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Tissue culture studies of human lymphocyte transformation and DNA synthesis utilizing tritiated deoxycytidine as specific DNA precursor

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TISSUE CULTURE STUDIES OF HUMAN LYMPHOCYTE
TRANSFORMATION AND DNA SYNTHESIS,
UTILIZING TRITIATED DEOXYCYTIDINE AS
SPECIFIC DNA PRECURSOR

By

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INTRODUCTION

In recent years evidence has been produced to show that the lymphocyte plays a major role in important immunologic mechanisms, including the mediation of delayed hypersensitivity, homograft rejection, antibody production and possibly in the body's defense against tumors (20). Despite participation of the lymphocyte in many immunologic processes, the potentials and the specific factors that may control the differentiation and function of these cells have not been fully elucidated (50). The function of lymphocytes has been shown to be impaired in numerous diseases, including Hodgkin's disease (59), chronic lymphatic leukemia (27), sarcoidosis (59), infectious mononucleosis (21), and ataxia telangiectasia (39). This increasing recognition of the important functions of the lymphocyte has provided impetus in the search for new ways to evaluate these cells. Improvements in tissue culture techniques and in methods for separation of lymphocytes from peripheral blood have allowed study of the function of the lymphocyte relatively free of the influence of other types of cells (12).

In 1955 Rigas and Osgood (48) showed that an extract of a red bean called Phaseolus vulgaris would agglutinate red blood cells rapidly, allowing the white cells to be readily obtained for culture.

The bean extract has been named phytohemagglutinin, and has been fre-
quently referred to by the abbreviation PHA. In 1960 Nowell (40)
demonstrated that cultures of peripheral blood cells obtained after
PHA agglutination showed mitotic proliferation which he was able to
relate to the activity of PHA. Since then, a large body of literature
has appeared which has substantially confirmed Nowell's original ob-
servation that lymphocytes, under the influence of PHA, transform into
large blast-like cells, some of which proceed into mitosis. In addi-
tion, numerous other substances have also been found to be capable of
inducing transformation in the normal lymphocyte (13). This phenomenon
of lymphocyte transformation has been utilized in the investigation of
lymphocyte function, and has been shown to be impaired in a variety of
diseases (27,36,44).

The exact mode of action of PHA remains uncertain, but differs
from that of most of the other substances in both being more rapid
acting and producing a larger percentage of transformed cells (12).
The agents that will cause lymphocyte transformation or blastogenesis
may be divided into four groups, as reported by Robbins (49):

1. General mitogenic agents, such as PHA, staphylococcal and
   pokeweed extract;

2. Specific antigens, to which the subject has been previously
   sensitized, such as purified protein derivative tubercu-
   lin, vaccinia vaccine and tetanus toxoid, where the lym-
   phocyte response is dependent upon the previous sensiti-
   zation.

3. Mixed lymphocyte cultures, wherein the mixing of peripheral
   blood white cells from individuals, other than identical
   twins, will show a transformation of the lymphocytes,
   apparently in response to the foreign white cell antigens.

4. Blastogenesis associated with antiserum, wherein the change
   is the result of culturing white cells with antiserum
   directed against white cells.

These specific materials may induce lymphoblastoid transformation in up to 30% of the cultured lymphocytes, whereas the non-specific stimulants such as phytohemagglutinin can cause over 90% lymphoblastoid transformation (17).

Four main ways of evaluating the lymphocyte response to stimulation have evolved (11,35). Initially, the transformation of the cells was judged by morphological standards, utilizing absolute cell counts and differential cell counts to determine the degree of blastogenesis. The results obtained by this method depended to a great extent upon the subjective assessment by the examiner of morphological changes in the normal and abnormal lymphocyte (58). Many investigators have encountered difficulties caused by the need to differentiate these cells from other large cells, termed epithelioid cells, macrophages, or lymphoid macrophages by different workers, which may appear in lymphocyte cultures even in the absence of any stimulant (6). In addition to being laborious and time consuming, especially when large numbers of cultures have to be studied, such procedures are also difficult to apply on a quantitative scale (9).

Subsequently, methods for assessing the transformation of lymphocytes in culture were based on counting the number of mitotic figures produced, the total being referred to as the Mitotic Index. This procedure was facilitated by the addition of a stathmokinetic agent, such as colchicine, that blocks cell mitosis in the metaphase (59). Mitotic figures were thus easily identified. Although this technique was more accurate than the cell counting method, there remained a need for a quantitative method of assessing the activity or transformation of lymphocytes in tissue culture in which there would be no need to identify

individual cells.

It has been established that synthesis of nucleic acids occurs during blastogenesis (22,28,34,38). In contrast, in the absence of PHA or another stimulating agent, little or no transformation occurs among cultured lymphocytes (49) and the rates of RNA and DNA synthesis are negligible (22). Determination of nucleic acids has, as a consequence, been chosen as an objective indicator of lymphocyte transformation and thus of lymphocyte function.

Determination of DNA, the most commonly selected index of blastogenesis, has been accomplished by chemical extraction techniques (26,28). These methods, however, have generally been very difficult to apply to small amounts of materials (9). As refinements in radioisotope techniques developed, these new investigative tools were soon applied to the problem of nucleic acid determination. It was found that isotopic nucleic acid precursors, such as tritiated thymidine, could be introduced into the tissue culture medium. The precursors were then utilized by the cell in the synthesis of new DNA in preparation for blastogenesis and mitosis. The extent to which the radioactive substances were incorporated into the nucleic acid, thus reflecting the rate of nucleic acid synthesis, was determined by earlier investigators by autoradiography.

Autoradiography has been accomplished by exposing smears of cells, which have been incubated with specific radioactive isotopes, to a film of photographic material for a prescribed length of time. The photographic film was then developed and the extent of isotope uptake by the cell could be evaluated by the degree of exposure of the film. Although a great deal of information has been acquired utilizing autoradiography, the technique has been found to be laborious, time consuming and less

accurate than more recently developed methods (1).

More quantitative methods have been developed; they are also based on measurement of the amount of radioactive-labeled nucleic acid precursors taken up by lymphocytes in tissue culture. Michalowski (38) and Aisenberg (1) have both described techniques in which the final radioactivity is measured by means of planchettes and an end-window counter. The method of Sell and Gell (55) for quantitative measurement of the incorporation of radioactive nucleotide by lymphocyte cultures makes use of a liquid scintillation counter system. This method has numerous technical advantages, and has been utilized by many investigators in the study of the normal and abnormal lymphocyte (11,55). The development of tritium-labeled thymidine, a specific deoxyriboside precursor of DNA (8), provided a powerful tool for the in vivo and in vitro study of the kinetics of DNA synthesis, and thereby the response of the lymphocyte to stimulation. Measurements of thymidine uptake under the closely controlled conditions of lymphocyte tissue culture are generally accepted as valid measurements of the rate of DNA synthesis (13).

