An Assay for the Mitogenic Activity of Phytohemagglutinin Preparations

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The addition of phytohemagglutinin, an extract of the kidney bean Phaseolus vulgaris, to cultures of human peripheral lymphocytes promotes the synthesis of γ-globulin, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA), and induces mitosis in a large fraction of lymphocytes.1 7 The present communication describes an assay in which the graded induction of DNA synthesis in leukocyte cultures by different levels of phytohemagglutinin has been used to measure this mitogenic principle quantitatively. In addition to its usefulness in isolating the mitogenic principle, the assay also affords a system for the demonstration and study of factors present in normal and pathologic sera that modify the ability of leukocytes to undergo replication.

Materials and Methods

Leukocyte Sus’penions

Blood from normal adults was drawn in heparinized syringes and 10–12 ml. aliquots were added to sterile 13 ml. screw cap centrifuge tubes containing 0.3 ml. of heparin (heparin sodium, 1000 U.S.P. units/cc., Upjohn Company, Kalamazoo, Mich.). The tubes were inverted 5–10 times to insure mixing and allowed to settle for 2 hours at room temperature. The leukocyte-containing plasma layer above the red cell boundary was withdrawn. The number of leukocytes per ml. of plasma was 70–80 per cent of that of whole blood, and there was less than 1 per cent contamination with erythrocytes. Normal distribution of cell types was observed in plasma from normal individuals. After determining the leukocyte count in a hemocytometer, the required volume of plasma was withdrawn and the cells sedimented for 7 minutes in a clinical centrifuge. The cell pellet was suspended in 5.0 ml. of 0.05 per cent sterile trypsin (Nutritional Biochemical Corporation, Cleveland, Ohio, 1–300) and incubated for 10 minutes at 37 C., during which time the cells were gently dispersed 3 times with a 10 ml. volumetric pipette. The gelatinous cell suspension was then centrifuged for 5 minutes in a clinical centrifuge and the cell pellet resuspended in culture medium for planting.

Culture Medium

The modified Eagle's HeLa medium (EHM) used in these experiments has been described previously.9 EHM supplemented with 10 per cent bovine serum is referred to as BEHM; EHM combined with serum from clinically normal persons is referred to as NEHM. Human serum was prepared by allowing whole blood to clot for 30 minutes at room temperature for personal use only.
followed by sedimentation in the cold at 1250 g in a model PR-2 International Centrifuge. The supernatant serum was then withdrawn and stored at -20 C. After combining, the medium was sterilized by passage through an 03 Selas porcelain filter candle. Phytohemagglutinin (PHA-M) (Difco) was used as a temporary standard in this work. The contents of each vial (100 mg. PHA-M) was dissolved in 5.0 ml. of sterile distilled water; 1.0 ml. of this stock solution was designated as 1.0 unit of activity. It is anticipated that further purification of PHA and assessment of the factors in sera which modify the lymphocyte response will permit definition of an absolute unit of activity. Several lots, all similar in activity, were used. PHA-M solutions were made up fresh for each experiment and further diluted with distilled water so that 0.1 ml. was used in each case.

**Assay Procedure**

Figure 1 graphically presents the assay sequence. Replicate cultures of leukocytes were prepared by suspending approximately 2.7 x 10^6 cells in 3.0 ml. of EHM in 16 x 150 mm. test tubes containing 0.1 ml. of PHA-M in water. (Difco Laboratories. Detroit, Mich.). All cultures were gassed with 5 per cent CO_2 in air and incubated on a 4° slant at 37 C. At the end of 48 hours of incubation, each culture received 0.1 ml. of a water solution of 3 x 10^-5 M amethopterin (ameth) (obtained as methotrexate sodium from Lederle Laboratories, Pearl River, N. Y.) and 1.5 x 10^-3 M adenosine (A) (A grade, California Corporation for Biochemical Research, Los Angeles, Calif.). After 16 hours of incubation in the presence of amethopterin. 0.1 ml. of thymidine-2-C_14 (Tdr-2-C_14) solution containing 30 gg/ml. (3.66 mc/mM, New England Nuclear Corporation, Boston, Mass.) was added to each culture, which was then incubated for an additional 6 hours to assess the capacity of the cells to incorporate Tdr into DNA. Each addition was made at 37 C. and the cultures were regassed with 5 per cent CO_2 in air before being returned to the incubator.

