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PHYTOHEMAGGLUTININ-INDUCED BLASTIC TRANSFORMATION AND DNA SYNTHESIS OF LYMPHOCYTE CULTURE

I. LYMPHOBLASTIC TRANSFORMATION

II. TRITIATED THYMIDINE INCORPORATION

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When blood leukocytes are incubated in an artificial medium over several days, they exhibit minimal or no growth at all. The granulocytes degenerated within a few days. while monocytes spread out on the surface of the culture vessel, assumming the appearance of cells indistinguishable from tissue macrophages. The majority of lymphocytes remain viable and morphologically unchanged. If the plant lectin, phytohemagglutinin (PHA), is added to the incubating medium, a sequence of dramatic morphorogic changes is initiated. The small lymphocyte undergoes transformation into a larges blast-like cell. (1-12) This morphological changes is accompanied by increased protein, RNA and DNA syntheses, (3, 4, 9, 10, 12) and is followed eventually by mitosis of the tansformed cells.

Lymphocytes can be stimulated in vitro by a wide variety of agents including viruses, bacteria, fungi, protozoa, and pollen as well as purified amino acid polymers and drugs. (13) The agents capable of striggering this chain of events may be catagorized as general stimulants (or nonspecific mitogens) and specific stimulants (specific mitogens). The mitogenic is characterized by its strict dependence on the prior sensitization of the cell doner to immunizing antigens, such as tuberculin, (14, 15) penicillin, (16) streptolysin O (17) tetanus and diphtheria toxoid, purtussis, polio virus and smallpox vaccines, (16, 18, 19) and protein-hapten

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conjugates. (20) These specific mitogens transform only a small portion of lymphocytes, usually 5 to 30 percent. Nonspecific mitogens transform 70 to 80 percent of small lymphocytes (21) isolated from normal individual regardless of prior exposure to each particular mitogen. The plant extracts (PHA, (1-12) pokeweed mitogen (22, 23)), antileukocyte sera, (23) antiallotype sera, (25, 26) and bacterial products (Steptolysin S., (17) staphylococcal exotozin, (26)), are well-known examples of nonspecific mitogens. (See Table I.)

TABLE I: MAJOR CHARACTERISTICS OF DIFFERENT TYPES OF MITOGENS

SPECIFIC MITOGENS	NON-SPECIFIC MITOGENS
1. Transform only 5–30% of small lymphocytes.	1. Transform 70 – 80 % of small lymphocytes.
2. Require prior sensitization of the cell doner to the particular mitogen.	2. Do not require prior exposure to each particular mitogen.
3. Examples: Tuberculin, Penicillin, streptolysin O; Tetanus and diphtheria toxoid; pertussis polio virus and smallpox vaccines.	3. Examples: Plant extracts (PHA, pokeweed mitogen), Antileukocyte sera, Antiallotype aera, Bacterial Products (Streptolysin S, Staphylococcal exotoxin) etc.

Because the magnitude of response to specific mitogen correlates closely with the state of hypersensitivity of the donor, it is believed that the vitro phenomenon may have immunologic significance. (28-30) The significance of the transformation induced with a nonspecific agent such as PHA is unclear. However, PHA-induced blasts bear morphologic features in common with the cells transformed by specific antigens, nd with the large pyroninophilic cells that develop during certain cellular immune reactions in vivo. (31) Thus, response to PHA has been used increasingly as a means of determining changes in proliferative protential and possibly functional state of lymphocyte populations in different physiological conditions such as chronic lyphocytic leukemia, (32, 36) Hodgkin's disease, (37-

39) Sarcoidosis (40-42) ataxia telangiectasia, (43, 44) Tymic aplasia, (45) Sjogren's syndrome, (46) and patients receiving cytotoxic therapy. (47) Circulating lymphocytes from patients described above react poorly to PHA and specific antigens.

Various modifications of the culture technique described by Moorhead et al (48) are widely used to culture of lymphocytes. We are describing a method of short-term in vitro culture of lymphocytes. This procedure involve the separation of lyphocytes from blood, and incubation of the cells in an appropriate artificial medium. Many of the steps are similar to those used in other laboratories. It is the reliable, reproducible and the most simple one to perform in order to study the aberration of cell-mediated immunity mechanism.

IN VITRO CULTURE AND STIMULATION OF LYMPHOCYTE

I. THE MORPHOLOGY STUDY

The degree of lymphocyte stimulation may be assessed either by morphologic changes or biosynthetic activities. The morphologic method entails the enumeration of blasts evolved per 1,000 - 2,000 cells, after a suitable incubation period. Cytologic features are best visualized in smear stained by one of the Romanowsky stains such as Wright's, Giemsa or May-Grunwald, but a nuclear stain, actic orcein (16) and acridine orange (49) have also been successfully employed. Cell growth may be assessed in viable culture by phase-contrast microscopy. (5, 50) Certain steps are essential to ensure viability and successful growth of the cultures: the cells should suffer minimum trauma in their preparation; the incubation milieu should provide adequate nutrients at a physiologic pH; and no potentially cytotoxic materials should be present in the system.

Despite its limitations the morphologic method provide the fast and convenient way of assessing lymphocyte stimulation where precise quantitative is not a necessity.

A—MATERIALS AND EQUIPMENTS.

I: MATERIALS

1. Sterile heparin solution (500 units/ml. in pyrogen-free saline for injection).

2. 5-10 ml. sterile syringe (preferably, disposable plastic syringes).

3. Sterile screw cap culture tube (13 × 35 mm) or sterile disposable plastic tubes 12 × 75 mm. with cap.

4. Sterile capillary pipettes.

5. 1 and 5 ml. sterile disposable plastic graduated pippettes.

6. Culture media: Stock TC-199 Earle's Base or MEM Hank's Base (10 x) Steriled by millipore filtration, kept at 4° C.

7. 6% dextran, sterile, (Abbott Lab.) kept. at 4° C.

8. 0.5 M and 0.25 M. Perchloric acid.

9. Penicillin 10,000 units per ml.

10. Phytohemagglutinin (Bacto - Phytohemagglutinin M. 5028, Difco

11. Stock Hepes buffer (Hydroxyethylpiperazine - N - 2 - Ethanesulfuric acid) pH. 8.1

12. Wright's stain.

13. May - Grunwald stain (Harleco, Phila).

14. Giemsa stain (Figher, Fair Lawn, N. J.

15. 0.85 % Na Cl solution, kept at 4° C.

