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Phytohemagglutinin Committed Lymphocytes: the Mitotic Activity of the Phytohemagglutinin Stimulated Lymphocytes[1] (38701)

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The peripheral lymphocytes are capable of undergoing blastoid transformation and subsequent mitosis when they are stimulated with mitogenic substances *in vitro*, e.g., phytohemagglutinin (PHA) or Pokeweed Mitogen. The cell division is the last stage of a sequence of events in the cell cycle. The first sign of active cell cycle begins about 5–10 hr after incubation with PHA when the lymphocytes cluster around a macrophage. This is followed by swelling of the small lymphocyte in about 24 hr. Between 24 and 48 hr, there is a steady increase in transition of large lymphocytes to more morphologically immature cells with large irregular nuclei and vacuoles in the cytoplasm. By 48 hr, there are many blastoid cells (transformation). An occasional mitotic cell can be seen now.

The maximum mitotic rate of the PHA stimulated lymphocytes occurs about 72 hr after incubation (1, 2). The process of the blastoid transformation and mitosis of PHA stimulated lymphocytes is regarded as a nonspecific response and is believed to involve thymus dependent (T) lymphocytes. This is in contrast to the specific immunologic response of lymphocytes to a specific antigen to which the cells were previously sensitive (3). These specific immunologic responsive lymphocytes appear to require a longer incubation period for their morphological transformation. We observed a marked decrease in the mitotic activity in PHA-stimulated normal lymphocyte cultures after prolonged incubation (4). Reported here is an extension of our previous observations concerning the mitotic activity of the lymphocytes.

Materials and Methods. The lymphocytes obtained from the peripheral blood of two normal individuals of each sex, between the ages of 20 and 40 were cultured with PHA-M (Difco, Detroit) according to the standard method used in our laboratory (5). Sixty-eight hours after incubation, ^{3}H-thymidine (^{3}H-TdR, specific activity 1.9 mCi/m*M*, conc. 1 mCi/ml, Schwarz Bio Res Inc., Orangeburg, NY) was added to make a final concentration of 1 μCi/ml of cell culture (6). The cells were incubated for another 4 hr. Each culture was then divided into three aliquots. To one of these aliquots, colcemid was added to the culture medium for one hour and the cells processed as described (5).

The cells from the other two aliquots of each donor were washed three times with chilled (5°) HBSS and recultured with autologous plasma and freshly prepared cell culture medium. PHA was added to one, but not the other subculture from ecah donor. All cultures were coded and incubated for another 3 days before they were terminated and processed as described (5). All cells were exposed to hypotonic (0.075 *M*, KCl) solution, fixed with aceto–alcohol (1:3) and the slides made with the air-dry method (6). The slides were processed for autoradiographic studies using NTB-2 emulsion (Kodak, Rochester, NY). After being stored in the refrigerator in the dark for 4 days, the slides were developed and then stained with Giemsa (6). The mitotic rate, the frequency of the silver-grain labeled cells (both at interphase or mitosis) were determined under direct microscopic examination at 450×. At least two slides and 10,000 interphases (4) from each culture were counted to compute the frequencies of the mitotic rate and the labeled interphases. One-hundred metaphases per culture were also analyzed to compute the frequencies of the labeled metaphases.

Results. Figure 1 graphically represents

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the results obtained from Donor 1. Table I summarizes the percent changes in the mitotic rate, the labeled metaphases and the labeled interphases of the 6-day cultures as compared to the 3-day cultures of each donor. All measured parameters decreased in the 6-day cultures when no fresh PHA was added during subculturing. Although the frequencies of the labeled cells were decreased in all subcultures, whether PHA was added or not during the subculturing, there was a significant difference between the frequencies among the two different types of cultures. The mean decrease in the labeled cells in the subcultures where no PHA was added was 75.5% (range 61.5% and 94%). However, the decrease was only 59.4% (range 52.7% and 73%) in the cultures where fresh PHA was added.

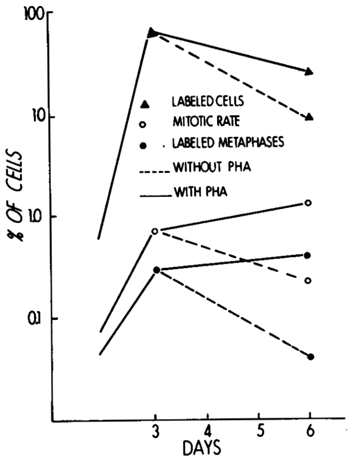

FIG. 1. Semilogarithmic curve of the percentage of the radioactive labeled cells, the mitotic rates (mitoses per 100 interphases) and the radioactive labeled metaphases in the PHA restimulated (——) and PHA nonrestimulated (- - -) of donor 1's peripheral blood lymphocytes cultures after three and 6 days' incubation.

The greatest differences between the PHA restimulated cultures and the PHA non restimulated cultures were the frequencies of the labeled metaphases and the mitotic rates. There was a 25.3% (range 18% and 33%) increase of labeled metaphases in the 6-day PHA restimulated cultures as compared to their 3-day PHA stimulated cultures. There was an increase of 81.2% (range 75% and 88%) in their mitotic rates between these two different types of cultures. In contrast to the PHA restimulated cultures, the frequencies of these parameters were decreased in the 6-day non-PHA restimulated cultures. The silver grains were seen in both sister-chromatids in the autoradiographic slides of the 3-day cultures. However, in the 6-day cultures, the silver grains were only seen in one of the two sister-chromatids. We found only two metaphases in the 6-day cultures having both chromatids labeled. The locations of the labeled segments suggested a chromatid exchange had occurred (7).