Other investigators have suggested, however, that the incorporation of radioactive thymidine into the DNA of lymphatic tissue, particularly thymus tissue, may not be an accurate measure of DNA synthesis. Hoffert and White (30) demonstrated rapid incorporation of tritiated deoxycytidine into thymus DNA, in contrast to low incorporation of tritiated thymidine. This observation was in accord with the rapid rate of incorporation of radioactive phosphate into thymus DNA, and the high mitotic index for this tissue (2,51). Tritiated deoxycytidine may thus provide a more reliable estimation of DNA synthesis in thymus than does the incorporation of tritiated thymidine. It was of particular interest that the incorpora-

tion of tritiated thymidine into peripheral lymphocytes may not be a reliable index of DNA synthesis under various conditions and thus not a reliable indication of lymphocyte stimulation. To investigate this possibility, this project was planned, first of all to establish a workable technique for viable lymphocyte cultures with good morphological evidence of consistent lymphocyte transformation. Subsequently, it was desired to study the relative incorporation of tritiated deoxycytidine and tritiated thymidine into normal and, later, abnormal lymphocytes in vitro under the influence of PHA or other stimulating agent.

MATERIALS AND METHODS

Population Studied

Peripheral venous blood was obtained from laboratory personnel and medical students, ranging in age from 20 to 40 years. All subjects were considered to be immunologically competent by history and free of known diseases. One additional sample was obtained from an individual with chronic lymphocytic leukemia.

Preparation of Lymphocytes

The separation of lymphocytes from peripheral blood was performed by the method of Rabinowitz (46) with slight modification, utilizing the attributes of glass beads found by Garvin (26). Glass columns were made from Pyrex glass tubing 16 cm in length by 1 cm in diameter, with a capacity to retain 5 ml of cell suspension. A rubber stopper was fitted to the column inlet; cell suspension and wash solutions were applied by syringe through a 19 gage hypodermic needle inserted through the inlet stopper. Speed of flow through the column was controlled by altering the weight placed on the plunger of the syringe. A sleeve-type rubber

stopper was used at the outlet, and a collecting test tube was inserted into the sleeve to provide a closed system. An air vent permitted flow from the column during collection of effluents. The columns were packed with glass beads (No. 100-15 Superbrite, Minnesota Mining and Manufacturing Co.), using a thin layer of glass wool for the bottom layer to prevent loss of the beads.

The glass beads were prepared for use by washing with concentrated nitric acid followed by multiple tap and distilled water rinses. All other glassware was washed with detergent followed by 10 tap water and 3 distilled water rinses. All glassware, including glass beads and glass wool, was siliconized with Siliclad (Clay-Adams, Inc.). All syringes, columns, stoppers, culture tubes and other glassware were autoclaved at 230° C for a minimum of 30 minutes. The entire procedure was carried out aseptically.

Blood samples were obtained from the cubital vein, drawn into 50 ml glass syringes which contained 500 units of sodium heparin and 10 ml of a 5% dextran solution. After replacing the needle cap, the syringe and its contents were fastened to a ring stand in an upright position. A small amount of stopcock grease was required to prevent leakage of blood around the plunger of the syringe. The blood was allowed to sediment by gravity in this position at 37° C for 30 minutes. After sedimentation, the leukocyte-rich supernatant plasma was expressed from the vertically held syringe into two capped culture tubes via a flexible polyethylene catheter. The volume of plasma thus obtained varied from 15 to 25 ml depending on the erythrocyte sedimentation rate. The leukocyte rich plasma was then centrifuged at 600 rpm for 10 minutes, and the supernatant cell-free plasma was then removed by pipette, leaving 5 ml of plasma

into which the cells were resuspended by gentle shaking. The excess plasma was temporarily stored in the incubator at 37° C.

The concentrated leukocyte rich plasma was aspirated into a syringe and then applied to the column of glass beads, the rate of application being controlled by varying the weight on the plunger. After 30 minutes incubation at 37° C, the cell suspension in the column was eluted into a collecting test tube, using 10 ml of fresh plasma obtained following centrifugation of the white cell suspension for the washout. Cell counts and smears were made of the elution to determine the efficiency and effectiveness of the column.

Fresh plasma was added to the lymphocyte-rich plasma to yield a volume of 7.5 to 15 ml. This was mixed with tissue culture medium 199 (Difco)* to produce a total volume of 50 ml, with the plasma concentration varying from 15-30%. The cells were kept well suspended at all times by constant agitation. The 50 ml volume of lymphocyte-rich culture medium was divided into two equal portions, one of which received 0.2 ml of phytohemagglutinin-M* or 0.05 to 0.20 ml of phytohemagglutinin-P*. The other portion received an equal volume of balanced salt solution and thus provided the control cultures. The control culture medium and the medium containing the PHA were pipetted separately into plastic culture dishes, 4 ml per dish, yielding five pairs of dishes which were covered with lids and placed in the incubator at 37°C in an atmosphere of either room air, 5% CO_2 and air, or 5% CO_2 and oxygen. Total time elapsed from obtainment of the blood until the cultures were in the incubator ranged from 2-4 hours.

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* Difco Laboratories, Detroit, Michigan

The cultures were harvested after 2 hours on the day they were established, and at 24-hour intervals thereafter over a period of four days. Two culture dishes, one control and one containing the PHA were chosen for each harvesting. Initially, the results were evaluated microscopically from smears of the harvested cultures stained with Wright's stain. When consistantly viable cultures were obtained and satisfactory lymphocyte transformation was observed, 0.33 micrograms of colchicine* per ml of media was added to the individual cultures two hours prior to harvesting to arrest mitosis in the metaphase. The mitotic index of the cultures was then easier to evaluate.

Attempts were made to study the kinetics of DNA synthesis in several of the later cultures, utilizing tritiated deoxycytidine and liquid scintillation counting. Each culture was pulsed with 1 micro-curie of the radioactive nucleotide two hours prior to harvesting. Harvesting of these cultures was accomplished by washing the contents of the culture dishes into a cold Millipore filter with cold normal saline. Each filter was rinsed with a total of 20 ml cold saline, taking care to avoid spillage. The filter was then placed in a Tri-Carb liquid scintillation counting vial containing 15 ml of Bray's counting solution (10). The Millipore filters rapidly disintigrated in the Bray's solution with minimal debris remaining in the bottom of the counting vial. The vials were stored in the dark at -10° C for 48-72 hours and then placed in a Packard Liquid Scintillation counter. All counts were in duplicate and were taken over a 10 minute period. The counting procedure was repeated 3 times. Average values were used for evaluating the results and were expressed as total counts per minute.

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* Difco Laboratories, Detroit, Michigan

RESULTS

During the initial series of twelve lymphocyte cultures several problems were encountered. Within 48 hrs. the first three cultures showed signs of bacterial contamination, i.e., color change, clouding and increased debris in the culture dishes containing PHA-M, while the unstimulated control cultures remained viable with no evidence of contamination. Smears of the stimulated cultures revealed the presence of Gram negative rods. Culturing the stock PHA solution in thyoglycollate media and on blood agar confirmed this to be the source of the contamination. It was subsequently noted that PHA-M is not a sterile preparation as produced by the manufacturer and that antibiotics are required in the culture medium if this agent is to be used in tissue culture studies. Rather than introduce antibiotics and the accompanying preservatives into the cultures, and thus additional unknown factors, the sterile and highly potent PHA-P was chosen as the stimulating agent.

