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THE EFFECT OF PHYTOHEMAGGLUTININ ON PROLIFERATION OF HUMAN CELL STRAINS IN VITRO

By

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Introduction

In 1958, Nowell *et al.* reported a method of the *in vitro* cultivation of leucocytes in human peripheral blood for the purpose of chromosome study in the presence of phytohemagglutinin. It is well known that human leucocytes maintained in tissue culture rarely go on to divide; indeed, they have usually been considered to be the end cell in the pathway of differentiation. However, the addition of phytohemagglutinin (PHA)—a protein fraction derived from the red kidney bean, *Phaseolus vulgaris*—to leucocytes cultured *in vitro* causes the striking transformation of small lymphocytes into large blast-like cells. More, PHA raises the increase of their over-all metabolic activity and mitosis. Thus, the emergence of large numbers of dividing cells, under the influence of PHA, has been widely utilized for the investigation of chromosome morphology in the peripheral blood of normal individuals. Since the method has established, cytogenetics of human being and other mammalians have been exceedingly developed during the past decade.

Although numerous studies on the effect of PHA on lymphocytes *in vitro* have been published, relatively little attention has been paid as to what effect, if any, PHA may have on other types of cells in culture. The effect of PHA is now considered to be limited depending on the cell type in blood. As Robbins (1964) stated, not all cells in peripheral leucocytes can divide. The monocyte does not undergo blastogenesis and not divide in the presence of PHA, neither polymorphonuclear leucocytes remains unchanged (Pogo *et al.* 1966). Besides blood cells, Ioachim (1966) found increased mitotic indexes in cell lines in tissue culture under the effect of PHA. A strong mitogenic action of PHA was confirmed on cultures of free-living soil amoebae by Agrell (1966). Gamble (1966) also observed a stimulatory response in all cell types in mouse spleen *in vivo*.

The problem on mitogenic effect of PHA still remains unsolved in related to its selective effect upon cell proliferation. The present study was designed to contribute knowledge in mitogenic factor of PHA and to define the possibility of an effect on the proliferation of cells which have gross mitotic potentialities *in vitro*.

Materials and Methods

The type of cultured cells used were those derived from the following normal and

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neoplastic sources : (1) Human amnion cell strain, established by Fernandes (1958) from normal human amnion, is a stable epithelial-like cell strain ; (2) the HeLa strain, which is known world-widely as a serial, long-term culture, originally derived from a specimen of an epidermoid carcinoma of the human uterine cervix (Scherer *et al.* 1953) ; and (3) the McCoy strain was derived by Pomerat (unpublished) and briefly described by Hsu and Moorhead (1957) and Awa *et al.* (1962), from cells of the synovial fluid in the knee joint of a patient suffering from degenerative arthritis in October 1955. The cells are typically spindle shaped. Culture fluid employed for their maintenance was Eagle's medium containing 10 per cent horse serum.

Bacto-Phytohemagglutinin (Difco Laboratories, Detroit, U. S. A.), of both types M and P, were used in the present experiment. Bacto-PHA-M, prepared according to the method of Rigas and Osgood (1955), is a stable, non-toxic, desiccated muco-phytohemagglutinin. It has shown by Hungerford *et al.* (1959) to possess the remarkable ability to initiate mitosis in cultures of leucocytes isolated from peripheral blood. Bacto-PHA-P is a desiccated, purified, highly potent, protein phytohemagglutinin from which the polysaccharide moiety has been removed. It is recognized having approximately 50 times more potent than PHA-M in its hemagglutinating capacity. Each vial contains about 50 mg dry substance. It was rehydrated by adding 5 ml of double distilled water, and a certain volume of either PHA-M or PHA-P was added to matched subcultures of different cell types.

T-30 flasks recieved an initial seeding of 100,000 cells of human amnion, HeLa, or McCoy strain, with 5 ml of the nutrient fluid containing 0.01-1.0 per cent PHA. In some experiments the PHA was added 24 hours after seeding, without obtaining significant differences in the final result. Controls were fed with nutrient medium alone. Cell populations in groups of flasks were estimated by means of the Coulter Cell Counter (Richar and Breakell 1959 ; manufactured by Coulter Electronics, Chicago) on the 2nd, 4th, and 6th days after seeding. This technique consisted of (1) removed of the culture medium, (2) washing with normal saline, (3) trypsinization for 10 minutes with 5 ml of 0.5 per cent trypsin to free the cells from glass ware, (4) dilution with saline to make a final volume of 20 ml, and (5) counting the number of cells in 0.5 ml of the suspension, which was repeated 10 times per a flask.

Results

Comparative Effect of PHA on the HeLa Cell Growth.

