Phytohemagglutinin: An Initiator of Mitosis in Cultures of Normal Human Leukocytes*

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SUMMARY

Possible factors responsible for the initiation of mitotic activity in “gradient” cultures of leukocytes from normal human blood were investigated. Variations of temperature, pH, oxygen tension, carbon dioxide tension, plasma and cell concentrations, as well as the amount of agitation, over at least as wide a range as might be encountered in vivo, produced only moderate quantitative changes in mitotic activity.

The mucoprotein plant extract, phytohemagglutinin (PHA), employed originally as a means of separating the leukocytes from whole blood in preparing the cultures, was found to be a specific initiator of mitotic activity: in its presence, cell division occurred; in its absence, no mitoses appeared. The studies suggest that the mitogenic action of PHA does not involve mitosis per se but rather the stage preceding mitosis—the alteration of circulating monocytes and large lymphocytes to a state wherein they are capable of division. The relationship of this mitogenic action of PHA to mitotic and premitotic processes in the body remains to be investigated.

In a recent paper (8) we have described the differentiation, in short-term tissue culture, of both normal and leukemic human leukocytes obtained from peripheral blood. In the course of this study, considerable mitotic activity was observed in the cultures of normal leukocytes as well as in the cultures of leukemic “blasts.” The dividing cells in the normal cultures were tentatively identified as monocytes and large lymphocytes which had become mitotically active in vitro after a 2-day latent period. Ordinarily, normal leukocytes do not divide in the peripheral blood. There is evidence, however, to suggest that some circulating white cells do have mitotic potentialities in the body (2). Experiments were, therefore, designed to attempt to determine what specific factor or condition in our culture system was responsible for activating this latent mitotic potential of circulating mononuclear cells. Physiologic factors such as oxygen tension, carbon dioxide tension, and plasma concentration were varied individually but proved to have only minor quantitative effects on mitotic activity in the cultures. Instead, an apparently nonphysiologic mitogenic agent was uncovered: the plant extract, phytohemagglutinin (PHA) (11). This substance was originally employed for its erythrocyte-agglutinating ability in obtaining leukocytes from whole blood. The present studies, however, indicate that it also has the ability to initiate mitosis among these leukocytes, apparently by stimulating the alteration of monocytes and large lymphocytes to a state wherein they are capable of division.

MATERIALS AND METHODS

Standard cultures.—The “gradient” technic for culturing leukocytes has been described in detail elsewhere (4, 8, 10). In preparing the “standard” cultures which were used as a base line for the present studies, phytohemagglutinin (PHA)

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ml. of PHA was added. After mixing, the blood was allowed to stand for 45 min. at 4°C and was then centrifuged at 350 r.p.m. for 10 min. to remove nearly all of the agglutinated erythrocytes. The supernatant plasma, and the leukocytes remaining suspended in it, were then utilized in setting up the cultures. No additional PHA was added. The cells were incubated at 37°C in a total volume of 6—20 ml. (15-20 per cent plasma). This gave a culture depth of approximately 20 mm. A piece of standard microscope slide (90 X 10 mm.) was placed in each bottle at an angle. The cultures were incubated without agitation, and the cells settled out and grew on the slide as well as on the bottom of the bottle. To examine the cells or to estimate mitotic activity in the cultures, the slide was removed and stained with Giemsa (8); or the entire culture was sacrificed and squash preparations made of the harvested cells after they were fixed and stained in acetic-orcein (4). Repeated examination of the same culture was made possible by simply replacing each removed slide with a new one and depositing cells on the new slide by brief agitation of the culture. Ordinarily, no changes of medium were made during the life of these cultures.

"Spinner" cultures of whole blood.—To investigate a number of variables simultaneously, cultures were set up consisting of 10 ml. of heparinized whole blood. PHA (0.2 ml.) was added to each of these cultures, and penicillin and streptomycin were also included. The cultures were incubated with constant agitation by means of a magnetic stirrer. The vigorous agitation not only maintained all the cells in suspension but also prevented any agglutination of the erythrocytes, despite the presence of PHA. To maintain the pH at physiologic levels, it was necessary to seal the bottles with paraffin to prevent loss of carbon dioxide.