Dextran Sedimentation

Sedimentation of the blood without dextran yielded inconsistent results, largely due to the low sedimentation rates of the predominately male donors, and significantly increased the time required for adequate separation of the red cells from the white cells and platelets. To determine what effects dextran would have on the number of lymphocytes in the final cell suspension, several blood samples were sedimented, half of the samples containing dextran and the others an equal volume of balanced salt solution. It was found that the supernatant leukocyte-rich plasma of the samples sedimented without dextran contained approximately 10% more lymphocytes than did the samples containing dextran.

However, after column separation of the cells it was found that the samples without dextran yielded only 60% of the lymphocytes entered into the column, in contrast to 93% from columns containing samples sedimented with dextran. As a result, the total number of lymphocytes available for ensuing culture studies was greater when dextran was used to hasten sedimentation. On this basis, 10 ml of 5% dextran was used in all subsequent blood samples.

Cell Counts

Following sedimentation the red blood cells from the peripheral blood, the supernatant plasma was expressed from the vertically held syringe. Samples of the leukocyte-rich plasma were obtained and total and differential cell counts were performed utilizing an improved Neubauer hemocytometer. The number of lymphocytes present varied from 6.2 x 10^6 per ml to 1.1 x 10^7 per ml with an average of 7.1 x 10^6 lymphocytes per ml. After passage through the column of glass beads, the mean number of lymphocytes was 6.6 x 10^6 per ml. The 5 ml of the eluted plasma were diluted with autologous plasma and tissue culture media 199 to a total volume of 50 ml with an average number of .57 x 10^6 lymphocytes per ml of culture medium. Lymphocyte counts were repeated on each aliquot following separation of the 50 ml volume into two equal portions. The difference between these cell counts was on no occasion greater than 15,000 cells/ml. Therefore, the control and stimulated cultures were considered to contain equivalent populations of cells at the onset of each culture series. Cell counts could not be accurately accomplished after the addition of PHA-P because of marked clumping of the cells. Differential counts revealed that the column separation of lymphocytes from other elements of peripheral

blood was quite variable, with the percentage of lymphocytes present in the final suspension ranging from 70-95% of the white cells.

Microscopic Evaluation of Lymphocyte Stimulation

The results of the initial cell culture series were determined by evaluating the morphological changes in the cultured cells. In the control cultures, the viable cells appeared very similar to lymphocytes as seen in smears of peripheral blood. The nucleus was centrally located with a definite nuclear membrane evident. The chromatin was coarse, granular, and deeply stained. Nuceloli were usually pale blue but were rarely seen in the unstimulated cells. The cytoplasm was sparse, appearing as a thin, homogenous light blue rim around the relatively large nucleus. These unstimulated cells generally remained unchanged in appearance during the culture period. There was, however, a substantial increase in the number of nonviable cells and in the amounts of cellular debris present at the end of the 96-hour culture period.

In the cultures containing PHA, the cells resembled those of the control cultures when examined within 3 hours after the cultures were established. At the end of 24 hours, however, numerous cells were seen which were the size of small lymphocytes but differed in that they had a more abundant, deeply basophilic cytoplasm. The nuclei were round and nucleoli were visible in a high percentage of these cells. In addition, large mononuclear cells were seen in the samples taken at 48 and 72 hours. These cells were larger than the original cells and reached diameters 1½ to 3 times that of mature lymphocytes. They had round, oval or indented nuclei, and abundant cytoplasm which fre-quently contained numerous distinct vacuoles. Both nuclear and

cytoplasmic basophilia varied greatly in intensity between different groups of cells, and from cell to cell within a given culture. The percentage of the lymphoblastoid cells present varied and was used as an indication of the suitability of the culturing conditions and to select the proper concentration of materials added to the culture media.

Eleven cultures were studied before a significant number of mitotic figures were visualized. Multiple changes in the concentration of PHA and autologous plasma were made. Initially the PHA-P as supplied by the manufacturer was dissolved in 5 ml of distilled water and 100 microliters was added to 25 ml of culture media. This amount of PHA (4 microliters/ml of media) was similar to that utilized successfully by Schrek and Rabinowitz (54) and by Richter and Naspitz (47). The first three viable cultures containing this concentration of PHA revealed moderate numbers of transformed lymphocytes (approximately 30%); however, there were no mitotic figures present. In view of this, the amount of PHA was increased to 0.2 ml per 25 ml of culture media (8 microliters/ml of media). Subsequent cultures were not as successful, with decreased numbers of viable cells and of transformed lymphocytes. No mitotic figures were noted. Therefore, the concentration of PHA was reduced in 1 microliter/ml of media decrements until optimum results were obtained. With the addition of 0.05 ml of PHA-P to 25 ml of media (2 microliters/ml), lymphocyte transformation occurred in over 90% of the cells and, after the addition of colcemide numerous mitotic cells were demonstrated (Figures 1-4).

Concentration of Autologous Plasma

The concentration of autologous plasma initially selected was 30%,
after the method of Hersch and Oppenheim (29), and of Borjeson, Gardell
and Norden (9). This amount of plasma was utilized for the first four-
teen cultures. It was noted, however, that many investigators advocate
the use of lower concentrations of plasma, ranging from 15% to 20%
(1,16,35). Therefore, the plasma concentration was reduced to 15% for
all subsequent cultures, including the cultures demonstrating a high
percentage of lymphocyte transformation and the presence of mitotic
figures.

Culture Environment

The cultures were originally placed in an incubator at 37° C with
a gaseous phase of 5% CO_2 and oxygen, to meet the carbon dioxide re-
quirements as noted by Dutton and Eady (19). In view of unsuccessful
cultures, the gas phase was changed to room air, and subsequently to
5% CO_2 and room air, as recommended by Cooper and Barkam (14). Viable
cultures with good cellular transformation and numerous mitotic figures
were obtained in both atmospheres.

Studies Utilizing Tritiated Deoxycytidine

The results of eleven cultures utilizing tritiated deoxycytidine
to quantitate the synthesis of DNA are shown in Figures 5-17. Cul-
tures 31, 32, 33 and 34 revealed significantly greater uptake of the
radioactive nucleotide by the cells stimulated with PHA than by the
control lymphocytes. In culture 31 (Figure 5), the counts per minute
gradually increased during the first 48 hours, with an increased rate
of uptake occurring from 48 to 72 hours. By 96 hours, the rate of

uptake began to decrease slightly. The controls in culture 31 revealed an uptake similar to the stimulated cells at 24 hours; however, the total counts decreased to low levels at 48, 72 and 96 hours. After an initially sharp decline, the uptake of tritiated deoxycytidine by the stimulated cells in culture 32 (Figure 6) increased rapidly during the remainder of the culture period. In contrast, the control cultures continued to utilize relatively small quantities of the nucleotide.