16. Microscope slides.

17. Cover glasses,

18. Permount (Fighes, Fair Lawn, N.J.)

II. EQUIPMENTS

1. Refrigerated centrifuge.
2. A 37° C.
3. A microscope.

II.I WORKING SOLUTIONS.

1. TC. 199-Hepes solution

Stock Earle's Base (10x)	10 ml.
1. N Sodium Hepes, pH 8.1	4 ml.
Penicillin	10,000 units

Sterile deionized distilled water is added to make a total volume of 100 ml.

2. Culture solution (freshly prepared)

	Solution A	Solution B
Plasma or serum	1.00 ml.	1.00 ml.
TC. 199-Hepes solution	3.75 ml.	4.00 ml.
PHA.	0.25 ml.	-

One ml. aliquots of both solutions are transferred to sterile culture tubes.

II. METHODOLOGY.

1. Five ml. of venous blood is drawn into a plastic syringe containing 0.5 ml. of heparin solution (500 units/ml.). This produces or final concentration of about 45 units heparin per ml. blood.

2. One ml. of 6% dextran is added into the syringe.

3. Incubate the syringe in an upright position at 37° C. (or room temperature) for 45-60 minutes.

4. The supernatant is then collected aseptically into a sterile plastic tube by using the bent needle technique.

5. Centrifuge the supernatant at 1,000 rpm, 4° C. for 10 minutes.

6. Remove the supernatant plasma. Most of the lymphocytes are in the cell button.

7. Resuspend the leukocyte cell button in the TC. 199-Hepes buffer to makes a final concentration of 2×10^6 cell per ml. Do the differential leukocyte count and per cent of viable leukocytes (using 0.2% eosin Y.).

8. Transfer 0.5 ml. of leukocyte suspension into 4 culture tubes containing 1 ml. each of culture solution A (2 tubes) or B (2 tubes). The final cell concentration is 1×10^6 leukocytes /1.5 ml. culture fluid.

9. Incubate these tightly stoppered

culture tubes at 37° C. for 72 hours, undisturbed.

10. After that, the total leukocyte count and differential count is done on each sample using Wright's stain or May-Grunwald - Giemsa stain.

II. BIOSYNTHETIC ACTIVITIES — TECHNIQUE

The incorporation ef ^{14}C or tritiated thymimedine (^{3}H TDR) into a trichloracetic acid precipitate of cells represents the most sensitive indicator of lymphocyte transformation. The parallel study using radioautography has shown that it is the transformed cell which incorporates the thymidine lable. This mothod has the advantage of a low spontaneous incorporation of custimulated small lymphocytes and macrophages do not usually proliferate in short-term cultures. (30) Since it is an ideal technique for detecting those lyphocytes which respond to stimulation by proliferation and following by the proliferation it obviously will not detect lymphocytes which respond to stimulation but do not enter into the S-phase of the cell cycle. Nevertheless, this tachnique has proved to be one of the best way to follow the response of lymphocytes in vitro.

A. MATERIALS AND EQUIPMENTS.

In addition to those required for the morphology study, it requires :

1. Tritiated thymidine (^{3}H - TDR) 10 uc / 20 ug / ml.

2. Hymine hydroxide.

3. Scintillation fluid is prepared as follow :

4.9 gm. of PPO (2, 5-diphenyloxazole)

0.1 gm. of POPOP. (1, 4 - bis (2 - (5-phenyloxazolyl)) - benzene.

120.0 gm. of Naphthalene.

1,000.0 ml. of dioxane.

METHODOLOGY.

In general, the first part of this method is the same as the transformation method described up until step 9. (It is noted that tritiated thymide method may required the leukocyte culture of 48 hours to 72 hours). Then process as follows :

10. After 48 hours of incubation at 37°C., 0.1 ml. of tritiated thymidine solution (10 uc/ml) is added into each culture tubes aseptically

11. Incubate these tubes for an additional 18 hours at 37°C.

12. Do the leukocyte count and differential count, then centrifuge at 800 - 1,000 rpm at 4°C. for 10 minutes. Discard the supernatant fluid in a special container.

13. Wash the cell button twice with 2.0 ml. (or more) of cold saline solution.

14. Add 1.0 ml. of cold 0.5 M. Perchloric acid (PCA) to the cell button, mixed.

15. Incubate the cell suspension at 4°

C., overnight (non-stoppered).

16. Wash the precipitate formed overnight with cold 0.25 M. PCA then allow to dry in air (room temperature) for an additional 1 hour.

17. Dissolve the moist residue in 0.5 ml. Hyamine. Added 0.2 ml. aliquots of this solution (in duplicate) to 14.8 ml. of the PPO and POPOP scintillant.

18. The radioactivity (beta emission) is measured in a Tri-carb Liquid Scientillation Spectrometer (Packed Instrument Co.).

TECHNICAL COMMENTS

1. Although heparin has been reported to inhibit the lymphocyte transformation response, many workers observed no such problem with heparin from Mann Research Lab., N.Y., and Connaught Research Lab., Toronto, especially when used at about 20 units/ml. of blood. It is avisable that heparin used should not contain phenol as a preservative. Heparin preserved with benzyl alcohol is also satisfactory for this purpose. Various doses of heparin have been employed ranging from 20 to 100 units/ml. of blood. The most widely used is 50 units/ml. of blood.

2. A fasting donor is not required, however, grossly lipemic plasma should be avoided. The donor's condition and drug administration must be considered in interpreting the ability and inability of his lymphocytes to proliferate in vitro. These include the upper respiratory tract infection, adrenal cortical steroid or cytotoxic therapy. Oral contraceptive has not been found to interfere with the proliferation of lymphocyte in vitro.

3. The preparation of a "pure" or "relatively pure" lymphocyte suspension generally requires the isolation of the leukocyte from the whole blood, followed by removal of contaminating monocytes and granulocytes. Other workers prefer leukocytes obtained from defibrinated blood. (51) It is either allowed to settle at 37°C. for 45 - 60 minutes or is contrifuged at low speeds (400 - 500 rpm for 10 minutes) to obtain a leukocyte-rich plasma fraction. Because of the low sedimentation rate of the whole blood obtained, dextran, gelatin, fibrinogen or methyl cellulose may then be added before allowed to stand at 37°C. for 30-60 minutes. The addition of such agents will enhance the sedimentation of erythrocytes. The sedimentation rate of heparinized blood at 37°C. is about twice those at 4 C.

