The Delayed Response of Chronic Lymphocytic Leukemia Lymphocytes to Phytohemagglutinin *in Vitro** (32769)

KLAUSS HAVEMANN AND ARNOLD D. RUBIN (Introduced by William Dameshek)
Department of Hematology, The Mount Sinai School of Medicine, New York, New York 10029

During a 3-day *in vitro* incubation with phytohemagglutinin (PHA) most normal lymphocytes enlarge into blast-like cells, replicate their DNA and divide (1–3). Under these conditions lymphocytes from patients with chronic lymphocytic leukemia (CLL) show little or no evidence of growth (4–6). Quaglino and Cowling (7) as well as Robbins (8) have suggested that a primary cellular defect renders the CLL lymphocyte totally unresponsive to PHA. The present report will show that many CLL lymphocytes are able to enlarge and proliferate when stimulated by PHA. However, the development of such a response requires intervals longer than the usual 3 days.

To demonstrate this phenomenon a kinetic study into the growth of PHA treated lymphocytes was undertaken. Rates of RNA, DNA, and protein synthesis were selected as the most sensitive indicators of cell enlargement and potential for undergoing mitosis.

Methods. All CLL patients included in the study exhibited lymph node enlargement as well as blood and marrow lymphocytosis. The initial experiments were restricted to CLL patients with lymphocyte counts in excess of 50,000/mm^3.

Peripheral lymphocyte suspensions (95–99% pure) were isolated from six normal individuals and from six CLL patients of comparable ages (9). Each culture contained 3 $\times$ 10^6 lymphocytes in 4 ml of medium TC 199 with normal human AB serum (15%) and antibiotics. After PHA (PHA-P, Difco 75 μg) had been introduced, the cultures were incubated at 37°C in stoppered, conical centrifuge tubes. Paired duplicate cultures received 4 μC of cytidine-^{3}H (2 C/mmole) or 4 μc of thymidine-^{3}H (3 C/mmole) 2 hours before harvesting. Stained smears were prepared from small aliquots of each culture, following which whole culture RNA and DNA were extracted quantitatively according to the method of Cooper and Rubin (9). The RNA was extracted from cytidine treated cultures and DNA from thymidine treated cultures. The entire procedure was carried out in the original culture tubes to minimize loss of material. Typical spectral patterns of ultraviolet absorption established the identity of the major constituent in each extract and eliminated the possibility of significant protein contamination (10). The RNA extracts of cultures exposed to thymidine (^{3}H) were not radioactive. In resting culture cytidine-^{3}H exposure labeled only the RNA extracts. The specific activity of each extract was expressed as cpm per μg of nucleotide RNA or DNA as calculated from standard curves of optical density employing lymphocyte RNA and DNA as standards.

Kinetic studies of protein synthesis were carried out in additional replicate cultures. Except for the omission of isoleucine, lysine, and serine from medium 199 in the original incubating solution, the remainder of the *in vitro* conditions were identical to the nucleic acid experiments. At specified times a 0.3 μC mixture of ^{14}C labeled isoleucine (250 C/mmole), lysine (247 C/mmole), and serine (120 C/mmole) was added to each culture. After 12-hour incubation with radioactive precursor the cultures were chilled, washed, and the cells were broken by repeated freezing and thawing. Radioactivity incorporated into protein was determined in trichloroacetic acid precipitates of whole culture lysates after dissolving the precipitates in hyamine.

Results. Normal lymphocytes. The PHA stimulated lymphocyte cultures prepared from

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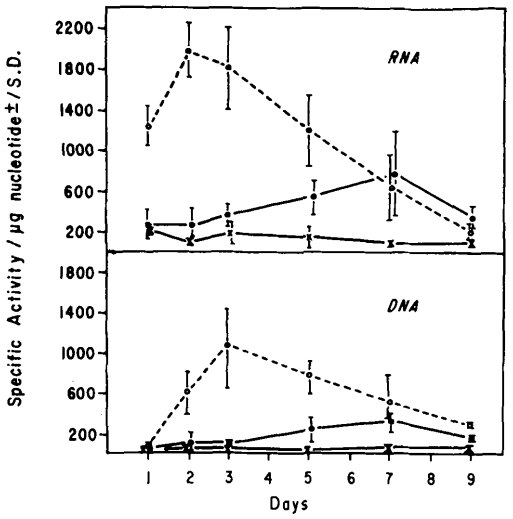

FIG. 1A. The kinetics of nucleic acid synthesis in cultured lymphocytes. Each point represents the average ± 1 SD of pooled data from 6 different individuals studied in duplicate. - - ○ - - normal cultures stimulated by PHA; —●— CLL cultures stimulated by PHA; -X- normal cultures unstimulated.