As described above, two hours after being pulsed with one micro-curie of tritiated deoxycytidine, the cells were washed unto a cold Millipore filter with cold saline. To determine the adequacy of this technique, an additional control and PHA-containing culture dish was established for cultures 33 and 34. No tritiated deoxycytidine was added to the extra dishes for culture 34, which were harvested and counted in the usual manner. This was done to provide baseline values and to determine the effects of additional factors (eg., light, tempera-ture) on the scintillating properties of Bray's solution. The extra culture dishes from culture 33 were pulsed with the nucleotide as previously described. These cultures were promptly harvested and counted in order to determine the adequacy of the washing procedure, and thus the amount of radioactive material retained on the filter but not incorporated into the cells. The results of these determinations are shown in Figures 7-8. The total counts from the unpulsed cultures were very low and compared favorably with the background counts. The total counts from the pulsed and immediately harvested cultures were also very low, indicating that only small quantities of the nucleotide were retained on the filter when inadequate time was allowed for in-corporation into the cells.

The total counts obtained from the stimulated cells of culture 33 (Figure 7) were very similar to those noted for culture 32, with an initially sharp decline at 24 hours, a rapid increase in uptake at 48 and 72 hours, followed by a leveling off at 96 hours. As in culture 32, the control cells of culture 33 revealed considerable utilization of the nucleotide at 72 and 96 hours, although to a lesser extent than the stimulated cells. Culture 34 (Figure 8) demonstrated a rapid increase in DNA synthesis in the PHA-stimulated cells at 24 hours and subsequent inactivity during the remaining culture period. The control cultures showed minimal activity. The mean values for cultures 31, 32, 33 and 34 are graphically depicted in Figure 9.

A patient with chronic lymphatic leukemia was also studied. The results of cultures of his lymphocytes are shown in Figure 10. Because of technical difficulties, samples were obtained only for 2, 48 and 96 hours, omitting determinations at 24 and 72 hours. It was of particular interest, although of questionable significance, that the PHA-stimulated cultures utilized large amounts of tritiated deoxycytidine and yielded very high total counts, compared to relatively low uptake by the controls.

Subsequent cultures (36-43) were accomplished in the same manner as cultures 31-35. The results, however, were markedly different. As seen in Figures 10-17, the total counts from cultures harvested just after they were established (2 hours after pulsing), were generally high in both the stimulated and control cultures. Succeeding values obtained at 24, 48, 72 and 96 hours revealed marked decrease in tritiated deoxycytidine uptake, with the PHA and control cultures utilizing similar amounts of the nucleotide. In view of the parallel decreases in

uptake in the control and stimulated cultures, the viability of the cultures was suspect.

DISCUSSION

Preliminary Observations of Lymphocyte Cultures

Cultures 31-34 appeared to be viable and showed good evidence of stimulation. The mean % maximum response curve of these cultures compared favorably with the composite curves of other researchers as shown in Figure 19. Cultures 36-42, however, which were accomplished in the same manner as earlier cultures, showed no evidence of stimulation and were probably not viable. No explanation was immediately evident, although it was possible that inadequate rinsing of detergent and acid cleaning solutions may have yielded concentrations which were toxic to the cells. Culture 35, which contained lymphocytes from a patient with chronic lymphocytic leukemia, yielded very high total counts when compared with the controls. The lymphocytes in chronic lymphocytic leukemia have been shown to be delayed in their response to phytohemagglutinin in vitro (10,43). Although purely speculative, it is possible that lymphocytes in chronic lymphocytic leukemia are capable of utilizing tritiated deoxycytidine to a greater extent than tritiated thymidine.

The technique for separation of lymphocytes from peripheral blood as described was found to be difficult to apply consistently. It was necessary that the glass columns and beads be as dry as possible. If not dry, the efficiency of the column was markedly reduced, producing a very low percentage of lymphocytes in the column elution and usually a high degree of contamination by polymorphonuclear leukocytes. To

accomplish adequate drying, the beads were autoclaved and oven dried separately; then, the columns were later assembled under aseptic conditions. In addition, the efficiency of the columns was affected by the compactness of the glass beads, the more loosely packed columns being unable to retain the 5 ml of cell suspension during the 30 minute incubation period, thereby encouraging granulocytic contamination. In retrospect it was evident from the more recent literature that the glass bead method of lymphocyte separation has largely been abandoned in favor of other techniques, such as sterile cotton columns (15) and sedimentation of phagocytic cells with carbonyl iron and methyl cellulose (13). In view of the difficulties encountered, future cultures would probably be facilitated by a more consistent separation procedure.

Initially, the cultures were incubated in an atmosphere of 5% CO_2 and oxygen, a readily available gaseous mixture. However, no viable cultures were obtained in the presence of high concentrations of oxygen, which was probably toxic to the cellular enzyme systems as reported by Forsdyke (25). Subsequent cultures were incubated in either room air or 5% CO_2 and room air with good results. The majority of recent publications advocate 5% CO_2 and air as the most desirable atmosphere for lymphocyte cultures (16,25,54).

The technique described for the study of DNA synthesis utilizing tritiated deoxycytidine and liquid scintillation counting was easy to apply and seemed to yield consistant results. Further experimentation would be necessary, however, to evaluate possible sources of error. Duplicate cultures should be studied to determine the adequacy of the saline washing procedure, the error introduced by exposure of the Bray's solution to light and changes in temperature, and the effect of the Millipore

filter in the counting solution. Correction for quenching should also be included in the determination of counting efficiency.

It would be desirable to evaluate the effects of other possible variables, including the consistency of PHA preparations, the effects of the culture medium depth, cell number, area of culture surface, and the degree of individual day-to-day variation. In addition, direct comparison of the various methods of quantitating lymphocyte response should be undertaken, with absolute cell counts, mitotic indices, tritiated thymidine and tritiated deoxycytidine determinations being done in parallel. Then perhaps definitive statements could be made as to which technique or combination thereof accurately reflects the response of normal and abnormal lymphocytes to stimulation.

DNA Synthesis in Leukocyte Cultures

In recent years many publications dealing with DNA synthesis in short-term cultures of human peripheral blood have appeared. DNA synthesis has been used as an indicator of metabolic and mitotic activity, but very few researchers have investigated the time sequence of DNA synthesis in the nuclei of proliferating lymphocytes. Determination of the DNA content of cells during the various stages of proliferation has been accomplished by several methods, with many improvements in technique made possible by advancing technology. The study of the kinetics of DNA synthesis was greatly facilitated by the development of tritium-labeled thymidine, a specific deoxyriboside presursor of DNA, by W. L. Hughes (32) in 1958. With a powerful tool for the in vivo and in vitro study of DNA synthesis and cellular kinetics in man and animals, new techniques were soon applied in the search for totipotential cells as hypothesized by Bond and Cronkite (7). In 1958 these investigators introduced

tritiated thymidine into cultures of normal peripheral blood leukocytes and found that after one hour incubation only a very small percentage (less than 0.06%) of the cells were synthesizing DNA, and were thought to be capable of division. Labeling of the cells was determined by autoradiography and was seen only in large mononuclear cells identified as monocytes, and large and medium-sized lymphocytes. No labelling of the myeloid series was found. These investigators were convinced of the existence in the peripheral blood of man of a small percentage (but large absolute number) of circulating leukocytes, either phagocytic monocytes and/or lymphocytes, that have the capacity to synthesize new DNA. They noted, however, that synthesis of DNA was presumptive but not direct evidence that the cell was destined to divide.