4. Purification of the lymphocytes suspension may then be achieved by one of the following techniques :

A. Surface adherence. The leukocyte fraction is incubated at 37°C for 1 to 2 hours on an appropriate surface to which monocytes and polymorphonuclear leukocytes (PMN) attach. The lymphocytes (with contaminating platelets and erythro-

cytes) remain unattached and can be removed virtually free of phagocytes. The materials used for phagocytic attachment are nylon fiber, (52, 53) cotton, (54) (55) glass wool; (23) beads, such as glass (56, 57) and polysterene; (58) or wide-surfaced containers, such as petri dishes. T flaskes, or Blake bottles. (59, 60)

B. Phagocytosis. Carbonyl iron filings added to the leukocyte suspension are ingested only by monocytes and granulocytes. The phagocytic cells then can be removed from the lymphocytes by centrifugation or by introducing a magnet into the cell suspension. (61, 62)

C. **Density gradients.** The leukocytes are sedimented through density gradients made of albumin, (63, 64) dextran, methylcellulose, Ficoll (65) or Isopaque. (66) Phagocytes and red cells, possessing greater specific gravities, sediment to the lower layer, while the less dense lymphocytes remain in the upper portion of the gradient. Some of these methods are time consuming and some albumin preparation may have a stimulating effect on lymphocytes. (63)

5. Centrifugal force in excess of 200× g are not advised. In addition to minimizing possible damage to the cells, gentle centrifugation of the supernatant after red cell sedimentation serves to eliminate most of the platelets. When suspending a centrifuged cell pellet, gentle mixing or trituration with a wide-bore pipette should be employed.

6. It is shown that tissue culture medium 199 supported growth in lymphocyte culture to a much greater extent than MEM. (67, 68) Valentine has found that RPMI 1640, a culture medium developed at the Roswell Park Memorial Institute is excellent for short term lymphocyte culture. (69)

7. In order to obtain a good proliferative response plasma or pooled human sera should be added into the culture medium. Fresh autologous plasma seems to work best (AB plasma, human serum are also adequate). Fetal calf serum is not recommended since it contains a growth-promoting factor that increases background ("spontaneous") transformation. (70) With human lymphocytes, a good response obtained with plasma or serum concentration of between 10% to 20%.

Lymphocyte transformation can be achieved in a serum-free medium, (71) but viability and degree of transformation are greatly reduced. Bergman et al (72) have reported that 6% dextran or 5% glucose can replace serum in the culture medium without diminishing the lymphocyte response to PHA.

8. Penicillin with is included in the culture medium to counteract bacterial

growth, may contribute to the culture response by stimulates the penicillin-sensitized lymphocytes. (16, 70) However, most workers have not experienced this problem.

9. When high concentration of cells are employed cell-to-cell contact will occur earlier than when low concentration are used, It is observed that cell population (or density) must often be at a certain level before cells will grow. In general, concentration of human cells below 0.25×10^6 per ml. should be avoided unless a very small culture vessel is used. With human blood leukocyte cultures $0.25\text{-}0.5\times 10^6$ lymphocytes per ml. will usually give a vigorous response. It is advise that a concentration of 0.5 to 2.5×10^6 lymphocytes per ml. in the original inoculum seems optimum in promoting maximum growth and transformation. (48, 60)

10. The maintenance of a physiologic pH (7.2 to 7.4) is essential for viability and growth of the culture. (73,74) During the final days of culture when considerable cell division occurs, the medium may become quite acid especially when large numbers of cells are employed. It is stressed that the lethal effect of failing to control pH of the medium at the onset would have been likely to effect the lymphocytes before the onset of transformation and later to the transformation cells losses of lymphocytes will occur. (75) The pH of the medium is commonly regulated with a bicarbonate buffer (7.5% solution) and the cell suspensions incubated in tightly stoppered containers (allowing an air phase of at least three times the depth of the fluid) or in lossely covered tubes under an atmosphere of 95% air and 5% CO_2. There is no enhancement of the response whed 7% oxygen is used but the oxygen concentration of greater than 20% (air) will decrease the proliferative response. (76, 77) Many workers find it rather incovenient to use a CO_2-bicarbonate buffer system. A method in which Hepes buffer can be substituted for the two-phase CO_2-bicarbonate system is developed with better results. (78) This single phase liquid buffer used together with medium 199 allowed assay of a mixed lymphocyte culture as early as 2-3 days more stimulation being observed on day 3-4.

11. Optimal growth is also dependent upon the source of lymphocytes and the mitogen used. Lymphocytes from human peripheral blood exhibit good growth and mitosis when stimulated with 0.05 ml. PHA-P per 3×10^6 cells. However, lymphocytes from different tissues vary with respect to the amount of PHA required to induce maximum growth. (79) Poor precision in the past has been attributable to the use of a PHA preparation having a high titer of leukoagglutinin (PHA-P), which

resulted in the formation of large clumps during incubation. The use of purified PHA (such as Burrough-Wellcome "Phytomitogen" MR 68) has greatly increased the precision. (80)

12. One of the initial events is the settling of the suspended cells to the bottom of the culture tube. The formation of this bottom of cells may be of importance for the subsequent immunological reaction. Cell-to cell interaction appears to be a requisite for growth especially during the first 36 hours of culture; (48, 81, 82) hence culture vessels with around or conical bottom with small surface area support better growth than those flat bottom flask or bottle with a large surface area.

13. It is a general practice to eliminate phagocytic cells because of the believe that the presence of the latter may influence the lymphocyte growth kinetic. Conflicting reports have attributed both an enhancing (83) and an inhibitory (84) effect on lymphocyte response to PHA, It is noted that lymphocytes appear to cluster around macrophages in these culture (87) and that if adherent sells are removed from leukocyte suspension the remaining. "Purified" lymphocytes responded poorly to stimulation by antigen and in mixed leukocyte cultures. (86, 88) The transformation response could be restored in the proportion to the number of phagocytes added to these cultures. (85, 86) Although the adherent cells (phagocytes) may be functioning in part as a feeder layer for the lymphocytes, their major function would seem to be in the presentation of antigen to the lymphocytes. However, the role of the macrophage in **PHA-induced** transformation remains largely obscure. "Pure" lymphocyte cultures and unseparated leukocyte cultures achieved a comparable level of transformation in response to PHA, (57, 86) but the possibility can not be ruled out that in "pure" lymphocyte cultures, a fraction of the lymphocytes transform into macrophages, (89. 90) or perhaps a few contaminating monocytes effect the transformation of other cells in the population.

14. Accurate pipetting of the lymphocyte suspension into the triplicate cultures, adequate mixing of the suspension between pipetting are important.