normal individuals exhibited an increased rate of RNA synthesis which rose to a maximum between 2 and 3 days of incubation (Fig. 1A). The RNA synthesis then declined steadily to control levels by the ninth day. A close parallel is seen in the kinetics of protein synthesis (Fig. 1B). The rate of DNA synthesis began to increase on the second day (Fig. 1A), reached a maximum on the third day, and then also dropped off to control levels. Meanwhile the percentage of blast-like cells, which was 63.5% in 3-day cultures, increased to 75.2% over the next 4 days of incubation. These observations implied that most small lymphocytes transformed during the first 3 days of incubation, yet the concentration of blast-like cells appeared greatest as metabolic activity approached baseline. Many complex factors determine the percentage of blast-like cells at any given time. In particular, it is not known how long a lymphocyte remains blast-like even after undergoing DNA replication and mitosis. Therefore, to enumerate the blast-like cells in PHA-stimulated cultures would reflect the dynamics of cell growth only partially. The rates of nucleic acid and protein synthesis emerge as more reliable parameters.

Further justification of the present experimental system was provided by a series of experiments, in which the incubating medium of PHA-stimulated cultures was changed every 2 days. This maneuver did not alter the growth pattern or the kinetics of nucleic acid synthesis, indicating that maximal proliferative activity was not limited by early consumption of essential nutrients or growth supporting factors.

It should be noted that normal, unstimulated cultures maintained barely detectable levels of RNA and protein synthesis throughout the period of incubation. In this respect unstimulated CLL cultures resembled normal cultures. Consequently only the normals are depicted in Fig. 1. The DNA synthesis in all unstimulated cultures was nil.

CLL lymphocytes. The rates of RNA, DNA, and protein synthesis increased only slightly in CLL cultures exposed to PHA for 3 days (Figs. 1A, B). Three-day cultures contained 10% blast-like cells. Upon extended incubation, the rates of synthesis increased significantly attaining maximal levels between 5 to 7 days. The pooled data of RNA synthesis showed greater variability because cultures from some CLL patients contained 26% cells which were enlarged and blast-like.

A variable loss of acid extractable nucleotides from PHA-treated cultures occurred over the 9-day incubation, but CLL cultures did not differ from normal cultures in this

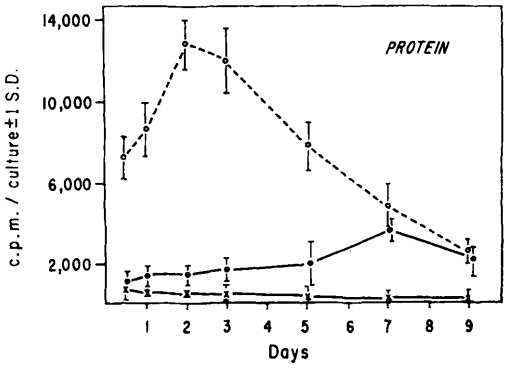

FIG. 1B. The kinetics of protein synthesis in cultured lymphocytes. Each point represents the average ± 1 SD of pooled acid precipitable counts per minute (cpm) from duplicate determinations performed on 3 individuals.

respect. Consequently the increased specific activity cannot be attributed to an accelerated rate of cell death within a large population of unresponsive CLL lymphocytes. There was a real increase in the metabolic activity of CLL cultures.

Thus, in the CLL cultures, a delay of several days preceded the development of maximal metabolic activity, a phenomenon never observed with normal cultures. Even then, the magnitude of the response was considerably less than the peak response observed in normal cultures at 3 days. The percentage of blast-like cells in CLL cultures at 7 days was less than one third of the percentage observed in normal cultures. However, after the first 3 days, the rate of blast cell appearance in normal cultures diminished while in CLL cultures, the rate increased.

To demonstrate that proliferation in 5 and 7 day cultures did not represent an outgrowth of a small population of normal lymphocytes whose growth had been retarded by the presence of a totally unresponsive population of CLL cells, equal mixtures of normal and CLL lymphocytes were placed in a single suspension. Under these circumstances, nucleic acid synthesis progressed unimpeded to maximal rates at 3 days. When CLL cultures received 1–5% normal lymphocytes in order to approximate a proportion which might actually exist in CLL patients, the kinetics of nucleic acid synthesis did not change. Clearly our technique was not sufficiently sensitive to detect the addition of small numbers of normal cells.

Additional evidence of delayed growth is provided by three cases of CLL in which the lymphocyte count was less than 30,000/mm^3. Such individuals responded to PHA with a prompt increase in the rates of nucleic acid synthesis (Fig. 2). The magnitude of the response approached the levels observed with normal cultures. However, cultures prepared from these cases differed from normal cultures as the development of maximal rates of nucleic acid synthesis still required 5 days. In the case depicted in Fig. 2 an apparent plateau in RNA synthesis at day 2 could reflect a small subpopulation of normal cells. The secondary rise in DNA synthesis at day 9 remains unexplained. Such a phenomenon was occasionally observed in normal cultures and possibly represents a second round of cell division.

