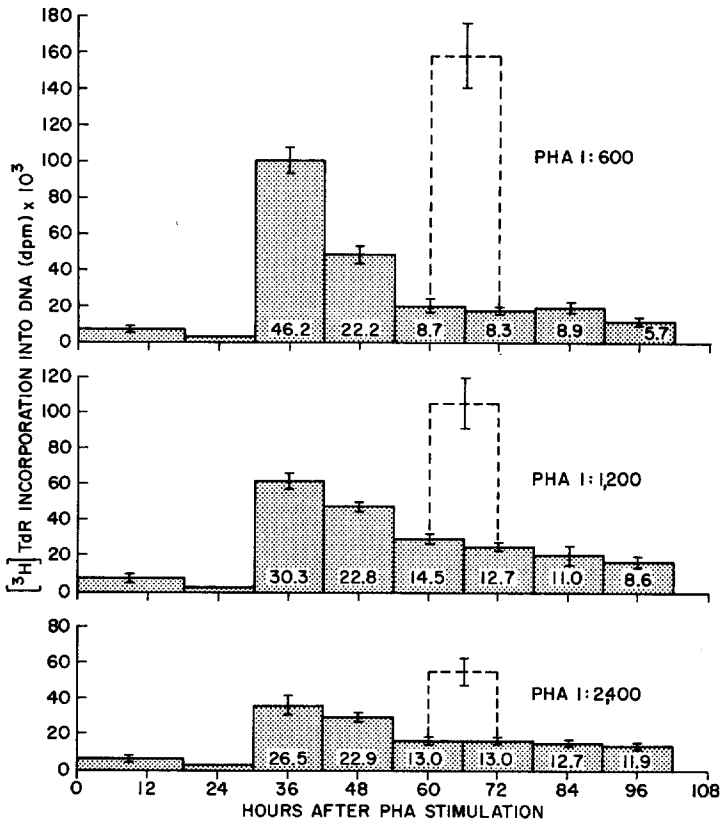

FIG. 4. DNA synthesis by first gon reseneratiponders in PHA-stimulated dog lymphocyte cultures. Quadruplicate cultures, grown in the presence of colchicine, were labeled during successive 12-h periods with [^{3}H]TdR. Thymidine uptake into DNA during any of these 12-h intervals is a measure for the number of first generation responders passing through S during the labeling period (see text for details). The shaded bars indicate the mean [^{3}H]thymidine uptake (± SD) during the respective labeling period. The figure at the bottom of each bar indicates what percentage of the total number of first generation responders (between 30 and 102 h after PHA stimulation) entered the division cycle during the respective labeling period. The broken bars illustrate the DNA-bound [^{3}H]TdR in control cultures grown without colchicine (labeled 60–72 h after stimulation). Top: Cultures stimulated with 0.1 ml of PHA 1:600 (the same result was obtained with a dilution 1:300); center: stimulation with 0.1 ml PHA 1:1,200; bottom: stimulation with 0.1 ml PHA 1:2,400.

DISCUSSION

Hydroxyurea has two separate effects on dividing mammalian cells: It selectively inhibits DNA synthesis and/or kills cells in DNA-reduplicating (S) phase of the division cycle (17, 18), and it inhibits cells from proceeding from G_1 to S (19). Lymphocytes in S are killed by hydroxyurea (unpublished observation).

All proliferating cells in G_2, M, or G_1 accumulate at the G1-S border after addition of hydroxyurea, provided the drug is present for a minimum time equal to $TG_2 + TM + TG_1$. Using these properties of hydroxyurea, randomly dividing Chinese hamster lung cells (18) and HeLa cells (19) have been successfully synchronized. The results of our experiments indicate that a high degree of synchronization is also obtained after hydroxyurea incubation of stimulated lymphocyte cultures.

As outlined in the introduction, knowledge of the cell cycle kinetics of stimulated lymphocytes permits conclusions as to the number of responding cells. Using in vitro stimulated, hydroxyurea-synchronized dog lymphocytes, we determined TC to be 14.0 ± 0.6 h, TS to be 8.3 ± 0.6 h, and TG_2 to be 2.0–2.5 h. TG_1 was then calculated to be about 2 h. The values were found to be identical whether lymphocytes were stimulated with PHA, or with allogeneic cells; there were no differences at low or high stimulation ratios. To our knowledge, few cell cycle kinetic data are available for stimulated lymphocyte cultures. These data are summarized in Table II.

The short TG_1 of 2 h found in our studies apparently is a parameter of a rapidly dividing cell population. In PHA-stimulated dog lymphocyte cultures, the doubling time is about 12 h (unpublished data). Marshall et al. (20) found doubling times in stimulated lymphocyte cultures to be shorter than the generation times (because cells enter the division cycle for the first time as late as 150 h after stimulation [6, 20, 21]). A doubling time of 12 h, and a generation time of 14 h are well in accordance.

