The Effect of Phytohemagglutinin on Leukocyte Cultures as Measured by $^{32}$P Incorporation in the DNA, RNA, and Acid Soluble Fractions

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With the technical assistance of Martha P. Vaules

VARIOUS tissue culture technics have been used to demonstrate cell division in cells derived from the peripheral blood of normal individuals.$^{1-3}$ However, the only consistently successful technic has recently been developed by those interested in chromosome morphology and involves the isolation of leukocytes from whole blood by phytohemagglutinin (PHA). Although long used for the isolation of white cells for the establishment of leukocyte cultures,$^4$ this mucoprotein, derived from the common red kidney bean (*Phaseolus vulgaris*) or navy bean (*Phaseolus communis*),$^5$ has only recently been demonstrated in critical experiments by Nowell$^6$ to be a factor necessary for cell division in cultures of this type.

The present study attempts to correlate the changes in the cell population with the metabolic events which take place in the presence and absence of PHA as measured by $^{32}$P incorporation into the DNA, RNA, and acid soluble fractions in the culture.

METHODS

Composition of cultures: Human venous blood from normal individuals and from several patients undergoing phlebotomy treatment for hemochromatosis or polycythemia vera was drawn into heparinized glass syringes or plastic collection bags. Part was then allowed to sediment in a silicized 150 x 35 mm. test tube at 37 C. until about 20 per cent of the volume could be withdrawn as a plasma-leukocyte suspension often containing less than one erythrocyte per leukocyte as contamination. The remainder was spun at 800 g for 20 minutes and the resulting plasma spun once again at 800 g for 20 minutes to insure that all of the leukocytes were removed. The plasma-leukocyte suspension was then diluted with cell-free plasma to yield a suspension containing $1 \times 10^6$ leukocytes/ml. The resulting suspension was tagged with 5 $\mu$c $^{32}$P/ml. (a mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$ at pH 5.5, the majority being NaH$_2$PO$_4$) and 1 ml amounts were pipetted into 16 x 125 mm. screw top tubes, each containing 4 ml. of culture media.

The media was prepared by combining 100 ml. of 10 times concentrated culture media† and 900 ml. sterile double glass distilled water to which 24 mEq. NaHCO$_3$, 250,000 units of penicillin, and 0.25 Gm. streptomycin had been added; 0.02 mg. of PHA† per ml. of culture was added to the indicated tubes. The PHA used throughout these experiments was a lot of proven activity as demonstrated by its ability to produce mitosis in cultures prepared with

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†TC 199: Difco Laboratories, Detroit, Mich.
‡PHA lot no. 448064: Difco Laboratories.
All tubes were incubated with loose caps in a 37°C incubator gassed with 5 per cent CO₂ and air. At the indicated times, tubes were removed and fractionated in triplicate.

**Fractionation:** The culture tubes were agitated on a mechanical mixer and spun at 2300 g at 4°C for 10 minutes. After aspiration, the cells were resuspended in 5 ml of 0.9 per cent saline and washed again. At this point the cells were quickly frozen at —23°C and stored until fractionation. Kahan's adaption of the Schmidt-Tannhauser technic to membrane filtration was used with slight modification.

To the washed cells, 0.75 mg of bovine albumen and 5 ml of 0.3 N perchloric acid (PCA) was added at 0°C. The tubes were spun at 1500 g and the supernatant was decanted. Two ml of a solution containing 10 mg/ml of acid-treated and extensively washed charcoal was added to the supernatant. The resulting suspension was agitated by means of bubbling compressed air into the tube with a soda straw. The acid soluble phosphorous fraction adsorbed on the charcoal at the end of 10 minutes agitation at 0°C was separated by cooling.

The residue remaining after acid precipitation was dissolved in 0.2 ml of 1 M NaOH and was treated in a boiling water bath for 10 minutes to solubilize the RNA. Subsequently 6 ml of a lipid solvent (2 parts chloroform to 3 parts ethanol) was added, and the tubes were held at 60°C for 15 minutes in a water bath in order to extract the lipoprotein fraction, as well as to reprecipitate the RNA and DNA. After cooling, the RNA and DNA fractions were collected on a millipore filter, and the filtrate containing the lipoprotein activity was discarded. The tube and filter were washed again with 6 ml of lipid solvent.