15. The inabillity to count lymphocytes in culture has been due to the aggregation of cells in tight clums by PHA, the accumulation of debris, and the agglutinated red cells (contaminated in the leukocyte suspension) become resistant to the lytic agent in the WBC counting fluid. White cell clumps in PHA-containing culture can be completely dispered by aspirating back and forth 4 to 5 times through a 25 G. disposable needle connected to a 2

ml. syringe, followed immediately by dilution of the aliquot for cell-counting.

Two methods have been described recently for counting aggregated lymphocytes in PHA-containing cultures. (91, 92) Both employ a detergent for disaggregating the leukocyte agglutinates. Stewart and Ingram (91) have found that a proteolytic enzyme, pronase (Calbiochem, L.A., Calif.) is superior to trypsin in digesting nonviable cells and debris and the cytolytic agent, Cetrimide (Cetryltri-methylammonium bromode) in the couting solution (optimum concentration 5 mg/ml.) effectively strips cytoplasm from cells so that accurate counts of nuclei and nuclear volume measurement can be obtained. However, a small number of macrophages often appearing in the cultures may be confused with blast cells. Since degenerating as well as dead cells may be susceptible to the lytic action of pronase, this method raises the added problem of determining what proportion of the cells represented on the smear should be included in the cell count.

Care should be taken to avoid loss of cell clumps above the level of medium.

Accuracy in cell counts may be improved by adding polystyrene particles to the cultures for subsequent identification of the phagocytic macrophages on stained preparations.

16. Other than Wright's stain, 2 other types of staining have been employed. An acid-orcein nuclear stain has proven useful especially in the hands of those accustomed to evaluating nuclear material. Others have experienced some difficulty in confusing macrophage and blast cell nuclei when using this stain and have obtained somewhat higher levels of "blast cells" in unstimulated cultures. Staining with Giemsa as a combination of May Grunwald and Giemsa provides the range of tinctorial properties of cytoplasmic as well as unclear staining which are quite useful in distinguishing cell type.

17. Wilson and Thomson (75) observed that in the absence of PHA, 10% or less of a leukocyte in the culture died in 2 days. Mortality of starting cells is significant as demonstrated by the presence of 150-300 pyknotic small lymphocytes per cu.mm. in 3 day culture. It is advisable to identify these pyknotic cells with non-transformed lymphocytes having died preferentially and progressively, both as a result of alteration in the environment produced by the transformed cells and from toxic action by non mitogenic impurities in the PHA preparation. There ean have been little cytocidal action by the PHA preparation on transformable lymphocytes prior to transformation.

18. With all of these methods one measured only the net result of all substances stimulating the lymphocytes and all

factors inhibiting or limiting their response.

19. The measurement of lymphocyte transformation by tritiated thymidine incorporation is extremely sensitive; however, it is particularly susceptible to errors arising from poor technique. Care with respect to sterility and accuracy must be observed at all stages. Infection in particular can give rise to abnormally high stimulation and therefore any contaminated cultures must be discarded.

REVIEW

Over the past decade considerable interest has been shown in the plant lectin, phytohemagglutinin (PHA), obtained from the red kidney bean - Phaseolus vulgaris. This lectin is a potent erythroagglutinin which is also capable of agglutinating leukocytes and apparently stimulating majority of small lymphocytes (long - live lymphocytes, thymus-dependent) in human peripheral blood culture to transform into large blast-like cells and undergo mitosis. Interest in PHA has been largely due to this latter activity, and numerous reports have documented stimulatory effect in vitro on lymphocyte RNA and DNA synthesis and on morphologic transformation and cell division.

The finding of Tunis (93) of a cytoagglutinin in preparations of PHA which agglutinated all nucleated cells tested indicated that PHA attaches to the surface of a wide variety of cell types. The leukoagglutinating and mitogenic activities of PHA can be separated from its erythroagglutinin by adsorption of the latter onto the surface of erythrocytes, (94-96) and or bentonite. (93) The leukoagglutinin and mitogen have not been separated and appear to reside in the same effective fraction (95, 96) and has in the past led to the conclusion that agglutination is a necessary prerequisite to stimulation. (79)

MECHANISM OF LYMPHOCYTE STIMULATION.

In the resting lymphocyte, RNA and protein syntheses proceed at low levels and DNA replication does not occur. Within minutes of contact, PHA disrupts this quiscent state and in the following few hours the small lymphocyte read justs its metabolism to support the active growth. The mechanism by which nitrogens initiate such profound alteration appears to be mediated in a triggerlike reaction, as washing cells free of the mitogen after only a few minutes exposure does not prevent subsequent transformation. (25, 98, 99) The cell surface has been implicated as the site of the trigger mechanism which becomes activated when PHA interacts when PHA interacts with receptors on the membrane. After a brief exposure to PHA, the electrophoretic mobility of the lymphocytes suggestive of alterations in the surface charge accured. (100) Although the receptors have not been iden-

tified, evidence suggested that they may take the form of gammaglobulin molecules risiding on the membrane. (25) Phospholipids also has been considered as possible surface reactive sites. (101) It has also been noted that lymphiod cells treated with wheat germ lipase as well as trypsin are unable to adsorb antibodies to their surface, thus, the receptor sites binding these cytophitic antibodies may be lipoprotien. (102) Certain sugars on the lymphocyte membrane also may play a role in lymphocyte activation. Fucose and N-acetyl-galactosamine on the membrane play an important role in the "homing" of infused lymphocytes to specific sites in lymphoid tissue. (103) Borberg et al (104) noted that N-acetyl-galactosamine inhibited both the mitogenic activities and the attachment of ^{131}I-PHA to lymphoid cells. This inhibition may due to competition with polysaccharide components at receptor sites on the lymphocyte membrane. An injury-mediated trigger mechanism is also considered as a possible mechanism of action of PHA at the cell membrane.

Enzymes such as trypsin, chymotrypsin and papain promote the transformation of a small percent of lymphocytes, probably by damaging the cell surface. (105) Microwave irradiation, (106) ultrasonic injury (107) and antigen-antibody complex (108) in the presence of complement are capable of stimulating nonsensitized lymphocytes, presumably through injury to the membrane.

Leukoagglutination and increased micropinocytosis that accompany lymphocyte activation is probably not essential to the trigger mechanism. (5) Transformation can be induced by staphylococcal filtrate and antiallotype serum (25) (these agents do not agglutinate lymohocytee while the Vi antigens-coated lymphocytes (thus, are prevented from agglutination) can be activated to from blast cells. (109) Increased micropinocytosis in activated lymphocytes may be secondary only to the membrane changes that have occurred.