Other factors in our standard culture system which were considered to be of possible critical importance in initiating mitosis were tested as follows:

Gas phase.—The effect of very high and very low concentrations of oxygen were tested by equilibrating standard PHA-containing cultures with 50 per cent oxygen, 100 per cent oxygen, or 100 per cent nitrogen at the time of planting and then sealing them with paraffin. Equilibration was obtained by gently bubbling the gas through the culture for 10—15 min. and then layering the gas over the surface. The effect of increased carbon dioxide tension was similarly tested by equilibrating cultures with varying concentrations of carbon dioxide gas up to 100 per cent (i.e., 5, 15, 50, and 100 per cent). During equilibration, the pH was continually adjusted by addition of 0.1 N sodium hydroxide. Perfusion with carbon dioxide was continued until no further pH adjustment was required, and the cultures were then sealed. To test low carbon dioxide levels, bicarbonate-free TC-199 (Difco) plus 10 per cent plasma was employed as the tissue culture medium. Cultures were planted in this medium and equilibrated with air in order to drive off as much carbon dioxide from the plasma component as possible. These cultures were then left unsealed and continuously agitated by a magnetic stirrer to prevent reaccumulation of carbon dioxide. In these cultures, the pH during the first day tended to rise and had to be adjusted with 0.1 N hydrochloric acid; thereafter, the pH remained constant.

Conditioned medium.—The importance of conditioned medium in initiating mitosis was tested on leukocytes which had been separated from whole blood by the usual PHA method. These cells were planted in cultures in which the liquid phase was replaced, completely or in part, by medium removed from 3-day-old standard cultures of actively dividing leukocytes.

Heparin and antibiotics.—The significance of these substances with respect to mitotic activity was investigated by setting up cultures free of both. The leukocytes were separated from oxalated blood with PHA and then planted in medium which contained heparin-free serum instead of plasma, and no antibiotics.

Phytohemagglutinin (PHA).—A variety of cultures were set up containing leukocytes separated from whole blood by some means other than PHA. In most of these, the cells were separated with dextran (19). Cells were also obtained by the fibrinogen method (12), as well as by simply centrifuging heparinized blood rapidly so as to yield leukocytes in the form of a "buffy coat." When it was observed that no mitotic activity occurred in any of these cultures, additional studies on the action of PHA were undertaken by means of the following culture systems:

1. Ten-ml. cultures planted with dextran-separated leukocytes, and hence free of PHA, were incubated at high and low levels of oxygen and carbon dioxide as described above. Similar cultures, to which 0.2 ml. of PHA was added at the time of planting, were employed as controls.
2. Ten-ml. spinner cultures of whole blood were set up exactly as described above, except that the PHA was omitted.

3. Ten-ml. cultures were planted with dextran-separated leukocytes, and 0.2 ml. of PHA was then added, either immediately, 1 day, or 2 days after planting.

4. Ten-ml. cultures were set up containing leukocytes which had been separated from whole blood with PHA as previously described but which were then washed 3 times and planted in PHA-free medium.

5. Cultures were set up containing leukemic "blasts" from the blood of patients with acute leukemia or containing normal human or rat bone marrow cells (7,8). The cells in these cultures were not exposed to PHA. Control cultures of similar cells, but containing PHA (0.2 ml/10 ml culture), were run simultaneously or at different times.

RESULTS

Standard cultures.—After incubation for 3 days, slides from the standard cultures, containing PHA, typically showed clumps of cells consisting of large mononuclear forms interspersed with small lymphocytes. Very few granulocytes remained. The majority of the large mononuclear cells had a uniformly round nucleus with one or more prominent nucleoli and finely dispersed chromatin. The cytoplasm was intensely basophilic and agranular, often with a pale area adjacent to one side of the nucleus. In all our experiments, such "large mononuclears" were frequent only in cultures in which mitoses were present.

In the standard cultures, after 3 days, the mitotic index on the slides and among the cells from the bottom of the culture was in the range of 0.5—1 per cent (number of mitoses/100 nucleated cells). The addition of colchicine (1 × 10^{-7}M) 18 hours prior to sacrifice, in order to accumulate mitoses, usually resulted in indices of 5—10 per cent (Fig. 1). These data are based on more than 50 standard cultures examined so far. Mitotic activity was generally uniform over the entire length of the slide. However, the cells harvested from the bottom of the culture often showed a slightly lower mitotic index than did those on the slide.

By the 3d day, in five standard cultures on which cell counts were done, the total cell number had generally decreased to 30—50 per cent of the original inoculum. From the 3d to the 5th day, during the period of maximal mitotic activity, the total cell number remained nearly constant or increased slightly. Thereafter, in cultures followed into the 2d week, the cell population gradually decreased.

Cultures of the dimensions noted above ordinarily required no pH adjustment. Good mitotic activity was observed over a pH range of 6.9—7.7, with occasional mitoses occurring at pH as high as 8.0. Temperature variation of 35°—39° C. also had little effect on mitotic activity; higher temperatures produced cell death.