With the introduction of labeled nucleic acid precursors, it soon became apparent that if DNA synthesis was to be utilized as an indicator of mitotic activity, the stability of the DNA molecule had to be determined. It was reasoned that if intracellular turnover of nucleic acid components occurred without synthesis of new DNA, then the uptake of tritiated thymidine would not be a reliable indicator of DNA synthesis (59). It was, however, reported by Watts and Harris (59) and Siminovitch and Grahm (56) that turnover of DNA adenine and thymidine could not be detected in mammalian cells in the absence of DNA synthesis. Later, other investigators showed this to also be true of peripheral human lymphocytes (14,57). It has generally been accepted that incorporation of specific nucleic acid precursors is a strong indication that de novo synthesis of DNA has occurred.

The remarkable finding reported by Nowell in 1960 that the bean extract phytohemagglutinin has mitogenic effects now represents the

beginning of a 10-year period of great interest in DNA synthesis by

lymphocytes. In 1961, one year after the discovery of Nowell, MacKinney,

Stohlman and Brecher (34) published a study of cultures of peripheral

leukocytes in which mitotic indices of 1% were observed by the third

day. The authors, utilizing the culturing technique of Hungerford,

failed to acknowledge the mitogenic effects of PHA which they had used

to facilitate the separation of leukocytes from erythrocytes for the

in vitro cultures. Their study was, nonetheless, the first investiga-

tion of DNA synthesis in cultures containing PHA. These investigators

thought that the mitotic cells originated either from the small number

of lymphocytes in DNA synthesis present in freshly drawn blood as re-

ported by Bond et al (8), or possibly from lymphocytes which manifest

their capacity for DNA synthesis and division only during culture. To

determine which population of cells was involved in mitotic activity and

thus DNA synthesis, they performed an experiment using tritiated thymi-

dine and autoradiography. At the beginning of the culture 0.1 to 0.5%

of the leukocytes (which were almost entirely lymphocytes after 24 hours

of incubation with PHA) were engaged in DNA synthesis. The addition of

tritiated thymidine to the cultures at intervals thereafter permitted

estimates of the proportion of cells entering DNA synthesis at various

times. There was no increase in the percentage of labeled cells between

0 and 24 hours, but thereafter the percentage of cells in DNA synthesis

increased rapidly. When tritiated thymidine was added at 48 hours, 10%

of the cells incorporated label; at 72 hours, 40-45% of the cells incor-

porated label. There was a parallel increase in the absolute numbers of

cells in DNA synthesis.

Two studies were done to determine if lymphocytes entering DNA synthesis between 24 and 48 hours were the progeny of those cells in DNA synthesis at time zero. In the first study tritiated thymidine was added at time zero; after 2-4 hours the medium was changed and non-radioactive thymidine was added to block further uptake of any residual tritiated thymidine. Aliquots were studied up to 45 hours, during which time the percentage of labeled cells increased very little, from an average of 0.2% to 0.4%. The authors were convinced therefore that the rapid increase in DNA synthesizing cells between 24 and 48 hours could not be due to proliferation of those cells in DNA synthesis at time zero, which would have yielded a much higher percentage of labeled cells. To further document their findings, they reasoned that if the cells in DNA synthesis at 48 hours were the progeny of the mononuclear cells labeled at zero time, an average of approximately five divisions in a 24-hour period would be necessary to account for the observed thirty fold increase in DNA synthesizing cells. If this were true, the addition of colchicine to cultures during the period of rapid increase in DNA synthesis should result in the collection of a substantial number of metaphases and a reduction in the number of labeled cells when tritiated thymidine was added after the colchicine. By blocking mitosis the number of cells dividing and again entering division would be reduced. The results of their experiments revealed that neither of these criteria were met, e.g., no mitosis were observed and the percentage of labeled cells in the controls and colchicine treated cultures were not significantly different.

To determine the time of onset of mitosis, colchicine was added to aliquots at successive time intervals and metaphase preparations were

made after 3-4 hours incubation. Mitoses were first seen between 40

and 45 hours and increased in number rapidly; at 65-70 hours the mitotic

index was 1% per hour of exposure to colchicine.

This study, therefore, indicated that the dividing cells in cul-

tures of peripheral blood must be derived from a relatively large popula-

tion of cells. Since granulocytes disintegrated during the first 24

hours, they concluded that lymphocytes were responsible for the synthesis

of new DNA in the cultures (34).

McIntyre and Ebaugh (37) attempted to correlate the changes in the

in vitro leukocyte population with the metabolic events which take place

in the presence and absence of PHA as measured by P^{32} incorporation into

DNA and RNA. Fractionation of DNA and RNA was accomplished utilizing

Kahn's (33) adaptation of the Schmidt-Thannhauser (52) technique. Their

total cell populations decreased due to loss of granulocytes by 24 hours.

No mitoses were seen during the examination of smears derived from cul-

tures not containing PHA. By the end of 96 hours, cultures without PHA

contained a preponderance of degenerating cells. In cultures containing

PHA, however, there appeared at 48 hours large transformed mononuclear

cells and at 48 to 72 hours, mitoses first appeared and reached a peak

at 96 hours when 1 to 1.5% of the cells were seen to be in mitosis.

These changes were reflected in the incorporation of P^{32} in the DNA

fraction. The authors noted that at about 24 to 48 hours there appeared

a significant increase in DNA P^{32}. This reached a peak in 4 to 8 days.

Logarithmic plots revealed the DNA doubling time to be 26 to 40 hours.

They assumed that P^{32} incorporation into DNA does not occur without

synthesis of a DNA molecule, despite the earlier observation by Hughes

(31) that there may be a turnover of DNA-P^{32} independent of that caused

by DNA synthesis.

In 1962 Bender and Prescott (5) confirmed the results of Bond et al (8) that a very small fraction of peripheral leukocytes of freshly drawn human blood are engaged in DNA synthesis, and suggested that the few cells that do synthesize DNA do so at a very low rate. To determine the time of onset of mitosis, they harvested colchicine treated cultures at 12-hour intervals and recorded the mitotic activity. No mitoses were seen in material fixed at 12, 24 and 36 hours, but numerous mitoses accumulated between 42 and 48 hours.

In an earlier study Bender et al (4) had demonstrated that when whole blood is cultured immediately after x-irradiation, only chromosome-type aberrations are obtained, but that chromatid-type aberrations are seen when cultures are irradiated shortly before the advent of mitosis. Therefore, the circulating leukocytes that divide in an in vitro system were thought to be in the pre-DNA synthesis (G_1) growth phase at the time the blood was drawn. To determine the time of DNA synthesis, further experimentation was conducted. Every 6 hours one culture was exposed to tritiated thymidine for 30 minutes. All cultures were treated with colchicine from the 42nd to the 48th hour after culture initiation and were then fixed, with interphases and mitoses being scored for labeling. These experiments established limits for the lengths of G_1, S and G_2[1], which were found to be a minimum of 24 hours, a minimum of 12 hours, and a maximum of 6 hours, respectively, in cells dividing in the first wave of mitoses. They found that the S period can be as long as 30 hours. They noted considerable variability in the time required to double DNA among cells of

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[1] G_1 : Pre-DNA Synthesis Period
S : Period of DNA Synthesis
G_2 : Period from Completion of DNA Synthesis to Onset of Mitosis.

the same genetic constitution; however, the observed lengths of G_1, S

and G_2 were of the same order of magnitude as those observed in other

mammalian cell types.