The processes in the rate of increasing cell populations were followed by the Coulter cell counter on the 2nd, 4th and 6th days after seedings of the HeLa cells in a T-30 flask. The initial cell population was provided at 10^5 cells per a flask. The rate of cell multiplication was indicated in Figure 1, which compared the time courses of cell proliferation in control and in cultures treated with 0.1 per cent PHA-M and -P. Each point of the graph represents the average number of cells in five T-30 flasks. The cell multiplication was registered on a logarithmic scale.

In the control condition HeLa cells usually represented a rapid and logarithmic cell multiplication. The increase in the total numbers of cells with PHA-M was closely paralleled by an equally striking increase in cells without PHA. The curve of cell

growth in the PHA-P-treated culture showed an initial depression of growth and thenceforth growth rate normally increased. The final cell population, however, on the 6th day with PHA-P was significantly inferior to the control group.

The addition of PHA, either M or P type, at the concentrations of 0.01, 0.1 and 1.0 per cent to the culture medium did not show any increase in cell number over the control curve (Figures 2 and 3). Figure 2 indicated the cells with three different concentrations of PHA-M have not been stimulated in cell multiplication more than the control cells although the initial depression was slightly detected in the cell growth only with higher concentration of PHA-M. The final cell populations of treated cultures on the 6th day were slightly over the population of the control, but it showed no meaningful difference in the rate of cell proliferation with or without PHA-M. The fact indicates that the mitotic indexes were nearly the same in 0.01 per cent PHA-M-treated culture and non-treated control.

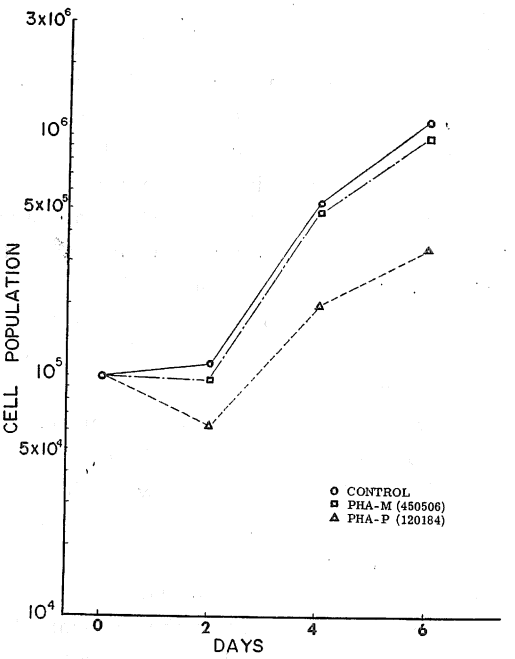
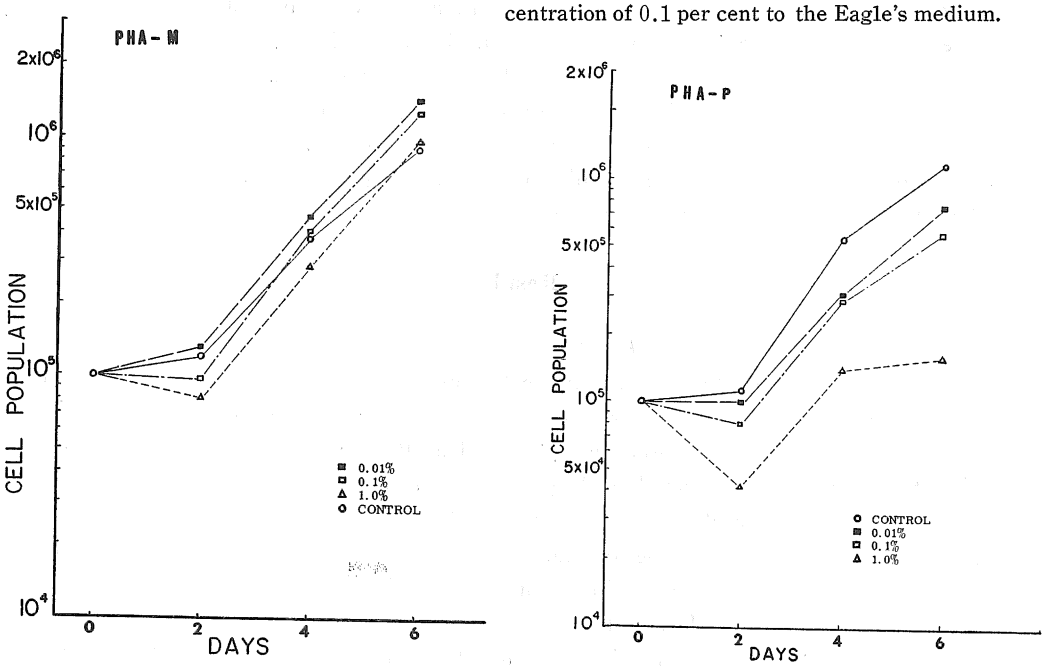


Figure 1. The cell growth of the HeLa strain in the presence of PHA-M and -P at the concentration of 0.1 per cent to the Eagle's medium.