"Spinner" cultures of whole blood.—In smears of the cells remaining in these cultures after incubation for three days, the red cells were uniformly dispersed as single cells and were nearly all intact. Although some leukocytes were degenerating, many more polymorphonuclear forms remained than in the standard cultures. Lymphocytes and occasional monocytes were also present, as well as many large mononuclear forms similar to those previously described. The leukocytes showed little clumping, although, occasionally, groups of three or four cells appeared together. Mitoses were present both in these small clumps (Fig. 2) and among the individually dispersed cells. In all of the four spinner cultures examined, the mitotic index, after colchicine, on the 3d or 4th day, was in the range of 0.5—1.0 per cent. This definite decrease in mitotic activity, as compared with the standard cultures (mitotic index = 5—10 per cent), is only partially accounted for by the greater number of mitotically inactive granulocytes surviving in the spinner cultures.

Gas phase.—Over a surprisingly wide range, variation of oxygen or carbon dioxide concentration had little effect on mitotic activity. At least two cultures were tested under each set of conditions. Standard cultures, containing PHA, incubated for 3 days in oxygen concentrations of 50 per cent and near zero (i.e., equilibrated with 100 per cent nitrogen) showed essentially the same mitotic index as standard cultures incubated in air (mitotic index = 5—10 per cent, after colchicine). Similarly, carbon dioxide concentrations of 5, 15, and 50 per cent had no effect on the mitotic index. However, the two extreme carbon dioxide concentrations tested, near 100 per cent and near zero, did reduce, although they definitely did not abolish, mitotic activity. Mitotic indices of only 1—2 per cent, after colchicine, were observed in these latter cultures.

Cultures incubated in 100 per cent oxygen showed no mitotic activity at all at the end of 3 days despite the presence of PHA. The cells in these cultures appeared healthy, but the number of large mononuclears was definitely lower than in standard 3-day-old cultures grown with air as the
gas phase. To investigate further this effect of 100 per cent oxygen, cultures of actively dividing cells which had been grown for 3 days in air were then placed in an atmosphere of 100 per cent oxygen. Mitotic activity in these cultures decreased only slightly over the subsequent 48 hours. On the other hand, when 3-day-old cultures which had been grown in 100 per cent oxygen, and hence showed no mitotic activity and few large mononuclears, were exposed to an atmosphere of room air, only occasional mitoses appeared in these cultures on the subsequent 2 days. However, after these cultures had been in an air atmosphere for 3 or 4 days, mitotic indices of 5–10 per cent, after colchicine, were observed. Apparently, the inhibitory effect of 100 per cent oxygen in our leukocyte cultures is directed more toward preventing the cells from undergoing the transition to a state capable of mitosis than toward the mitotic process per se.

**Conditioned medium.**—Leukocytes separated with PITA and then planted in conditioned medium (i.e., medium removed from mitotically active leukocyte cultures) showed, on the 3d day, mitotic activity equal to that of standard cultures. However, no mitoses were observed during the first 2 culture days. In fact, none of the wide variety of experimental conditions employed in our studies of leukocytes from normal peripheral blood ever resulted in mitotic activity on the 1st or 2d culture day.

**Heparin and antibiotics.**—Two leukocyte cultures which contained PHA but did not contain either heparin or antibiotics showed mitotic activity after 3 days, comparable to that seen in standard cultures.

**Phytohemagglutinin (PHA).**—As indicated above, cultures containing leukocytes which had been obtained from whole blood by any method other than that using PHA uniformly failed to show mitotic activity. Generally, the cells in these cultures looked healthy and underwent the same population changes over the first few days as did those in the PHA-containing cultures, except that typical large mononuclears appeared only in very low numbers. Mitotic figures were not observed, even after colchicine, in two PHA-free cultures of fibrinogen-separated cells, or in three cultures of leukocytes obtained from PHA-free “buffy coats.” In 23 of 25 cultures of cells separated with dextran, not a single mitosis was found on the 3d day on any of the culture slides despite careful search of the many thousands of cells on each slide. In the remaining two cultures, a single mitosis was found on each slide.

The various PHA-free cultures of dextran-separated cells incubated at high and low oxygen and carbon dioxide concentrations, as well as the spinner cultures of whole blood from which the PHA was omitted, all failed to show any mitotic activity. The control cultures, to which PHA was added at the time of planting, showed mitotic indices comparable to those previously observed under similar conditions in standard cultures of cells separated with PHA. At least two experimental and two control cultures were tested under each set of conditions.

Addition of PHA to a culture of dextran-separated cells either immediately after planting, or on the 1st or 2d day following planting, resulted in the appearance of mitoses, in normal numbers, on the 3d, 4th, or 5th culture day, respectively. Only very rarely was a mitosis observed in these cultures earlier than the 3d day following the addition of PHA.

Two cultures of PHA-separated, washed leukocytes planted in PHA-free medium showed definite, though somewhat decreased, mitotic activity on the 3d day. Cultures of both leukemic “blasts” and normal bone marrow cells regularly showed considerable mitotic activity, first appearing on the 1st or 2d culture day. Mitotic indices were the same whether or not the leukemic or marrow cells had ever been exposed to PHA.