To determine the minimum G_2 period, Cooper and Barkhan (14) studied

actively dividing cells after 3 days in cultures containing PHA, which

were colchicinized and tritiated thymidine added to the cultures at

various times prior to harvesting of the cells. It was found that a

minimum of 2 hours must elapse between the addition of the tritiated

thymidine and harvesting in order to obtain labeled mitotic figures. If

tritiated thymidine was introduced into the culture within 2 hours prior

to harvesting, the cells had already completed DNA synthesis and no

tritiated thymidine was utilized. A longer period of exposure allowed

uptake of the radioactive nucleotide during the remaining S period. Thus,

the minimum G_2 period was found to be 2 hours. In these experiments, the

average percentage of cells (0.2 to 0.5%) that incorporated tritiated

thymidine in suspensions of leukocytes freshly drawn from the patient

was higher than that reported by Bond et al (8). This was thought to be

due to the differences in incubation temperatures.

Michalowski (38) found the timing of DNA synthesis more difficult

to quantitate. He related this to the inhomogeneity of the cells in

leukocyte cultures. He observed, as have others (45), that at least two

distinct types of cells become labeled on the addition of tritiated thy-

midine, lymphocytes and "large cells" at that time thought to be unrelated

to lymphocytes. The cells of the various kinds may contribute to the DNA

synthesis of the whole population at different times. Thus, accurate de-

terminations of G_1, S, and G_2 would be difficult to obtain. The incorpora-

tion of ^{14}C-2-thymidine into human leukocytes maintained in the PHA-

containing medium as reported are shown in Figure 20. This investigator noted a drop in the rate of incorporation after about 66 hours of incubation, followed by another rise at 72 hours, which he attributed to the inhomogeneity of the cells synthesizing the DNA. He assumed that when some of the cells of one type enter their post-synthetic (G_2) period the bulk of the cells of the other type approaches the synthetic (S) period to bring the second peak of DNA synthesis at 72 hours. The number of mitoses was also determined and two peaks, one in the 65th hour and one in the 77th hour, respectively, made the curve similar to that of the incorporation of thymidine. By comparing the two curves, it was estimated that the modal duration of G_2 was approximately 5 hours for the two different cell types. This value was very similar to that found by Bender and Prescott (5), who reported that G_2 lasts shorter than 6 hours in cultures of human leukocytes.

Epstein and Stohlman (22) studied RNA synthesis in cultures of normal human peripheral blood in the presence of PHA. Parallel control cultures were prepared in which DNA synthesis was evaluated by the incorporation of tritiated thymidine to establish that the growth patterns were similar to those previously reported. The agreement was good and the results are included in Figure 18. DNA synthesis occured in 0.2% of the cells during the first 24 hours of culture. The percentage of cells synthesizing DNA increased rapidly after 24 hours so that by 48 hours, 13% were in DNA synthesis. After 72 hours, 45 to 50%, and after 96 hours, 55% of the cells were synthesizing DNA. In the absence of PHA there was virtually no RNA or DNA synthesis nor evidence of lymphocyte transformation. The initiation of RNA synthesis 24 hours prior to DNA synthesis and the absence of DNA synthesis in cultures without PHA were

considered evidence that DNA synthesis and transformation of the lymphocytes are necessary steps in the initiation of DNA synthesis.

Hayhoe and Quaglino (44) investigated RNA and DNA metabolism of human leukocytes cultured with PHA, utilizing uridine-5-^{3}H as a specific precursor of RNA and tritiated thymidine as precursor of DNA. They found a pattern of DNA synthesis similar to that previously reported. By comparing the appearance time of mitotic figures with extent of tritiated thymidine labeling, these authors suggested that the G_2 period of rest intervening between the end of DNA synthesis and the onset of mitosis in the cultured cells was approximately 3 hours. This value compared favorably to those previously discussed (5,14,38).

Chalmers et al (13) reported that the small lymphocyte is capable of undergoing the morphological changes of transformation without parallel synthesis of DNA. However, when the incorporation of tritiated thymidine was compared to the total numbers of transformed cells, good correlation was observed. They emphasized the importance of measuring the lymphocyte reactivity at the time of optimum response, since the induction of DNA synthesis does not occur simultaneously in all the transformed cells, even when the lymphocytes are exposed to the stimulant for only a brief period. Their accuracy in quantitation of the lymphocyte response to stimulation was greatest when comparisons were made only between the responses of aliquot of lymphocytes from a single blood sample. If results of experiments run on one day were compared with those from experiments run on another day, further sources of variation were introduced. The response of an individual's lymphocytes seemed to show a natural variation from day to day and the composition of the serum and other factors may have influenced the rate of incorporation of tritiated

thymidine into the DNA.

The findings of the above studies and of several other are depicted graphically in Figure 18. It can be generally stated that lymphocytes cultured in vitro synthesize only very small quantities of DNA in the absence of stimulation. With the addition of stimulants, such as PHA or other antigens, the lymphocytes respond in a predictable fashion. It is to be noted that while lymphocytes characteristically synthesize DNA in the presence of PHA with respect to time, the quantitative aspects of DNA synthesis depend on a multitude of additional factors. For example, Cooperband et al (16) reported that synthesis of DNA and protein by human peripheral lymphocytes is partly dependent on serum factors. He demonstrated that the maximum synthesis of DNA occurs in cultures containing 15% serum in the culture medium. In cultures containing greater or lesser quantities of serum the effects of PHA on DNA synthesis were inhibited. Since different researchers utilized widely different concentrations of serum in their cultures, the absolute quantities of DNA synthesized are not directly comparable.

In addition, the response of lymphocytes to PHA in vitro has been shown to vary with the concentration and source of the PHA used (47). Lymphocytes of different individuals or even the same individual react with varying intensity to PHA. The absence of identical or even similar response of cells of different individuals to the same preparation of PHA suggests that there are multiple mitogenic agents in PHA, exhibiting different reactivities with cells of different individuals. This variability further negates the application of quantitive determinations of DNA synthesis to the comparison of normal and abnormal lymphocyte function. Evidence also has been presented that lymphocytes may be functionally

divided into two groups, e.g., there may be a population of PHA respon-
sive lymphocytes and a population of non-responsive lymphocytes (37).
Variation in the relative number of each type of lymphocyte would clearly
affect quantitative determinations of DNA synthesis in the presence of
PHA. Other variables have been reported that affect the reactivity of
lymphocytes in vitro, including the culture atmosphere, PHA/serum ratios,
depth of the culture medium (38), cell number (47), duration of culture
(47), area of culture surface (47) and prevous irradiation (18,23). The
presence of steroids and other compounds, and possibly the choice of
radioactive precursor (47) have also been shown to be important consid-
erations.