Figures 2 and 3. The effect of PHA-M and -P at different concentrations on the HeLa cell culture.

The treatment of PHA-P resulted remarkable depression of the HeLa cell multiplication (Figure 3). Cells with 1.0 per cent PHA-P did not increase cell number over the initial population and seemed to have the toxic effect at the concentration. Other smaller quantities of PHA-P showed no such typical depressive response in cellular multiplication. But a great number of cells in the PHA-P-treated culture were disturbed the attachment to glass surface after seeding. In 0.01 and 0.1 per cent PHA-P cultures, the cell number remained at the same level of the initial seeding on the second day and gradually cells multiplied. However, no stimulative response was observed in cell growth of these experimental cultures as compared with those of non-treatment. From the experiment described above, it is concluded that the addition of PHA-M and -P to the HeLa cell cultures led no increase in the rate of cell proliferation, neither showing inhibition nor stimulation at the concentration of 0.01 and 0.1 per cent. Exceptionally, the inhibitory effect was observed in the culture treated with 1.0 per cent PHA-P.

Effect of PHA-M on Different Strain Cells.

In order to know there may be occurred, if any, some differences of the PHA effect on different cell strains, the HeLa, human amnion and McCoy cells were cultivated with and without treatment of PHA-M. At the initial seeding, 0.01 or 0.1 per cent of PHA was added to the culture medium. Each experimental group consisted of five T-30 flasks, in which cells were seeded in equal number in respective group. Each of the single diagrams represents an experiment with cells from the same stock culture (Figure 4). The rate of increased cell population were marked on both 2nd and 6th days. Cultures of three different strains showed logarithmic cell multiplication within 6

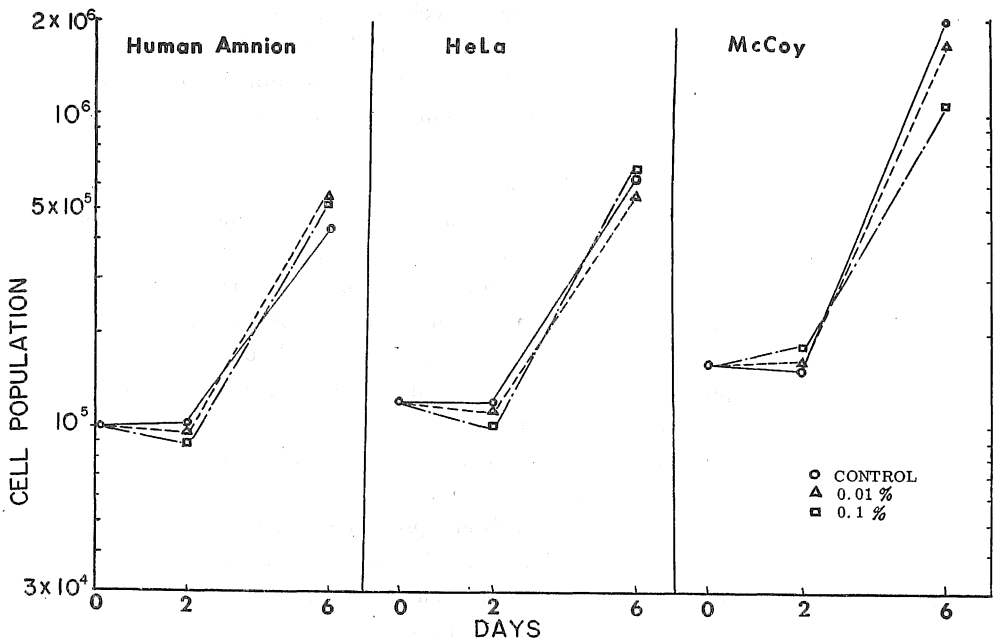


Figure 4. The comparative rate of an increasing cell number among three different cell strains ; human amnion, HeLa and McCoy, after treatment of 0.01 and 0.1 per cent PHA-M.

days under the ordinal condition. Even when the cultures were treated either 0.1 or 0.01 per cent PHA-M, no significant difference in the pattern of cell growth was noted among the HeLa, human amnion, and McCoy cell strains.

Though the graphs showed a slight initial depression of the growth on the 2nd day, the final cell population calculated on the 6th day indicated that these treated strain cells have multiplied without inhibitory response to PHA-M. Neither they did show any advantageous growth as compared with non-treated cells. The McCoy culture was seemed to proliferate better than other two strains, and those cells undergoing active multiplication might respond rather negatively to PHA. However, it is not clear, from the present experiment, whether the difference in cell proliferation between the treated and non-treated cultures in the McCoy strain was of significance or not.