Although the cell membrane plays an important role in the activation process, implementation of cell growth most probably proceeds through intra-cellular mechanisms. The controlling site may reside within the nucleus or more likely, the cytoplasm. Hischhorn and Weismann's findings (53, 100) have suggested that the action of PHA may proceed through mobilization of lysomal bound hydrolytic enzymes in the cytoplasm. These enzymes may then proceed into the nucleus for the purpose of stripping DNA of proteinaceous repressor materials that in turn allows for widespread gene activation in terms of DNA-directed RNA synthesis. Drugs such as chloroquin (111,112) and corticosteroids,

(113) which inhibit lymphocyte transformation, may act by stabilizing lysosomal membranes and thus prevent the release of lysosomal hydrolases. (114) Streptolysin S renders lysosomal membranes labile, thereby inducing mitosis in the manner similar to PHA. (17)

Studies utilizing PHA fractions labeled with tritium, (115) 1251. (116), fluorescein isothiocyanate, (117) and mercury show that PHA is localized initially within the cytoplasm. From controlling sites residing in the cytoplasm derepressors may be transmitted to the nucleus.

Direct nuclear activation by PHA remains a distinct possibility. Within minutes after lymphocytes are exposed to PHA, the number of dramatic biochemical events occur in the nucleus. The arginine - rich histones become rapidly acetylated, (119) and the binding of orange to the phosphate groups of DNA is markedly increased. This early nuclear events represent essential steps in gene activation and transcription.

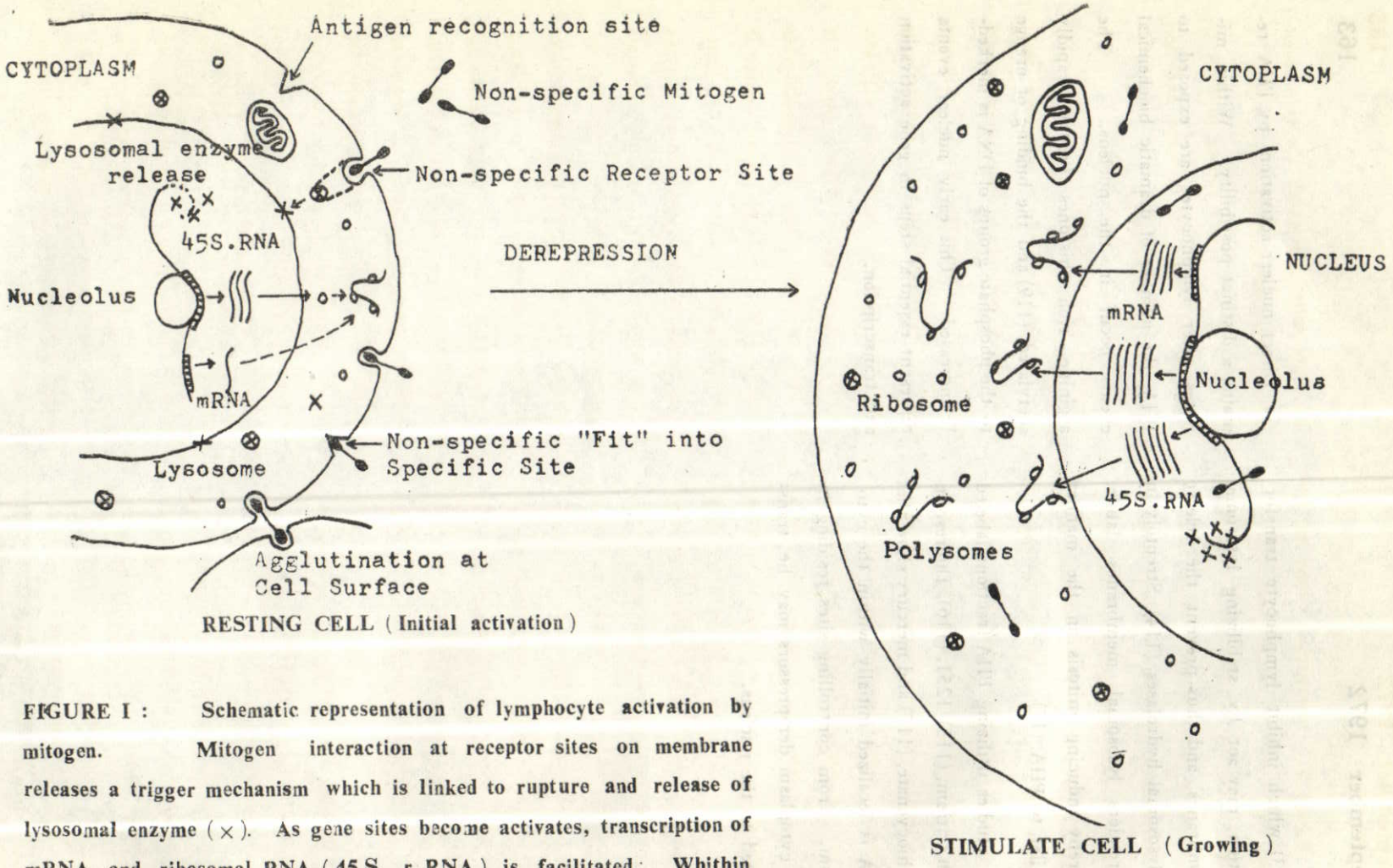


FIGURE I : Schematic representation of lymphocyte activation by mitogen. Mitogen interaction at receptor sites on membrane releases a trigger mechanism which is linked to rupture and release of lysosomal enzyme (×). As gene sites become activates, transcription of mRNA and ribosomal RNA (45 S., r. RNA) is facilitated. Whithin cytophasm ribosomes aggregate along r. RNA strands, now termed polysomes where proteins are synthesized.

Figure I. summarizes some of the views regarding the mechanisms that may operate to control lymphocyte proliferation in vitro. It is not known wheter specific and nonspecific mitogens activate lymphocytes by similar mechanisms. Conceivably, nonspecific agents interact with a wider spectrum of receptor sites, while specific mitogens may engage specific "recognition" sites on the lymphocyte surface. Recent studies indicated that some antigenic determinant sites on the PHA molecule and the lymphocyte surface were shared, as lymphocytes treated with anti-PHA antibodies were unable to respond to PHA (121) There appear to be a growing acceptance that the sequence of events in the normal immune response may commence when antigenic material is phagocytozed by a macrophage. (122) Subsequently, the antigen or antigen fragment is bound nonspecifically to machanism from that of the antigen-induced reaction in which the macrophage plays a crucial role.