CONCLUSION

The function and potentials of peripheral blood lymphocytes remain
largely unknown. Study of lymphocyte transformation can yield informa-
tion that cannot be obtained from the study of established tissue cul-
ture cell lines. Although lymphocyte transformation is subject to
many variables that interfere with accurate quantitation, it seems that
moderately accurate quantitation is possible if the limitations of the
methods utilized are constantly borne in mind. Elucidation of the kine-
tics of DNA synthesis by lymphocytes timulated with mitogenic agents
such as phytohemagglutinin has made possible the use of DNA synthesis
as an indicator of lymphocyte function. The introduction of radioactive
precursors of DNA has greatly facilitated the study of lymphocyte trans-
formation and it is anticipated that future research will further
delineate the lymphocyte responses to stimulation, and that accurate
quantitation will be achieved.

SUMMARY

The function of lymphocytes, important in numerous immunological mechanisms, has been shown to be impaired in many disease states. The extent of DNA synthesis by stimulated and unstimulated lymphocytes has evolved as a reliable indicator of lymphocyte competence. DNA synthesis has been most easily and accurately determined by measuring the uptake of tritiated thymidine by the cells in culture, utilizing liquid scintillation counting techniques. Recently, however, it has been suggested that the uptake of tritiated thymidine may not be a reliable indicator for all tissues, especially thymus tissue, and that tritiated deoxycytidine may more accurately reflect DNA synthesis by lymphocytes. The results of preliminary experiments utilizing tritiated deoxycytidine in the evaluation of lymphocyte cultures containing PHA were included. It was found that tritiated deoxycytidine was incorporated into the DNA of peripheral blood lymphocytes similar to the incorporation of tritiated thymidine as reported by other investigators. Large amounts of tritiated deoxycytidine were utilized by the cells of a patient with chronic lymphocyte leukemia. It was suggested that tritiated deoxycytidine may more accurately reflect DNA synthesis in these cells than does tritiated thymidine, which has been shown to be poorly incorporated by lymphocytes from patients with chronic lymphocyte leukemia. A review of the available literature on the kinetics of DNA synthesis by lymphocytes in vitro, cultured with PHA, was also included. It has been found that lymphocytes cultured in vitro synthesize only very small quantities of DNA in the absence of stimulation. With the addition of mitogenic agents, such as PHA, the lymphocytes usually respond in a predictable fashion, undergo lymphocyte transformation and

synthesize DNA in a characteristic manner with respect to time. DNA synthesis, while negligible at zero time and only slightly increased at 24 hours, increases rapidly from 48 to 72 hours. Synthesis then gradually decreases at 96 hours, depending on the state of the culture media and on other variables. Strictly quantitative aspects of DNA synthesis have been found to be dependent on many variables which must be further investigated in order to achieve accurate evaluation of lymphocyte function.
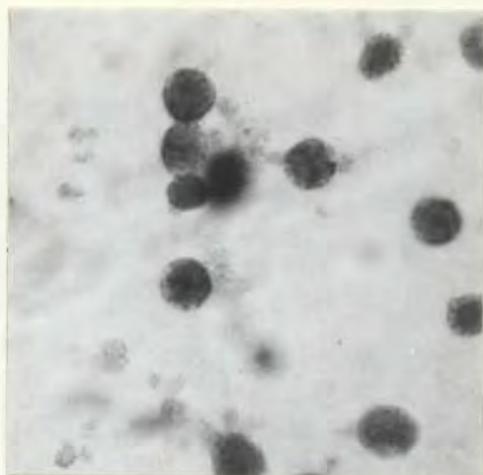
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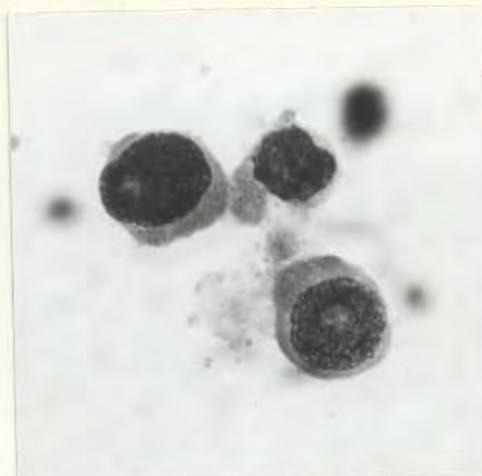
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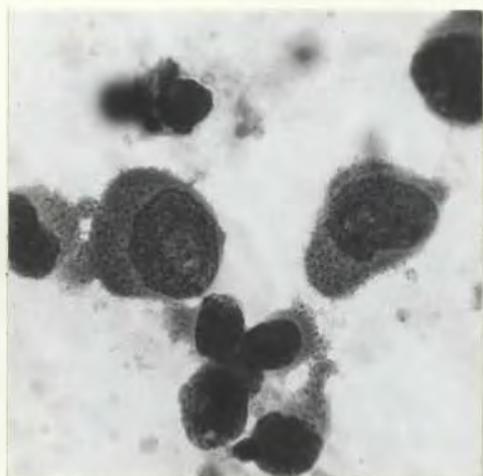
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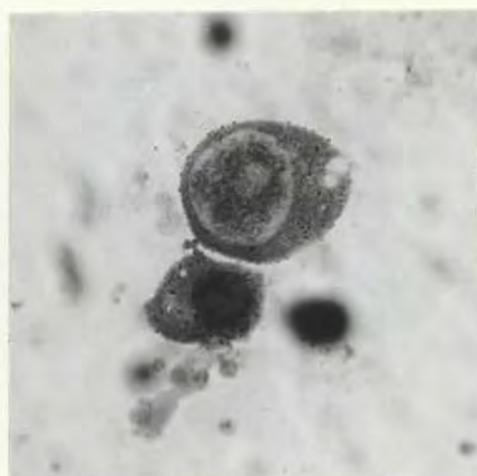
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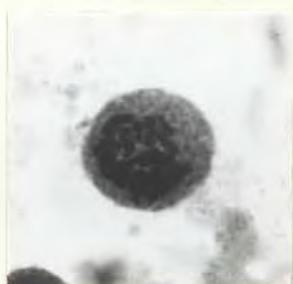
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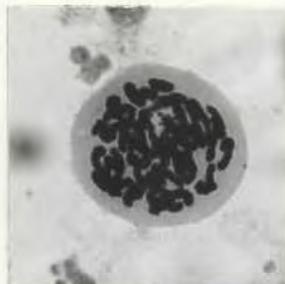
Figure 3b.
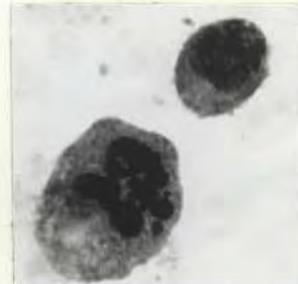
Figure 4.