Although no detailed quantitative studies were made, it appeared that in the cultures of dextran-separated normal leukocytes, as well as in the spinner cultures of whole blood, a minimal PHA concentration of approximately 0.2 ml in a 10-ml culture was required for mitotic activity to be initiated. Heating PHA at 100° C. for 30 min. completely abolished its mitogenic action as well as its ability to agglutinate erythrocytes; heating at 56° C. for 30 min. did not affect either activity.

**DISCUSSION**

The mechanism by which the mitogenic effect of PHA is exerted is not clear, although it would appear to be a direct action on the leukocytes themselves. The fact that PHA initiates mitosis in spinner cultures of whole blood rules out the possibility that its mitogenic action is an indirect one resulting from the removal of red cells through agglutination. In these spinner cultures, the red cells were not removed but remained present in normal numbers. PHA agglutinates erythrocytes by linkage of the euglobulin portion of the mucoprotein PHA molecule with a polysaccharide on the red cell surface (11). Perhaps the action of PHA on leukocytes also involves the cell surface. Possibly, it alters the cell membrane to permit entrance of some substance from the culture...

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**Note:** The above text is a continuation of a scientific manuscript discussing the initiation of leukocyte mitosis by phytohemagglutinin (PHA). The text details experiments involving the effect of oxygen concentration and PHA on the mitotic activity of leukocytes. It highlights the inhibition of mitotic activity at 100% oxygen and the stimulation by PHA, with a focus on the difference between PHA-containing and PHA-free cultures.
medium which, in turn, initiates the mitotic process. Our studies have not ruled out the possibility that some such essential factor, either serum component or cellular product, does exist in our cultures, although the fact remains that it alone, in the absence of PHA, does not initiate mitosis.

The present findings further suggest that PHA acts on leukocytes not to stimulate mitosis per se but rather to initiate the conversion of monocytes and large lymphocytes to a state capable of division. Certainly the cells as they come from normal peripheral blood are not mitotically active. Neither conditioned medium nor any other variation of the culture conditions, with or without PHA, was capable of stimulating these normal leukocytes to divide immediately. On the other hand, bone marrow cells and leukemic blasts from peripheral blood, under the same culture conditions, showed mitotic activity within a few hours. This difference may be the result of what Swann has recently called "long-latent-period" and "short-latent-period" responses to mitogenic stimuli (13). Tissues which normally have a relatively high mitotic index in vivo (e.g., bone marrow) and thus always contain many cells capable of division, respond to stimulation within a few hours; cells which normally show very low mitotic activity (e.g., liver) respond only after a latent period of several days, during which time differentiated cells of the involved organ are "switching over" from their usual specific functions to the synthesis of mitototic protein and other materials for division.

The consistent failure to observe mitoses in cultures of normal leukocytes before the 5d day in vitro would seem to fit this concept of a "long-latent-period" response; and the studies with PHA suggest that it is the substance, in our culture system, which initiates this "switching-over" process. The change to a mitotically active state of dextran-separated cells in our PHA-free cultures did not begin until PHA was added, as indicated by the fact that mitoses never appeared until 3 days after the addition of PHA regardless of the total age of the culture. Leukocytes in a state already capable of division, on the other hand, (i.e., marrow cells, leukemic blasts) did not require the presence of PHA for the mitotic process itself.

There must be a number of other factors which can operate both in vivo and in vitro to stimulate or inhibit the mitotic potential of circulating leukocytes (5, 9). Not only have previous workers obtained some mitotic activity in leukocyte cultures, in the absence of PHA (1, 3), but there is also at least indirect evidence that mononuclear leukocytes from the circulating blood can undergo mitosis in the body (e.g., at sites of inflammation) (6). However, our present attempts, in vitro, to uncover evidence of a physiologic control mechanism have been unsuccessful. In the absence of PHA, none of the variety of conditions studied resulted in the appearance of mitoses in our cultures; and, with PHA present, variation of these conditions, over at least as wide a range as might be encountered in vivo, uniformly failed to prevent the initiation of mitotic activity.

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REFERENCES


Fig. 1.—Three mitoses in clump of "large mononuclears" and lymphocytes on slide from 4-day "standard" culture of normal human leukocytes, containing phytohemagglutinin. Cells arrested in metaphase by colchicine treatment (18 hours). Swelling of cytoplasm of all cells results from water rinse before air drying and staining with Giemsa. ×720.

Fig. 2.—Mitotic figure in smear from 4-day "spinner" culture of whole blood. Apparently healthy lymphocytes are present, as well as degenerating forms. Erythrocytes are mainly intact and not agglutinated, despite presence of phytohemagglutinin. Colchicine treatment—18 hours. Wright's stain. ×720.
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