Discussion. Morphologically, there are small, medium and large lymphocytes in the

TABLE I. PERCENT CHANGES IN THE SIX-DAY'S AND THE THREE-DAY'S LYMPHOCYTE CULTURES EITHER WITH OR WITHOUT THE SECOND DOSE OF PHYTOHEMAGGLUTININ.

Normal donor	Labeled cells[a]			Mitotic rate[b]			Labeled metaphases[c]		
	$\bar{S}$[d]	$\bar{C}$	Δ	$\bar{S}$	$\bar{C}$	Δ	$\bar{S}$	$\bar{C}$	Δ
1	−73.3[e]	−53.9	+19.4	−72.0	+88.5	+160.5	−83.5	+21	+104.5
2	−61.5	−58.0	+3.5	−66.7	+75.2	+141.9	−71.5	+29	+100.5
3	−94.0	−73.0	+21	−78.0	+78.5	+156.5	−92.0	+18	+110
4	−68.6	−52.7	+15.9	−56.0	+82.4	+138.4	−78.0	+33	+111
Ave.	−75.5	−59.4	+15	−68.2	+81.2	+149.3	−81.3	+25.3	+106.5

[a] Interphase nuclei contained four times more silver grains than the background in the 3-day cultures and two times more silver grains than the background in the 6-day cultures.

[b] Number of mitoses per 100 cells.

[c] Metaphases contained four times more silver grains than the background in the 3-day cultures and two times more silver grains than the background in the 6-day cultures.

[d] $\bar{S}$: Subcultures where no PHA was added; $\bar{C}$: Subcultures where PHA was added; Δ: Percent changes.

[e] A (−) sign indicates decrease and a (+) sign indicates an increase in the percentage.

human peripheral blood. Recently, using newer techniques, at least two functional subpopulations of lymphocytes can be distinguished, namely: the T (thymus dependent) and the B (bone marrow derived) lymphocytes. The T lymphocytes, with ability to form rosettes with sheep erythrocytes, are believed to be the cells involved in the cell mediated immunity. The B lymphocytes produce immunoglobulin. These different lymphocytes appear to respond to different mitogens *in vitro* (8–10).

The decrease in the mitotic rate in prolonged cultures in the normal person reported previously (4) was not due to a deficiency in the essential materials in the culture medium that are needed to sustain a maximum cell response; nor was it due to an accumulation of the metabolites that might inhibit further division of the lymphocytes. This interpretation is supported by our present observations that a similar phenomenon is seen in the cultures where the old culture medium was replaced by a freshly prepared medium but without the addition of PHA. However, this phenomenon is not seen when PHA is added along with the freshly prepared culture medium. Therefore, the decrease in the mitotic rates observed previously (4) and seen in the present experiments must be due to a mechanism other than the two mentioned above. It is possible that there is a limitation in the number of generation cycles in which a single dose of PHA can stimulate the lymphocytes to undergo mitosis *in vitro*. An extension of this would be that the T lymphocytes can undergo a limited number of mitoses *in vitro* after a single PHA stimulation since there is evidence to suggest that PHA stimulates the T lymphocytes *in vitro* (8–10).

The increase or decrease of the mitotic rate in our present experiment was accompanied by an increase or a decrease in the labeled metaphases in the PHA and the non-PHA stimulated subcultures. This suggests that it was probably the original PHA sensitive cells that had been restimulated to undergo further division. This interpretation is strengthened by the observation that in the 6-day subcultures, both stimulated and nonstimulated by PHA, the silver grains were only in one chromatid. This type of labeling pattern has been interpreted as seen only in the second or more generations after the cells were initially labeled (11). Although the mitotic rates and the labeled metaphases were both increased in the subcultures where fresh PHA was added, the increase in the mitotic rates was far greater than the increase in the labeled metaphases (81.2% vs 25.3%). This suggests that some of the mitotic cells seen in the subcultures where fresh PHA was added must have been originated from cells that were not labeled in the previous cell cycle. Thus, it appears that PHA may be able to stimulate some of the previously noncommitted lymphocytes to undergo mitosis, and in so doing, help to maintain the *in vitro* lymphocyte population.

Summary. The purpose of these experiments was to find out why there was a decrease in the mitotic activity in the prolonged PHA stimulated lymphocyte cultures. The present observations suggested that the decrease in the mitotic rate in the prolonged PHA-stimulated lymphocytes was not due to a deficiency in the essential material in the culture medium to sustain a maximum mitotic response, nor was it due to an accumulation of the metabolites that might inhibit the lymphocytes to undergo further division. However, the results obtained suggest that there may be a limitation in the number of generation cycles in which the T lymphocytes can be stimulated with the PHA. They also suggest that a second dose of PHA may stimulate some of the original noncommitted lymphocytes to undergo mitosis thus attempting to maintain the *in vitro* lymphocyte population.

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