Determinations of the incorporated Tdr-2-C_14 were carried out on the acid-insoluble cell residues obtained in the following way. After 70 hours of culture the cells and medium were decanted into conical centrifuge tubes. The tubes containing the cells sedimented at 725 g for 10 minutes at 0–3 C. in a Model PR-2 International Centrifuge, and the original culture tubes containing adherent cells, were stored at -20 C.

Extraction was carried out at 0 C. by successive suspension and centrifugation of the cell residues: twice in 10 per cent trichloroacetic acid (TCA), once in 80 per cent ethanol, and once in a 1:1 mixture of absolute ethanol and ethyl ether. During the TCA washes the cells adhering to the original culture tubes were scraped free with a rubber policeman and combined with the respective sedimented cell residues of the assay tubes.

The air-dried, acid-insoluble residue was dissolved in 0.5 ml. of 88-90 per cent formic acid, and a 0.2 ml. aliquot of each sample was either plated on a stainless steel planchet for counting in a gas-flow proportional counter or added to 20 ml. of ANPO (α-naphthylphenyloxazole, Packard Inst. Co., LaGrange, Ill.) for counting in a Packard Tricarb Liquid Scintillation spectrometer. Results are expressed as counts per minute per aliquot. Sensitivity of the fraction to DNAse showed that the radioactivity in the residue is in DNA.
Fig. 2.—The response of lymphocyte cultures to graded levels of PHA-M. Replicate cultures of human peripheral lymphocytes were grown in the presence of varying concentrations of PHA-M and assayed for their ability to incorporate Tdr-2-C\(^{14}\) into DNA as described under Methods. Results are expressed as counts per minute of Tdr-2-C\(^{14}\) incorporated into the acid-insoluble fraction. Each point represents a separate culture.

RESULTS

The Induction of DNA Synthesis by Graded Levels and Treatments with Phytohemagglutinin-M

Figures 2 and 6 show the typical sigmoid dose response curve obtained when normal human peripheral leukocytes were cultured in the 70-hour assay system with different levels of PHA-M. No significant stimulation of DNA synthesis occurred until a certain level of PHA-M had been added. The intercept value was measured routinely; it appears significant in the characterization of certain pathologic sera. Above this level the response increases in a nearly linear manner over a range of additional increments of PHA-M until a plateau is reached with saturating levels of PHA-M. At high levels of PHA-M the response curve may fall off, suggesting the existence of secondary toxic factors in the commercial PHA-M preparations or in individual cell systems.

Using normal human leukocytes in BEHM or NEHM, an intercept value and a plateau response were usually obtained in the 70-hour assay system with .002–.003 and .007–.01 units of PHA-M, respectively. The character of the dose response curve and the reproducibility of the response permit a highly accurate assay of the mitogenic principle in different plant extracts at dif-
The effect of varying periods of treatment with PHA-M on the ability of leukocyte cultures to incorporate Tdr-2-C\textsuperscript{14} into DNA. In curve 1 (anterograde treatment) replicate cultures were planted in a medium containing 0.01 units PHA-M and the medium replaced with a PHA-M-free medium at 12, 24, or 48 hours. In curve 2 (retrograde treatment) replicate cultures were planted initially in PHA-M-free medium and 0.01 units of PHA-M added at 12, 24, 48, or 58 hours after the start of the incubation. The ability of such cultures to incorporate Tdr-2-C\textsuperscript{14} into DNA was assayed over the last 6 hours of the 70-hour culture period as described in Methods. The response in terms of counts per minute is plotted against the total hours of exposure of each culture of PHA-M.

To test whether the continued presence of PHA-M was required throughout the 70-hour assay, replicate cultures were planted in medium containing 0.01 units of PHA-M. After 12, 24, or 48 hours of incubation the cells were centrifuged down, the PHA medium was replaced with PHA-free medium, and the assay was continued as described in figure 1. The response was a function of time of exposure to medium containing PHA-M and PHA-M must be present continuously to elicit a maximal response (fig. 3).