To the filter, now containing the RNA and DNA fractions, 5 ml of 0.1 N PCA was added, and 10 seconds later suction was applied to separate the acid soluble RNA mononucleotides from the insoluble DNA fraction which remained on the filter. The filtrate was collected in tubes containing 2 ml of the charcoal solution. After 20 minutes mixing by air bubbles (as above), the adsorbed RNA fraction was collected by filtration, and the phosphoprotein fraction in the filtrate was discarded.

The filters containing the acid-soluble, RNA, and DNA fractions were glued with small amounts of a polyvinyl cement to 30 mm aluminum planchets. Counting was done using a Geiger-Müller tube in an automatic sample changer to an accuracy of 5 per cent.

The validity of the DNA separation used in the fractionation of these cells was tested by means of DNAase as follows: Three of nine tubes containing identical cultures were fractionated as described above. The cells in the second three tubes were disrupted by ultrasound and then fractionated, whereas the cells in the final three tubes were disrupted by ultrasound and were incubated with DNAase prior to fractionation. The cultures sonicated were suspended in 2 ml of 0.02 M MgSO₄ at pH 6.5 and were treated for 1 minute with an M.S.E. ultrasonic probe. One mg. of DNAase was added to the last three tubes, and all the sonicated tubes were incubated for 4 hours at 37°C. At the end of this time, 2 ml of 0.6 N PCA and 0.75 mg of bovine albumen were added to each tube. One mg. of DNAase was added at this point to the sonicated tubes incubated without DNAase, and all tubes were fractionated. It may be seen that treatment with DNAase was able to remove about 60–70% of the DNA.

*Midway through this work some inactive lots of Difco PHA were encountered, so an extract of PHA was made according to the technic of Li and Osgood. An extract of Difco PHA was used.*

Nit A. 25 mm. Hydrasol assay type Millipore filters with a pore size of 0.45 µ purchased from Millipore Filter Corp., Bedford, Mass.

After this work was done, Kahan recommended that instead of collecting the DNA and RNA by filtration from the lipid solvent, that centrifugation at 1500 g be used instead, since it is difficult to get all the DNA and RNA precipitate off the wall of the test tube.

The resulting lipid supernatant is then discarded and the DNA-RNA pellet is dissolved in 2 ml of 1 M NaOH at room temperature. This alkaline solution is chilled and acidified with 4 ml of 0.75 M PCA and allowed to stand at 0°C for ten minutes. The DNA precipitate is then collected on a Millipore filter and the RNA filtrate treated with charcoal as before.

**Desoxyribonuclease, 1 x crystalized; Worthington Biochemical Corp., Freehold, N. J.**
Table 1.—Purity of DNA Fraction

<table>
<thead>
<tr>
<th>Hours of Culture (no. of exp.)</th>
<th>Per cent added P(^{32}) in DNA (\times 10^{-3}) mean ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96 (6)</td>
</tr>
<tr>
<td>1 No PHA</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>2 PHA</td>
<td>32.0 ± 10.0</td>
</tr>
<tr>
<td>3 PHA + sonication</td>
<td>30.0 ± 12.0</td>
</tr>
<tr>
<td>4 PHA + sonication + DNAase</td>
<td>13.0 ± 4.3</td>
</tr>
<tr>
<td>5 % removed by DNAase (3-4/3) x 100</td>
<td>66.0 ± 4.5</td>
</tr>
</tbody>
</table>

per cent of the activity originally present on the filter. The significant drop of DNA recovered after sonication may be due to the destruction of DNA by sonication or by intrinsic cellular DNAase during the four hour incubation. In any case, the method of calculation of percentage DNA actually present in the fractionation technic employed represents a minimum figure (table 1).* Almost all the activity (95 per cent) could be eliminated by treating the filter with 10 per cent TCA at 100 C. for 20 minutes.