METABOLIC CHANGES IN PHA-STIMULATED LYMPHOCYTES

RNA. SYNTHESIS

Acceleration of RNA synthesis has been detected in lymphocyte cultures within 60 minutse after the exposure to PHA. (123) and increased logarithmically for the next 24 hours. It reaches maximal rates between 48 and 72 hours after exposure following which the rate receded toward prestimulation levels by 9 days. (36, 124, 125) As a consequence of PHA stimulation, synthesis of ribosome and non-ribosomal RNA increased.

PROTEIN SYNTHESIS

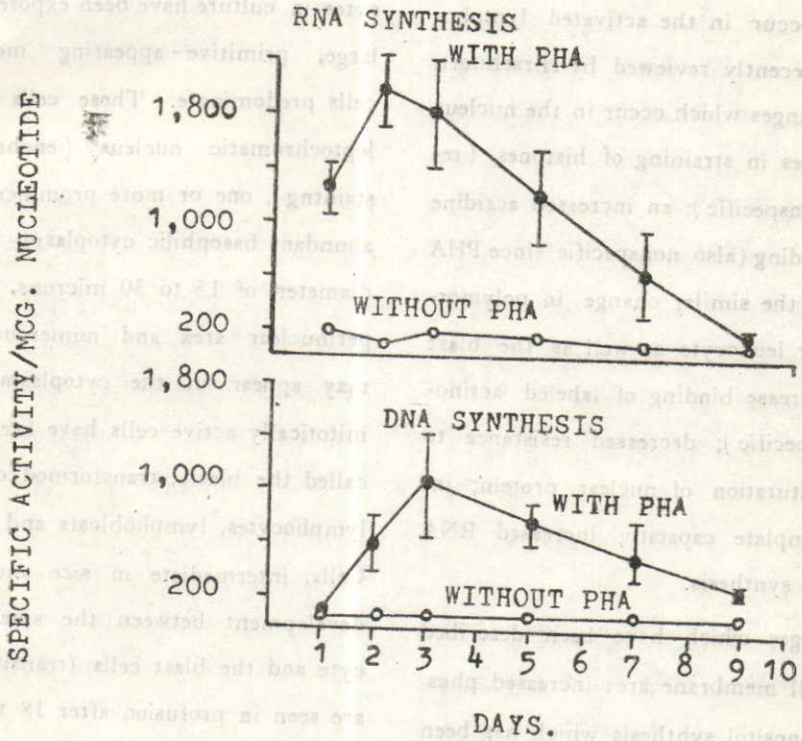
Resting lymphocytes produce small amounts of proteins, commensurate with their quiescent metabolic rate. Following exposure to PHA, protein synthesis is significantly elevated by 3 hours (125) and reaches a maximum at 2 to 3 days. It has been estimated that 10^6 lymphocytes produced about 10 ug of protein (about 1 percent of their weight) within 24 hour. (16) Protein synthesis is essential to lymphocyte transformation, as blast cells fail to develop in the presence of protein inhibitors. (74) Several workers agreed that freshly isolated lymphocytes incorporated labeled amino acids into many classes of protein, haptoglobin, and a variety of substances including all classes of immunoglobulins. (23) PHA-stimulated lymphocytes seem to produce all the varities of proteins synthesized by unstimulated cells, but in greater amounts. Although earlier investigators had identified gamaglobulins as the predominant protein synthesized by PHA-stimulated lymphocytes, most of the recent workers have found that the major protein of these proteins is nonimmunologic. (26, 126, 128, 131, 133) Of the im-

munoglobulins produced, PHA-treated lymphocytes showed either a small increase (131, 132) or no significant increase above the controls. It was postulated that the lymphocytes responding to the nonspecific mitogens must represent a population distinct from that primed to respond to specific antigens. It may be concluded at the present time that PHA (and also the antigen) -stimulated lymphocytes produce vast quantities of protein of which immunoglobulins constitute but a minute fraction. While specific antigens may induce antibody synthesis directly, PHA may enhancing the production of antibodies only in antigen -sensitized lymphocytes in which specific antibody production is already programmed. It is yet to be determined whether PHA does in fact enhance immunoglobulin production in a cell already geared for such synthesis, or whether PHA causes the recruitment of new cells into an immunoglobulin-synthesizing pool.

DNA SYNTHESIS

It appears that only blast cells synthesize DNA and proceed to mitosis. In the circulating blood only a small fraction (less than 0.5 percent) of morphologically small lymphocyte incorporate tritiated thymidine into the DNA. (137, 138) When incubated with PHA, the majority of the cells replicate DNA. Within 18 to 24 hour after exposure to PHA, DNA synthesis remains at a low level, then attain a maximum at 2 to 3 days (Figure 2-3). Almost all of the cells synthesizing DNA have been identified as blast cells on the autoradiograms. Percent transformation and labeling indices show good correlation in these cultures. (139) It is also noted by many workers that even in actively synthesizied cells, the number of cells incorporating thymidine (autoradiograph) is always small than the blast-like cells. This is probably due to the presence of an asynchroncus population of lymphocytes in cultures rather than the possibility of the presence of a factor (s) inhibiting thymidine incorporation. These asynchronous lymphocytes go into s-period (which is rather short period in these cells, approximately 6 to 8 hours) at different times. In addition, it is probably that not all cells that become activated will engage in DNA synthesis and not all blastogenenesis leads to replication. (140) Therefore it depends upon the technique of measuring the thymidine incroporation, the specific activity of tritailed thymidine used, the total amount of thymidine, and the duration of incubation in labeled thymidine, the results may be enormously diferens. (140) The kinetics of RNA syntheses can be investigated by exposing alternate cultures to ^{3}H uridine or ^{3}H-thymidine, respectively. Metabolic response may be

assessed in individual cells by autoradiographic processing of cultures exposed to ^{3}H or ^{14}C-labeled precursors. This technique has the advantage of differentiating cell types that are active in metabolic synthesis and is particularly useful in delineating the pattern in which macromolecular synthesis evolves the cells. (6, 142)



FIGURES 2 AND 3 KINETICS OF NUCLEIC ACID SYNTHESIS BY NORMAL HUMAN LYMPHOCYTES CULTURE IN VITRO.

RELEASE OF LYSOSOMAL ENZYMES. Shortly after stimulation of lymphocytes by mitogens, within 24 hours, there is an increase of lysosomal enzymes. The only enzyme that has been found in supernates following PHA stimulation is acid phophatase. Earlies it was thought that it was released from the cell through toxicity or cell death. It has recently been shown that, following phagocytoses, and concomittant with it, there is release of lysosomal enzymes into the supernate. This is not accompanied by release of other cytoplasmic enzymes, therefore, appears to be relatively specific. Shortly after stimulation of lymphocytes (within 18 minutes) there is an increase of endocytosis, and it was speculated that, similar

to the phagocytosis, there is a release of lysosomal enzymes. (143)

OTHER METABOLIC CHANGES.