In the same experiment a series of replicate cultures was planted in PHA-free medium. PHA-M was added after 12, 24, 48 or 58 hours of incubation. Cultures receiving PHA-M at 58 hours, for example, were exposed to PHA-M only during the last 12 hours of the 70-hour assay. The response to this retrograde pattern of treatment with PHA was very similar to that obtained with the anterograde pattern of treatment with PHA-M, and the response supported
the same conclusions. It was apparent from both experimental approaches that no significant stimulation of DNA synthesis was obtained with less than 24 hours of exposure to an adequate level of PHA-M.

In accord with these results it was observed that replicate cultures of leukocytes planted in the presence of PHA-M and exposed to successive 12-hour periods of Tdr-2-C14 from 0 to 120 hours incorporated no significant amount of Tdr prior to 36 hours. A rapid acceleration of DNA synthesis occurred between 36 and 60 hours, with a subsequent decline. In a number of experiments it was observed that a second dose of PHA-M administered to the cultures after 60 hours of incubation again accelerated DNA synthesis. Apparently PHA becomes limiting if the cultures are carried beyond 70 hours; perhaps the leukocytes utilize PHA-M. Further experiments on this aspect are in progress.

The Role of Amethopterin in the Assay System

Previous work with HeLa cells9,10 showed that endogenous synthesis of thymidine and the concomitant synthesis of DNA could be blocked with amethopterin under culture conditions which permitted the continued synthesis of RNA and protein. Cells in such cultures become triggered for DNA synthesis, yet are prevented from synthesizing DNA by the thymidine deficiency. The reversal of the deficiency state after 16 hours by the addition of exogenous Tdr is attended by a synchronous wave of DNA synthesis which involves almost all cells in the population.

An attempt was made to increase the sensitivity and uniformity of the assay by the application of this concept to the leukocyte cultures during the period of rising DNA synthesis. Despite the metabolic pool afforded by the breakdown of myelocytic cells4,11 early in the period of culture, it was verified (fig. 4) that the same principles do apply, at least in part, to the leukocyte cultures, since the Tdr-2-C14 incorporation into amethopterin-treated cultures was significantly greater than in control cultures without amethopterin. Figure 5 shows that the effect of amethopterin holds for all levels of PHA-M which induce leukocytes to replicate DNA. Since it was found in these experiments that sera from patients with certain pathologic conditions inhibit normal lymphocyte replication, an assay with such a serum (PEHM) from a patient with chronic myelocytic leukemia was included in this experiment. As in the case with the normal serum, a thymidineless state was similarly produced, even though a much higher level of PHA-M was required to overcome the strongly inhibitory effect of the serum on the cellular response to PHA-M.

Cell Factors Affecting the Assay

During the course of experiments 20 different cell sources were tested. Eighteen of the individuals had no known illness at the time, one donor had trichinosis, and another had nonspecific infectious cervical lymphadenopathy of several months duration. Cells from each of these donors responded similarly to increasing increments of PHA-M after the initial intercept dose was attained. The response of two different donors' cells is shown (fig. 6). In certain cases the total incorporation of Tdr achieved at high levels of PHA-M was dif-
Fig. 4.—The influence of amethopterin on the assay system for PHA-M. Replicate cultures of human peripheral leukocytes were grown in the presence of 0.007 units of PHA-M for the indicated periods of time. In the control cultures, Tdr-2-C\(^{14}\) was added 6 hours prior to harvest of the cultures. In the case of amethopterin-treated cultures, amethopterin was added 22 hours and Tdr-2-C\(^{14}\) 6 hours prior to harvest of the cultures. Data are expressed as counts of C\(^{14}\) per minute in the acid-insoluble residue at the indicated culture time.

Different, but the fractional response to graded levels of PHA-M was very similar. As shown in figure 7, the Tdr-2-C\(^{14}\) incorporation was proportional to the amount of cells in the culture up to \(8 \times 10^5\) cells per ml of culture. The linear relationship holds up to \(1.2 \times 10^6\) cells per ml of culture; however, when cell levels were raised to \(1.4 \times 10^6\) per ml, the medium was exhausted prior to the conclusion of the assay and the results were erratic. Accordingly, an initial cell density of approximately \(9 \times 10^5\) cells per ml of culture was chosen so as to afford as many assays as possible from one donor and still retain high accuracy.

No variations in the response have been attributable to A, B, O, or Rh blood groupings, female hormonal cycles, sex of the donor, blood corticosteroid levels, or heparin level. Trypsinization was used to disperse completely the leukocyte suspensions. More recent experiments have shown that the step is unnecessary for this purpose; in fact, the total incorporation is somewhat higher if trypsinization is avoided. However, trypsinization may reduce variability between cell preparations by removal of certain inhibitory factors.