**Phosphorus determination:** After agitation of the culture tube, 0.2 ml. of cells and culture media was added to a 12 ml. heavy-walled conical centrifuge tube containing 0.2 ml. of 10 N \(\text{H}_2\text{SO}_4\). The tube was heated to 150 C. in a sand bath to wet-ash the contents and cooled prior to the addition of two drops of concentrated \(\text{H}_2\text{NO}_3\). The temperature was brought to 150 C. once more, and the process repeated until the contents became perfectly clear. At this point the tube was taken to 205–220 C. for 1 hour in order to eliminate the \(\text{H}_2\text{NO}_3\). The phosphorus was then determined by the method of Fiske and SubbaRow.9

**Preparation of cells for cytologic study:** Gentle centrifugation was used to separate cells from the media. The cells were then smeared directly on slides and stained with Wright’s stain or with orcein by the technic of Moorehead et al.10

**RESULTS**

No mitoses were seen during the examination of numerous smears derived from cultures not containing PHA. By the end of 96 hours, cultures without PHA contained degenerating cells as evidenced by pyknotic nuclei and cellular debris. A few small lymphocytes and erythrocytes remained intact. In cultures containing PHA, however, there appeared at 48 hours large mononuclear cells as described by Nowell.6 By 96 hours there were clumps of these cells with a finely granular chromatin network in a large nucleus usually containing prominent nuclei. The sparse cytoplasm was quite basophilic and in many respects the cells resembled the “blast” cells seen on examination on bone marrow (fig. 1). Mitoses first appeared at 48 to 72 hours and reached a peak at 96 hours when 1 to 1.5 per cent of the cells were seen to be in mitosis. These changes are reflected in the incorporation of \(\text{P}^{32}\) in the DNA fraction. In figure 2, the per cent \(\text{P}^{32}\) incorporated into the DNA fractions at various times is indicated for leukocytes derived from two individuals, both of

*Hughes15 has shown that after two seconds exposure of DNA to the MSE ultrasonic probe, the average molecular weight decreases from \(9 \times 10^6\) to \(0.5 \times 10^6\), largely due to the shearing force. Continued treatment results in further decrease in DNA m.w. due to the effect of free radicals. Thus the per cent DNA removed by DNAase in table 1 might better be calculated by subtracting item 4 from item 2 and dividing by item 2, resulting in the following per cent DNA removed by DNAase: 62 ± 11, 91 ± 1, 85 ± 9, for 96, 120 and 144 hours respectively.
Fig. 1.—Cells present in PHA treated culture at 96 hours.

Fig. 2.—$^{32}P$ incorporation in DNA fraction of cultures with and without PHA.
PHA EFFECT ON LEUKOCYTE CULTURES

Fig. 3.—P³² incorporation in the RNA fraction of cultures with and without PHA.

whom had asymptomatic hemachromatosis with normal liver function tests. When compared with the activity of this fraction in cultures not containing PHA, it becomes evident that at about 24–48 hours there appears a significant increase in DNA P³². This reaches a peak in 4 to 8 days. These two representative experiments (run 36 and run 40) are typical in that they show differences in percentage added P³² incorporated into DNA and RNA and the time that a maximum P³² DNA and RNA is achieved. For this reason the effect of various environmental and chemical conditions on this culture system should only be analyzed within each run utilizing internal controls. It should be emphasized that the phosphorus content of culture tubes in each experiment was the same and was not influenced by the addition of the agents described below. The variation in activity from experiment to experiment could not be explained by variation in the total phosphorus concentration which averaged 1.97 with a range of 2.17 to 1.82 μM/ml.

The RNA label often appears somewhat earlier and usually, but not always, reaches higher activity than in the DNA fraction (fig. 3). There is a prompt and marked incorporation of P³² in the acid soluble fraction during the first 24 hours (fig. 4). This occurs in cultures with and without PHA. However, as time progresses, there appears an appreciable difference between values ob-
tained from the treated and non-treated cultures, the treated culture having the higher activity.