Mitotic arrest by colchicine was utilized to prevent clonal proliferation of responding lymphocytes, in order to determine DNA synthesis exclusively in first generation responders. Using different concentrations of stimulant, one can thus quantitatively compare the cellular response. Our studies show that greater numbers of cells respond to greater PHA stimulation. This is accompanied by a shift to earlier proliferation of the responsive cells with higher doses of stimulant. Similar experiments with allogeneic cells as stimulant were inconclusive because [^{3}H]T dR uptake in the absence of clonal responder proliferation never exceeded background values. This implies that the number of lymphocytes responding to allogeneic cells is small as compared to the number of cells responding to PHA. Bach et al. (23) and Wilson et al. (21) reported that in human MLCs 1–3% of the inoculated cells undergo proliferation. Similar figures have been reported for mouse MLCs (22, 27), compared to 11–26% of the cells responding to PHA stimulation (27).

Our experiments demonstrate that stimulated lymphocytes cannot divide more or less rapidly; once a dog lymphocyte is triggered into proliferative response, it will divide with a generation time of about 14 h. Higher [^{3}H]T dR uptake has to be attributed to earlier proliferation of greater numbers of responding cells, which by their nature divide with the same generation time as lymphocytes in weakly stimulated cultures. It may be concluded that a variable

TABLE II

Literature Data on Lymphocyte Cell Cycle Kinetics

Lymphocyte source	Stimulation with	Hours					Reference
		TC	TS	TG_1	TG_2	T/2*	
Human p.bl.‡	tuberculin	7.5–38 (12.3 ± 3.4)§					20
Human p.bl.	SK-SD‖	11.8–22 (16.9 ± 3)					
Human p.bl.	PWM	9.3–10					
Human p.bl.	Allog.cells	8.5–22 (15.0 ± 3.6)					
Rat p.bl.	Allog.cells	8.1–16.5 (11.5 ± 2.8)					
Human p.bl.	Allog.cells	8–21	9		>3	17	23
Rat p.bl.	Allog.cells	9	6			9–10	21
Rat p.bl.	PHA	>20	11–13				
Human p.bl.	Allog.cells	13–14	10				
Human p.bl.	PHA	>20	11				
Human p.bl.	PHA				5	10	24
Human p.bl.	PHA		>12 (->30)	>24	<6		25
Human p.bl.	PHA				>2		28
Human p.bl.	PHA	22	11	6	3		26
Mouse (source unspecified)	PHA, Con A, Allog.cells		9		2		27

* T/2, doubling time.

‡ p.bl., peripheral blood.

§ Numbers in parentheses indicate means ± SD.

‖ SK-SD, streptokinase-streptodornase; PWM, pokeweed mitogen; Con A, concanavalin A; Allog. cells, allogeneic lymphocytes.

sensitivity to a particular stimulating agent exists among the clone of responsive cells, some cells being exceedingly sensitive and others rather insensitive.

SUMMARY

Lymphocytes, stimulated with different doses of plant mitogens or allogeneic cells, incorporate varying amounts of [^{3}H]thymidine. Theoretically, this may be due to different numbers of responding cells, to earlier proliferative response of these cells, and/or to their more or less rapid transit through the cell cycle. Dog peripheral blood lymphocytes were stimulated in vitro with different doses of

phytohemagglutinin (PHA), or with allogeneic lymphocytes. After their synchronization by incubation with hydroxyurea (4 mM), the mean durations of the cell cycle, and of the different cell cycle phases were constant and unrelated to strength or type of stimulation. PHA-stimulated lymphocyte cultures were maintained in the presence of colchicine, to prevent clonal proliferation of responding lymphocytes. DNA uptake in this setting, attributed to first generation responders, was related to the strength of proliferative lymphocyte response in control cultures without colchicine. Furthermore, cell proliferation occurred earlier with greater stimulation. It is concluded that higher [^{3}H]thymidine uptake in vitro by stimulated lymphocytes is due to greater numbers of responding cells, which are triggered into proliferative response earlier, and not to a more rapid transit of the responding cells through the cell cycle.