These early changes which are observed to occur in the activated lymphocytes are recently reviewed by Hirschhorn (143). Changes which occur in the nucleus are: changes in straining of histones (relatively nonspecific); an increased acridine orange binding (also nonspecific since PHA will cause the similar change in polymorphonuclear leukocyte as well as the blast cells); increase binding of labeled actinomycin (specific); decreased resistance to heat denaturation of nuclear protein; increase template capacity; increased RNA and DNA synthesis.

Changes which have been described in the cell membrane are: increased phosphatidyl inositol synthesis which has been detect as early as 15 minutes after exposure to PHA; increased in phosphatidyl choline and adenyl cyclase activities with an increased in absolute level of cyclic AMC.

MORPHOLOGIC CHANGES ACCOMPANYING LYMPHOCYTE STIMULATION.

Two or three days after the lymphocytes in culture have been exposed to PHA, large, primitive-appearing mononuclear cells predominate. These cells possess a leptochromatic nucleus (enchromatically staintng), one or more prominent nucleoli, abundant basophilic cytoplasm and a cell diameter of 15 to 30 microns. A clear perinuclear area and numerous vacuoles may appear in the cytoplasm. These mitotically active cells have been variously called the blasts, transformed or activated lymphocytes, lymphoblosts and PHA cells. Cells, intermediate in size and cytologic development between the small lymphocyte and the blast cells (transitional cells) are seen in profusion after 18 to 30 hours of culture with PHA, but may be found thoughout the culture period.

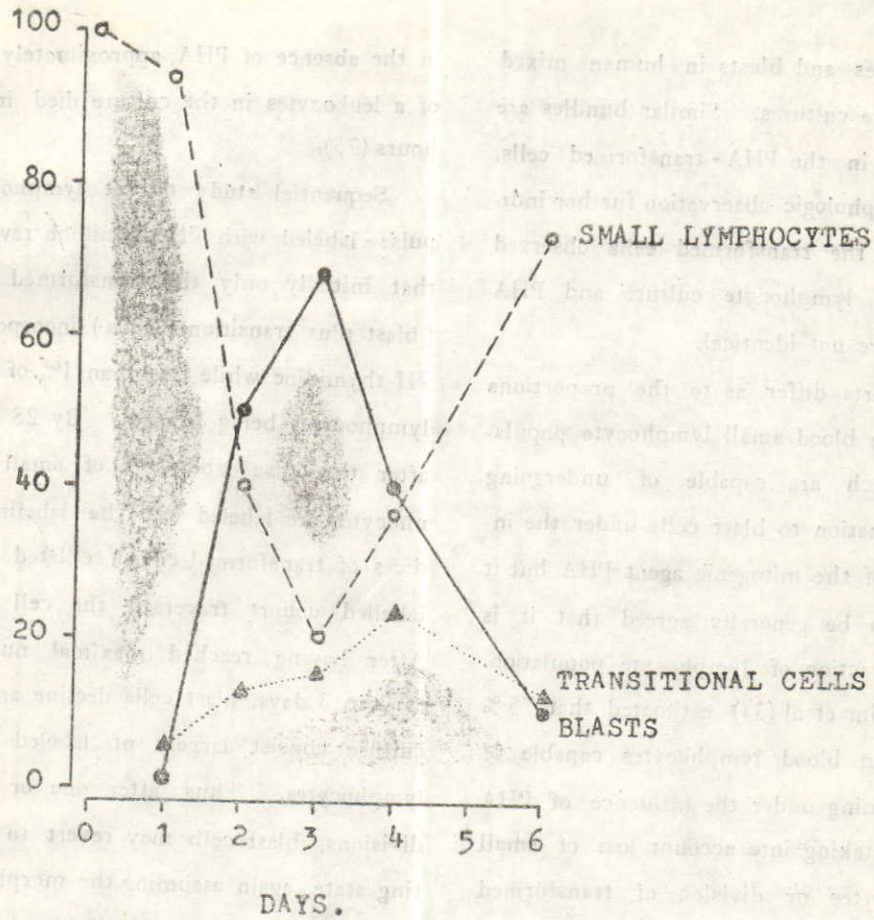


FIGURE 4: PERCENTAGE OF DIFFERENT TYPES OF LYMPHOCYTES IN CULTURE DURING STIMULATION WITH PHA.

The blast cells that develop in PHA-treated cultures exhibit an enormous increase in ribosomes, an hypertrophied Golgi apparatus, and a scant amount of rough-surfaced endoplasmic reticulum. The transformed cells are quite active in micropinocytosis, which accounts for numerous vesicles within the cytoplasm (5). Mitochondria are increased in numbers and many electron-densed acid phosphatase bodies are located in the proximity of the Golgi apparatus. It was observed earlier that both transformed lymphocytes from tuberculin sensitization (144) and the blasts seen in the mixed leukocyte culture (145) exhibit the fine structures that are indistinguishable from those observed in the PHA-transformed cells. Parker et al (146) has recently found that bundles of cytoplasmic fibrils are observed in activated

lymphocytes and blasts in human mixed lymphocyte cultures. Similar bundles are not seen in the PHA-transformed cells. This morphologic observation further indicate that the transformed cells observed in mixed lymphocyte culture and PHA culture are not identical.

Reports differ as to the proportions of human blood small lymphocyte population which are capable of undergoing transformation to blast cells under the influence of the mitogenic agent PHA but it seems to be generally agreed that it is only a fraction of lymphocyte population. Oppenheim et al (34) estimated that 75% of human blood lymphocytes capable of transforming under the influence of PHA without taking into account loss of small lymphocytes or division of transformed cells. However, the lower average value of 60% (45-73%) is observed when loss and gain are also considered. (75) In fact, in the absence of PHA, approximately 10% of a leukocytes in the culture died in 48 hours (75).