Discussion

Phytohemagglutinin is eminently suited for all hemagglutination techniques such as those described by Li and Osgood (1949). Moreover, PHA has been shown to possess the remarkable ability to initiate mitosis in cultures of leucocytes isolated from peripheral blood. And application of this technique has rapidly become of the utmost importance in the characterization of chromosomes. An interest was also extended to its mitogenic effect on various type of cells. In the present study, the HeLa, human amnion and McCoy strain cells, treated with the usual quantities in the leucocyte culture of phytohemagglutinin, failed to exhibit any detectable growth stimulation. These strain cells regularly show vigorous mitotic activity which greatly exceeds that of elements from peripheral blood. Since the growth rate of cells in strain cultures is at maximum. PHA probably could not enhance this function. Moorhead *et al.* (1960) reported that PHA is essential for mitotic activity of cells from normal peripheral blood but is not required for cells from leukemic peripheral or bone marrow sources, in which cells multiply actively *in vivo*.

Nowell (1960) demonstrated that PHA possesses the remarkable property of being able to initiate mitoses in normal human lymphocytes *in vitro*. Since this observation was made, a number of reports from different laboratories have analysed the properties of PHA. Previously Rigas and Osgood (1955), who purified PHA from red kidney beans, have described that PHA agglutinates erythrocytes by linking the euglobulin portion of the mucoprotein PHA molecule with a polysaccharide on the red cell surface. De la Chapelle (1961) reported that the mitogenic effect is independent of hemagglutinin activity. Nowell (1960) described that although the mitogenic mechanism of PHA is not clear, possibly it initiates mitosis by means of altering the cell membrane to permit the entrance of substances from the culture medium.

On the other hand, evidence has since been presented which identifies the small lymphocyte as the cell undergoing mitosis in the presence of PHA and numerous reports, reviewed by Robbins (1964), have documented its stimulative effect *in vitro*. In the presence of PHA, these small lymphocytes decreased in number and simultaneously many blastoid cells appeared, prior to the onset of visible cell division. Cooper *et al.* (1963) reported that glucose was utilized during blastogenesis and lactic acid was produced. Extensive studies by Polgar *et al.* (1968) suggested that glycolysis might be

the main energy source for blastogenesis by the PHA activation. Radioactive tracers have been used for studies of the synthesis of nucleic acids during the blastogenesis by many investigators (Bender and Prescott 1962, McIntyre and Ebaugh 1962, and others). Results of these studies supported the conclusion that both ribonucleic and deoxyribonucleic acids were being actively synthesized by the cells in response to the blastogenic action of PHA in culture.

More recent works have proved the mechanisms of PHA actions on cell stimulation on molecular basis. An increase in both DNA and RNA syntheses was demonstrated by several workers. Pogo *et al.* (1966, 1967) discovered that in small lymphocytes the acetylation of some histones occurs at a greatly increased rate, within minutes after PHA was added to white cell suspension, and it appeared to proceed the increase in the rate of nuclear RNA synthesis.

As far as the present work is concerned, it seems reasonable to conceive that phytohemagglutinin have no detectable stimulating effect on the growth of established cell strains under ordinary conditions of culture. The result may be correlated with the finding of Nowell (1960) who stated that PHA probably does not stimulate mitosis *per se* but acts as an initiator of mitosis in cells which have a high growth potential. It should be also noted that cytotoxic effects of PHA on rapid growing cells *in vitro* and *in vivo* were showed by Andrew and Gabourel (1966).

An interesting fact was obtained that when PHA-M was added to cultures of human amnion cells which were growing poorly the stimulation was observed (Seto, unpublished). This condition was due to a change-over with the substitution of 10 per cent horse serum for the 10 per cent human serum which had been added to the Eagle's medium employed maintenancing this strain. The response of cells growing in horse serum to treatment with a mitogenic agent might have been due to a factor that the agent may have been utilized metabolically by the cells. This fact might be concerned with the finding of Polgar *et al.* (1968) that the increased cellular activities following PHA activation are dependent on glycolysis rather than on respiration.

Summary

The mitogenic effects of phytohemagglutinin on stable cell strains *in vitro* have been studied by counting the multiplication of cells seeded in T-30 flasks. The treatment for 6 days with PHA-M or -P to the HeLa, human amnion and McCoy strains was not found to enhance growth of these cells.

PHA, known to have mitogenic effects on peripheral lymphocytes in tissue culture systems, has not shown its stimulative effect on strain cells which have a gross proliferative ability.

An inhibitory effect of PHA-P on rapidly growing cells *in vitro* was demonstrated, since disturbance of cell attachment to the glass was occurred in an initiation of the culture. Concentration of PHA-P required for 50 per cent inhibition of the HeLa cell growth was approximately 0.1 per cent in culture medium, and did not differ significantly with other two strains.

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