REFERENCES

1. Freiersleben, Sorensen S. 1972. The mixed lymphocyte culture interaction. Techniques and immunogenetics. *Acta Pathol. Microbiol. Scand. Sect. B. Suppl.* 230.
2. Daguillard, F. 1972. Immunologic significance of in vitro lymphocyte response. *Med. Clin. North Am.* **56**:293.
3. Oppenheim, J. J. 1972. Wherefore the mixed leukocyte reaction. *In* Transplantation Antigens. Markers of Biological Individuality B. D. Kahn, and R. A. Reisfeld, editors. Academic Press, Inc., New York.
4. Gordon, J. 1972. The mixed leukocyte culture reaction. *Med. Clin. North Am.* **56**:337.
5. Wilson, D. B. 1966. Analysis of some of the variables associated with the proliferative response of human lymphoid cells in culture. *J. Exp. Zool.* **162**:161.
6. Ling, N. R., and P. J. L. Holt. 1967. The activation and reactivation of peripheral lymphocytes in culture. *J. Cell. Sci.* **2**:57.
7. Weber, W. T. 1970. Mixed lymphocyte interaction and PHA-response of chicken spleen cells in a chemically defined medium. *J. Reticuloendothel. Soc.* **8**:37.
8. Sorensen, S. F., F. Kissmeyer-Nielsen. 1969. Studies on the quantitation of the lymphocyte response in vitro. A family study. *Acta pathol. Microbiol. Scand.* **77**:117.
9. Albertini, R. J., and F. H. Bach. 1968. Quantitative assay of antigenic disparity at HL-A—the major histocompatibility locus in man. *J. Exp. Med.* **128**:639.
10. Ling, N. R. 1971. Lymphocyte Stimulation. North-Holland Publishing Co., Amsterdam, Holland. 181.
11. Salzman, N. P., and J. Mendelsohn. 1968. Isolation and fractionation of metaphase chromosomes. In Methods in Cell Physiology. D. M. Prescott, editor. Academic Press, Inc., New York. 277.
12. Fry, R. J. M., S. Lesher, H. I. Kohn. 1962. A method for determining mitotic time. *Exp. Cell Res.* **25**:469.
13. Henry, J. L., J. Mayer, J. P. Weinmann, and I. Schour. 1952. Pattern of mitotic activity in oral epithelium of rats. *Arch. Pathol.* **54**:281.
14. Reiskin, A. B., and M. L. Mendelsohn. 1964. A comparison of the cell cycle in induced carcinomas and their normal counterpart. *Cancer Res.* **24**:1131.
15. Widner, W. R., J. B. Storer, and C. C. Lushbaugh. 1951. The use of X-ray and

nitrogen mustard to determine the mitotic and intermitotic times in normal and malignant rat tissues. *Cancer Res.* **11:**877.

16. Swift, H. H. 1950. The desoxyribose nucleic acid content of animal nuclei. *Physiol. Zool.* **23:**169.
17. Young, C. W., and S. Hodas. 1964. Hydroxyurea: Inhibitory effect on DNA metabolism. *Science (Wash. D. C.).* **146:**1172.
18. Sinclair, W. K. 1965. Hydroxyurea: Differential lethal effects on cultured mammalian cells during the cell cycle. *Science (Wash. D. C.).* **150:**1729.
19. Pfeiffer, S. E., L. J. Tolmach. 1967. Inhibition of DNA synthesis in HeLa cells by hydroxyurea. *Cancer Res.* **27:**124.
20. Marshall, W. H., F. T. Valentine, and H. S. Lawrence. 1969. Cellular immunity in vitro. Clonal proliferation of antigen-stimulated lymphocytes. *J. Exp. Med.* **130:**327.
21. Wilson, D. B., J. L. Blyth, and P. C. Nowell. 1968. Quantitative studies on the mixed lymphocyte interaction in rats. III. Kinetics of the response. *J. Exp. Med.* **128:**1157.
22. Wilson, D. B., and P. C. Nowell. 1970. Quantitative studies on the mixed lymphocyte interaction in rats. *J. Exp. Med.* **131:**391.
23. Bach, F. H., H. Bock, K. Graupner, E. Day, and H. Klostermann. 1969. Cell kinetic studies in mixed leukocyte cultures, an in vitro model of homograft reactivity. *Proc. Natl. Acad. Sci. U. S. A.* **62:**377.
24. Michalowski, A. 1964. Time-course of DNA synthesis in human leukocyte cultures. *Exp. Cell Res.* **32:**609.
25. Bender, M. A., and D. M. Prescott. 1962. DNA synthesis and mitosis in cultures of human peripheral leukocytes. *Exp. Cell Res.* **27:**221.
26. Sasaki, M. S., and A. Norman. 1966. Proliferation of human lymphocytes in culture. *Nature (Lond.).* **210:**913.
27. Jones, G. 1973. The number of reactive cells in mouse lymphocyte cultures stimulated by phytohemagglutinin, concanavalin A or histocompatibility antigens. *J. Immunol.* **111:**914.
28. Cooper, E. H., P. Barkham, and A. J. Hale. 1963. Observations on the proliferation of human leukocytes cultured with phytohaemagglutinin. *Br. J. Haematol.* **9:**101.