Effect of Serum on the Assay

The type of serum used in the assay plays an important role in the response. Using sera of clinically well individuals, 27 out of 32 gave the normal response.
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**AN ASSAY FOR MITOCENIC ACTIVITY**

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Fig. 5.—The influence of amethopterin on the assay system. Replicate cultures of human peripheral leukocytes were planted in medium supplemented with 10 per cent bovine serum (BEHM) or medium supplemented with 10 per cent serum from a patient with chronic myelocytic leukemia, a pathologic serum (PEHM), in the presence of varying levels of PHA-M. Half of the cultures with each type of serum were treated with amethopterin and assayed for Tdr-2-C\(^{14}\) incorporation as described under Methods; in the other half the Tdr-2-C\(^{14}\) incorporation was measured without previous amethopterin treatment.

which was similar to that obtained with 10 per cent bovine serum. However, sera from 18 of 29 patients with hematopoietic disturbances were inhibitory to the PHA-M assay system (i.e., fig. 5). A further description of the nature of the inhibitory principles in these pathologic sera will be the subject of a subsequent paper.\(^{12}\)

**DISCUSSION**

The control of replication and replacement of the various cell types in the human body, although profoundly important in the maintenance of structure and function, is but little understood. The normal lymphocytes of the peripheral circulation constitute a population of cells whose replication is under some metabolic restriction. When in culture, however, these cells can be stimulated to reproduce specifically by certain antigens\(^{13,14}\) or generally by the mitogenic principle in PHA extracts.\(^{14,15}\) The present studies with PHA-M showed that the induction of DNA synthesis leading to mitosis* requires both

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Fig. 6.—The response of leukocytes from two different normal donors in the 70-hour assay system in BEHM with varying levels of PHA-M. Tdr-2-C\textsuperscript{14} incorporation into DNA is measured as described under Methods.

an adequate level of PHA-M and an adequate period of exposure. The observation that 24 hours of contact with PHA-M fails to induce subsequent DNA synthesis seems to indicate that the initial contact of PHA-M with the cell sets in progress a sequence of events leading to DNA synthesis, but does not act directly as a mitogenic trigger. As will be shown elsewhere, a rapid acceleration of RNA and protein synthesis occurs during this period, even though the cells do not follow through to DNA synthesis.\textsuperscript{3} Thus it becomes important to identify the acceptor site for PHA-M and to determine how interaction with this site activates the genetic expression mechanisms leading to cell replication.

With this assay it has been possible to demonstrate factors in human serum which have a stimulatory or inhibitory effect on the response of leukocyte cultures to PHA-M.

The levels of PHA-M found to stimulate DNA synthesis optimally are lower than those used by most workers in studying mitosis. In some of these reports the PHA has been added to whole blood. Although the initial amounts were high, the concentration of the available mitogenic principle in the leukocyte suspension is unknown under these conditions. Experiments with high levels of PHA have also suggested the possibility of selective cell damage, and accordingly a possible cell selection in the mitotic study. In this connection it is important to report that the levels of PHA used in these experiments have also been found optimal for the induction of RNA synthesis in leukocyte cultures.\textsuperscript{8}
Fig. 7.—The effect of cell level on the assay system. Replicate cultures with the indicated numbers of human peripheral leukocytes were planted in BEHM in the presence of 0.005 or 0.01 units of PHA-M and assayed in the 70-hour system as described under Methods.

SUMMARY

The addition of increasing levels of phytohemagglutinin to amethopterin-treated cultures of human lymphocytes results in the induction of DNA synthesis which can be measured by the incorporation of exogenous radioactive thymidine. The graded nature of this response permits the assay of mitogenic activity of such preparations as well as the assessment of antimitogenic factors in sera.

SUMMARIO IN INTERLINGUA

Le addition de crescente quantitates de phytohemagglutininina a culturas de lymphocytos human pre-tractate con amethóptera resulta in le induction de un synthese de acido disoxyribonucleic le qual pote esser mesurate per le incorporation de exogene thymidina radioactive. Le graduate character de iste responsa permette le essayage del activitate mitogene de tal preparatos e etiam le evalutation de factores antimitogene in seros.

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