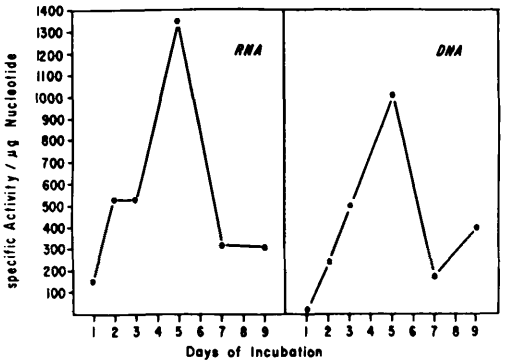

FIG. 2. Kinetics of nucleic acid synthesis in PHA-stimulated lymphocytes from a patient with low count CLL. Each point is the average of duplicate determinations.

Discussion. From the present data it appears that normal lymphocytes have completed a phase of increased nucleic acid and protein synthesis by the third day in culture. Blast-like cells may persist in these cultures even though metabolic activity has declined. Normal lymphocytes stimulated by PHA enter into DNA synthesis as a cohort. Autoradiographic experiments have demonstrated the greatest number of cells incorporating thymidine-^{3}H days after original exposure to PHA (11). If the blast-like cells which developed in stimulated CLL cultures were derived from a residual population of normal lymphocytes, as suggested by others (7,8,12, 13) the maximal rates of nucleic acid and protein synthesis should have been observed within 2–3 days. The CLL lymphocytes did not seem to inhibit the response of normal cells. Therefore, it is quite possible that the blast-like cells appearing in 5–7 days in CLL cultures were recruited from a population of previously unreactive, leukemic lymphocytes.

Lymphocytes from the patient with "low count" CLL were more reactive than the lymphocytes from patients with "high count" CLL. This would be consistent with the findings of Schrek and others who have reported that CLL patients whose absolute lymphocyte counts are only moderately elevated yield a greater percentage of blast-like cells in PHA-stimulated cultures than do patients

whose lymphocyte counts are markedly elevated (12,13). Schrek concluded that CLL lymphocytes are heterogeneous with respect to PHA responsiveness and that "low count" CLL is associated with a greater percentage of PHA responsive cells. However, the present data suggest that even in "low count" CLL, the PHA-responsive lymphocytes were not normal as the development of maximal rates of nucleic acid synthesis was still delayed. This delayed response to PHA may represent a primary proliferative abnormality of CLL lymphocytes evident during the early phases of the disease. Progression of the disease, as manifested by increasing lymphocytosis might be accompanied by total loss of reactivity.

Summary. The kinetics of RNA, DNA, and protein synthesis were analyzed in normal and CLL lymphocytes stimulated by PHA *in vitro*. Normal cultures exhibited maximal increases in lymphocyte nucleic acid and protein synthesis 2 to 3 days after exposure to PHA. Under identical conditions the maximal response on the part of the lymphocytes in CLL cultures occurred between 5 to 7 days. The data suggest that many CLL lymphocytes are capable of responding to PHA but the response is distinctly delayed.

TABLE I. Percentage Blast Cells ± 1 SD in Unstimulated and PHA-Stimulated Cultures from 5 Normals and 5 Patients with CLL after 3 and 7 Days of Incubation.

		3 days	7 days
normal (5)	unstimulated	0.6 ± 0.5	3.1 ± 0.6
	PHA stimulated	63.5 ± 4.2	75.2 ± 6.1
CLL (5)	unstimulated	0.8 ± 0.6	1.1 ± 0.8
	PHA stimulated	10.8 ± 4.2	26.0 ± 5.4

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Effect of Amygdaloid Lesions on Plasma and Pituitary Thyrotropin Levels in the Deermouse*† (32770)

B. E. ELEFTHERIOU AND A. J. ZOLOVICK (Introduced by M. X. Zarrow)
Department of Zoology, Kansas State University, Manhattan, Kansas 66502

The importance of the hypothalamus in the modulation of hypophyseal secretion is well documented. In recent years, however, increasing evidence indicates that other areas of the brain may be involved. Specifically, the amygdala, a part of the rhinencephalic-limbic system, has been implicated in the regulation of gonadotropic and corticotropic activity (1–5). More recently, it was found that another hypophyseal hormone, thyrotropin, is not affected by destruction of the amygdala (6–7).

However, since the latter two studies did not indicate which area of the amygdala was involved, and since the amygdala is composed of several nuclear groups, it was decided to repeat these works.

The present work deals with the effects of

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