The activity of the DNA fraction at 96 hours was used to compare the "growth stimulating potential" of PHA with various other substances. Fetal calf serum in concentrations of 2-20 per cent of the culture medium was found to be ineffective. Similarly, a small clot, conjugated estrogens* 0.1 to 1.0 γ/ml., traumatic acid 0.2 mg./ml., indoleacetic acid 2 γ/ml., kinetin 0.2 γ/ml., and the combination of the latter two at the given concentrations, did not cause DNA synthesis in the absence of PHA.

Figure 5 shows the changes resulting in the activity of the 10 per cent TCA soluble and acid insoluble residue after adding $1 \times 10^{-2}$M cyanide to PHA treated cultures. Increasing concentrations of chloramphenicol above $6.1 \times 10^{-4}$ M caused progressive decreases in the label contained in all fractions as shown in table 2. Table 3 shows the progressive decrease in the activity of the DNA fraction in cultures of three individuals with concentrations of diphenylhydantoin greater than $5 \times 10^{-5}$ M. Subject 3 had an aplastic anemia due to diphenylhydantoin and was recovering following cessation of the drugs.

*As Premarin, Ayerst Laboratories, New York, N. Y.
at the time his leukocytes were obtained for culture. Table 4 demonstrates the inhibition of uptake in all fractions when 6-mercaptopurine in a concentration of $1.3 \times 10^{-3}$ M is added to the culture.

When tubes containing washed erythrocytes or washed group A or B erythrocytes agglutinated by group specific antibodies are incubated in TC 199 for 24 hours, the red cells are found sedimented in the bottom of the tube. On the other hand, tubes containing washed erythrocytes agglutinated by PHA are found to contain a film of erythrocytes attached to the glass so firmly that, after agitation on a mechanical mixer and centrifugation, only a few can be sedimented (fig. 6).

**Discussion**

The difficult question of defining "growth" in the tissue culture system of this investigation needs first to be discussed. In a system such as this, where the total cell population decreases due to loss of granulocytes, cell counting can not be used in assessing growth.\(^{11}\)

The determination of the mitotic index is not in every case a measure of growth of a cell population\(^{11}\) and in this system would be difficult if not im-
Table 2.—Effect of Increasing Concentrations of Chloramphenicol on $^{32}$P Incorporation in Fractions of a Leukocyte Culture at 96 Hours

<table>
<thead>
<tr>
<th>Chloramphenicol Conc.</th>
<th>DNA</th>
<th>AS</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.8</td>
<td>55.0</td>
<td>47.3</td>
</tr>
<tr>
<td>$6.1 \times 10^{-4}$ M</td>
<td>15.9</td>
<td>54.0</td>
<td>40.0</td>
</tr>
<tr>
<td>$3.05 \times 10^{-3}$ M</td>
<td>4.16</td>
<td>27.1</td>
<td>13.0</td>
</tr>
<tr>
<td>$6.1 \times 10^{-3}$ M</td>
<td>1.42</td>
<td>10.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 3.—Effect of Diphenylhydantoin on DNA Synthesis as Measured by $^{32}$P Incorporation in Cultures from Three Individuals at 96 Hours

<table>
<thead>
<tr>
<th>Diphenylhydantoin concentration</th>
<th>% $^{32}$P x 10$^{-2}$ inc. in DNA subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$ M</td>
<td>11</td>
</tr>
<tr>
<td>$2.5 \times 10^{-4}$ M</td>
<td>9.1</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$ M</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 4.—Effect of 6-MP on $^{32}$P Incorporation in Fractions of a Leukocyte Culture at 96 Hours