Sequential study of rat lymphocytes pulse-labeled with ^{3}H-thymidine revealed that initially only the transformed cells (blast plus transitional cells) incorporated ^{3}H-thymidine while less than 1% of small lymphocytes being labeled. By 28 hours after the pulse label 26% of small lymphocytes are labeled but the labeling indices of transformed cells oscillated as the labelled cohort traversed the cell cycle. After having reached maximal numbers at 2 to 3 days, blast cells decline and the culture consist largely of labeled small lymphocytes. Thus, after one or more divisions, blast cells may revert to a resting state, again assuming the morphologic features of small lymphocytes. These quiescent cells can be restimulated to yield a second spurt of blast development. (148)

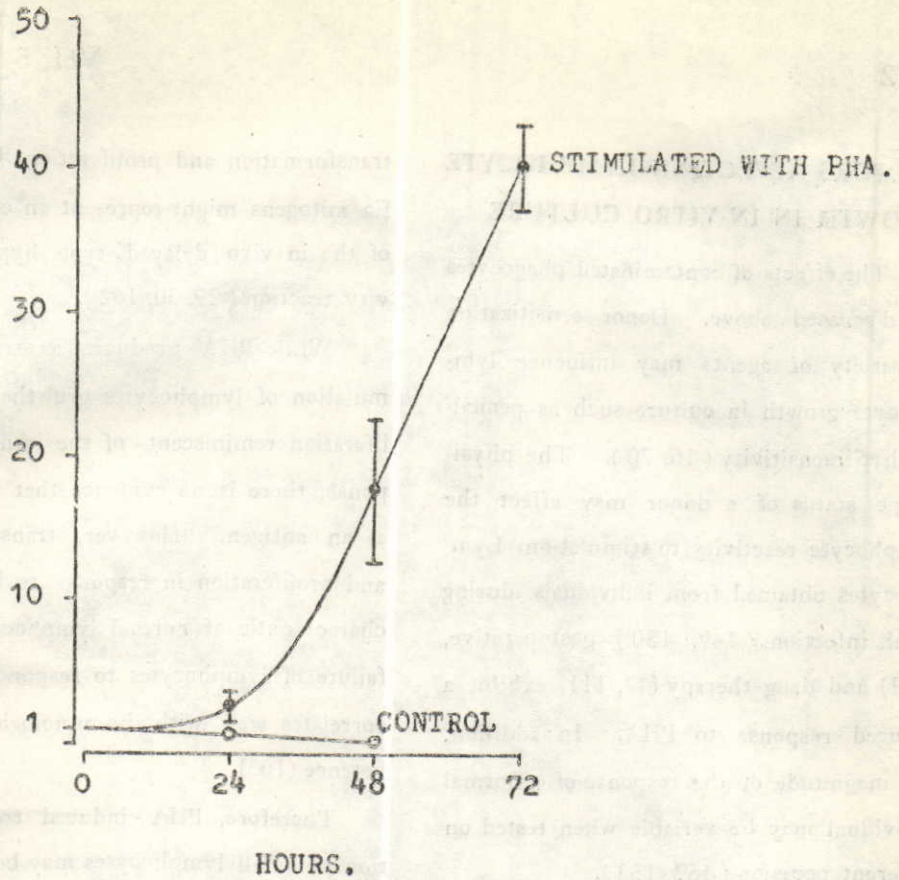


FIGURE 5 : LYMPHOCYTE UPTAKE OF TRITIATED THYMIDINE.

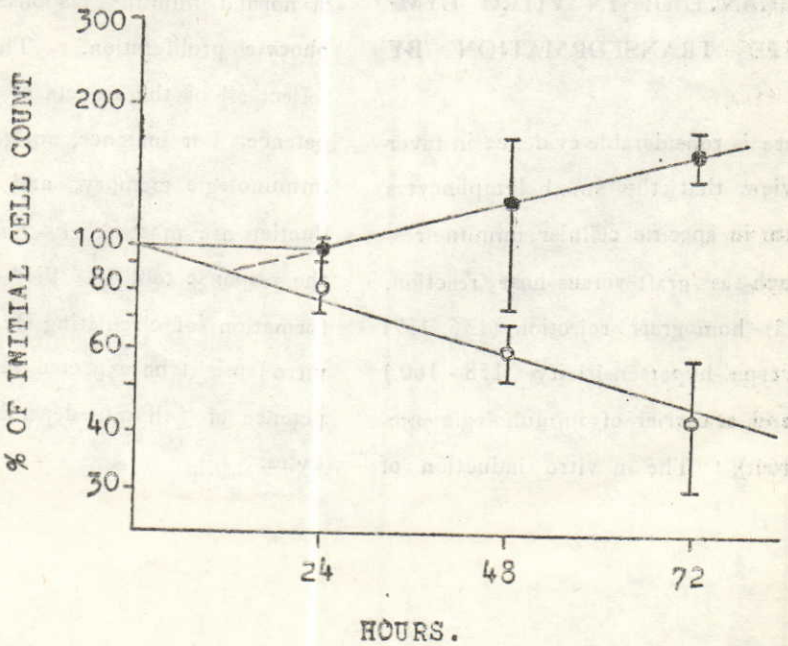


FIGURE 6 : EFFECT OF PHA ON LYMPHOCYTE PROLIFERATION.

FACTORS AFFECTING LYMPHOCYTE GROWTH IN IN-VITRO CULTURE

The effects of contaminated phagocytes are discussed above. Donor sensitization to variety of agents may influence lymphocyte growth in culture such as penicillin hypersensitivity (16, 70). The physiologic status of a donor may affect the lymphocyte reactivity to stimulation. Lymphocytes obtained from individuals during viral infection, (149, 150) postoperative, (151) and drug therapy (47, 111) exhibit a reduced response to PHA. In addition, the magnitude of the response of a normal individual may be variable when tested on different occasion (152. 153).

SIGNIFICANCE OF IN VITRO LYMPHOCYTE TRANSFORMATION BY PHA

There is considerable evidence in favor of the view that the small lymphocytes participate in specific cellular immune response such as graft-versus-host reaction, (154, 155) homograft rejection, (156, 157) delayed type hypersensitivity, (158 - 160) and acts as a carrier of immunologic memory (161). The in vitro induction of transformation and proliferation by specific mitogens might represent an expression of the in vivo delayed type hypersensitivity reaction (29, 30, 162).

While PHA produces a striking stimulation of lymphocytes growth and proliferation reminiscent of the immune response, there is no evidence that PHA acts as an antigen. However, transformation and proliferation in response to PHA is a characteristic of normal lymphocytes, and failure of lymphocytes to respond to PHA correlates well with immunologic incompetence (163).

Therefore, PHA - induced transformation of small lymphocytes may be regarded as an in vitro model of at least one phase of normal immune response-namely lymphocyte proliferation. This would not reflect all of the aspects of immune competence. For instance, antigen recognition, immunologic memory, and antibody production are most likely not reflected in the response to PHA. PHA-induced transformation of circulating lymphocytes (in vitro) might be expected to test the competence of " thymus-dependent " lymphocytes.

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