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>DNA</th>
<th>AS</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>54</td>
<td>69</td>
<td>101</td>
</tr>
<tr>
<td>$1.3 \times 10^{-5}$ M</td>
<td>55</td>
<td>74</td>
<td>110</td>
</tr>
<tr>
<td>$1.3 \times 10^{-4}$ M</td>
<td>50</td>
<td>81</td>
<td>105</td>
</tr>
<tr>
<td>$1.3 \times 10^{-3}$ M</td>
<td>18</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>$1.3 \times 10^{-2}$ M</td>
<td>3.3</td>
<td>3.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

possible to determine quantitatively for technical reasons. For the above reasons we have selected $^{32}$P incorporation into DNA as an index of "cell growth." We thus are assuming that $^{32}$P incorporation into DNA does not occur without synthesis of a DNA molecule. The burden of proof would appear to be on those who say there is a turnover of DNA-$^{32}$P independent of that caused by synthesis. We then are determining only DNA synthesis. It is likely that the observed DNA synthesis is associated with cell division but we have no direct proof that this is so. Logarithmic plots reveal DNA doubling time of 26 and 31 hours for runs 36 and 40 respectively (fig. 2). These figures are with the limits of 25 to 74 hours for average human myeloid cell generation times calculated by Cronkite on the basis of cell mitotic time of from 0.5 to 1.5 hours.

We have considered several possible mechanisms of action for PHA. One of these is that the addition of PHA produces mitoses merely by promoting division of cells which already contained the necessary DNA for cell division at the time they were placed in the culture. In other words, at the time of culture these cells were in the post DNA synthetic phase which persisted an unusually long period. This hypothesis is not consistent with the demonstration of DNA synthesis in these cultures as indicated by the above data and by the work of MacKinney, Stohlman, and Brecher, who demonstrated DNA synthesis...
Fig. 6.—Effect of PHA on washed erythrocytes maintained in TC 199 media. All tubes contain equal numbers of erythrocytes. Note that erythrocytes in tube containing PHA are firmly bound to the glass and that, after vigorous agitation and centrifugation, only a few cells have been sedimented.

in 40 per cent of cells in similar cultures at 72 hours by the use of H\textsuperscript{3}-thymidine for DNA labeling.\textsuperscript{13}

Secondly, there exists the possibility that PHA promotes growth because of the surface active properties witnessed in the above described erythrocyte experiment. The leukocytes in this tissue culture system tend to grow in thin films attached to the glass surface rather than as a sedimented “button” on the bottom of the test tube or as a suspension of free cells in the plasma. This was precisely the effect of PHA on the distribution of red cells in this culture system.

Thirdly, the ability of PHA to promote growth may depend on the ability of this agent to provide some as yet unrecognized factor necessary for DNA synthesis and mitosis of these cells. The present experiments do not indicate which of the latter two possibilities is the more likely. It is hoped future experiments will help to explain the action of this unique substance.

Moreover, the quantitation of P\textsubscript{32} incorporation into DNA in these cultures enables in vitro studies of drug effect on cells derived from human peripheral blood as herein demonstrated. This technic could be used to study the mechanism of drug resistance in cases of human leukemia treated with various antimetabolites.

**Summary**

PHA, when added to human leukocyte cultures, results in DNA synthesis and cell mitosis. The addition to these cultures of fetal calf serum, indoleacetic acid, kinetin, estrogens, or a small clot did not duplicate the effect of
PHA. Concentrations of chloramphenicol $3.05 \times 10^{-3}$ M, diphenylhydantoin $5 \times 10^{-5}$ M, and 6-mercaptopurine $1.3 \times 10^{-3}$ M resulted in inhibition of growth. Possible mechanisms of action of PHA have been considered. In addition, the possible use of the method described as an in vitro means of evaluating drug sensitivity in leukemia has been suggested.

**Summario in Interlingua**

Quando phytohemagglutinina es addite a culturas de leucocytos human, le resultato es synthese de ADN e mitosis. Le addition, a iste culturas, de sero de fetos bovin, de acido indolacetic, de cinetina, de estrogenos, o de un micro coagulo non duplicava le effecto de phytohemagglutinina. Concentraciones de $3.05 \times 10^{-3}$ M de chloramphenicol, de $5 \times 10^{-5}$ M de diphenylhydantoina, e de $1.3 \times 10^{-3}$ M de 6-mercaptopurina resultava in un inhibition del crescentia. Possibile mechanismos del action de phytohemagglutinina es discutite. In plus, es suggerite le possibilitate de utiliar le methodo describite como un medio pro evaluatar in vitro le pharmaco-sensibilitate presente in casos de leucemia.

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**REFERENCES**


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