Figures: 1. Unstimulated control lymphocytes;
2a, 2b. Typical transformed lymphocytes;
2c. Transformed lymphocytes demonstrating prominent nucleoli and cytoplasmic vacuoliation;
3a, 3b, 4. Typical mitotic figures cultured in the presence of colchicine.
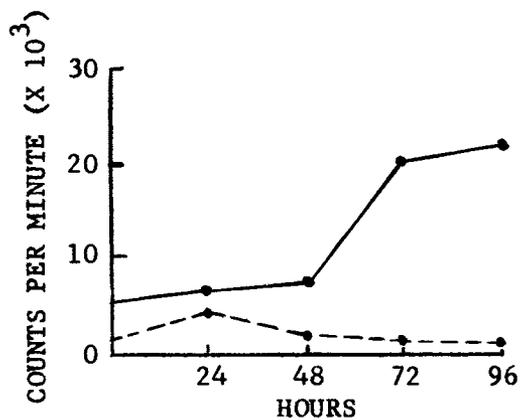
Figure 5. Culture 31
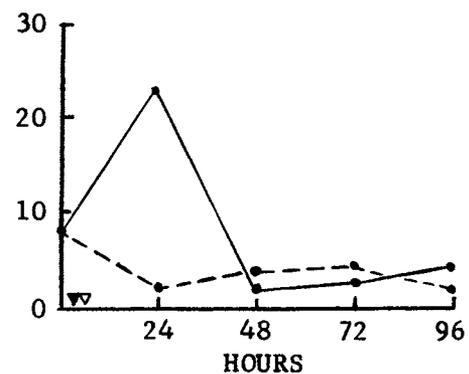
Figure 8. Culture 34

▼ Unpulsed Control Cells
▽ Unpulsed PHA Culture
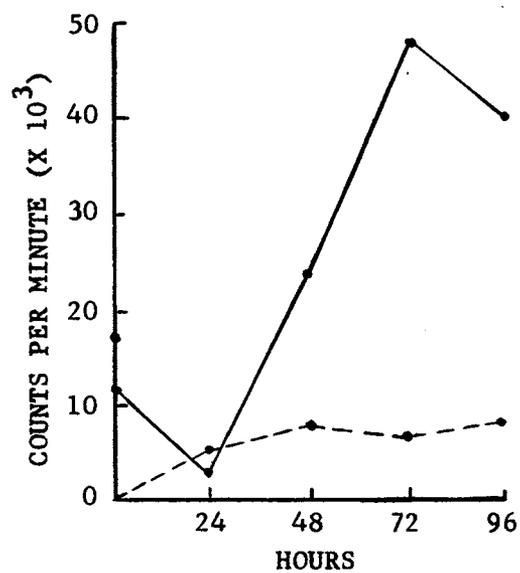
Figure 6. Culture 32
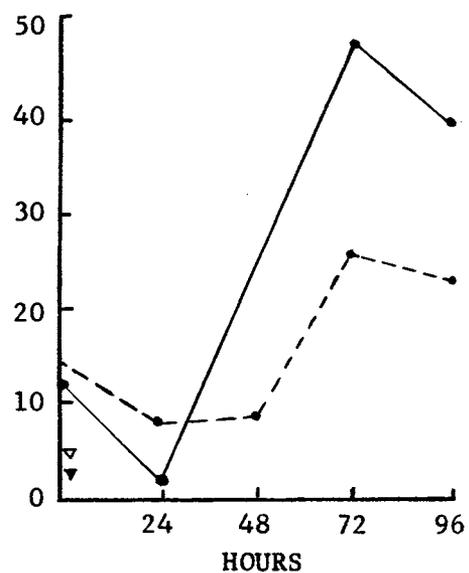
Figure 7. Culture 33
Pulsed, Harvested after
5 minutes: ▼ Controls;
▽ Stimulated Cultures.

Legend for Figures 5-18:
●----● Unstimulated control cultures
●——● Cultures stimulated by PHA
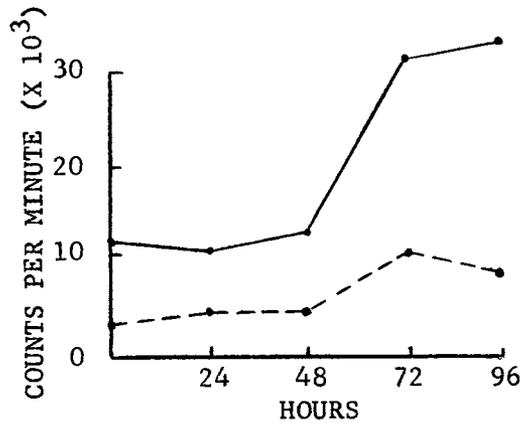
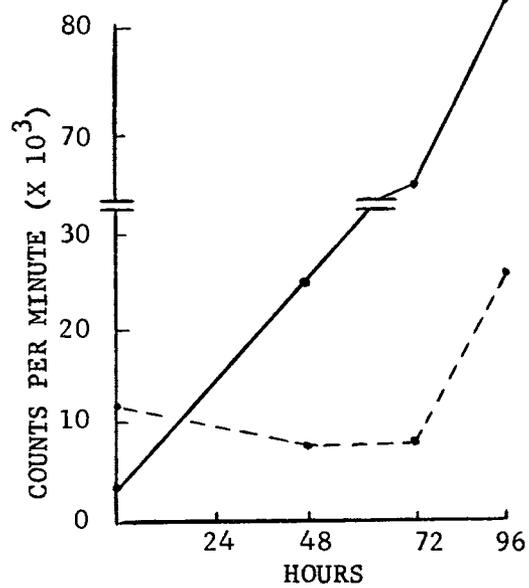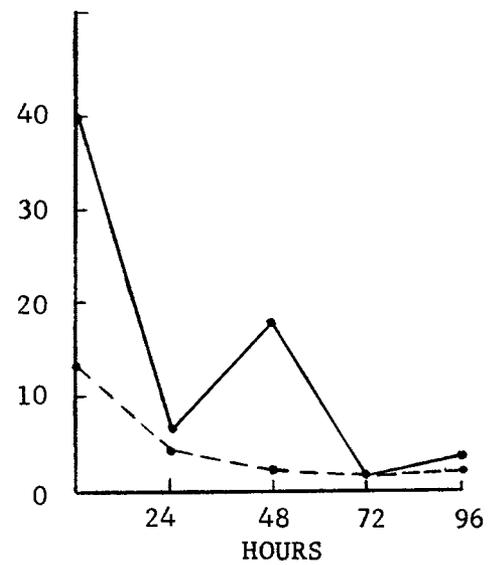Figure 9. Mean Curve
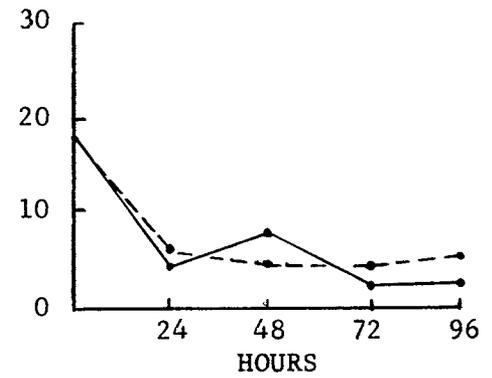
Figure 11. Culture 36

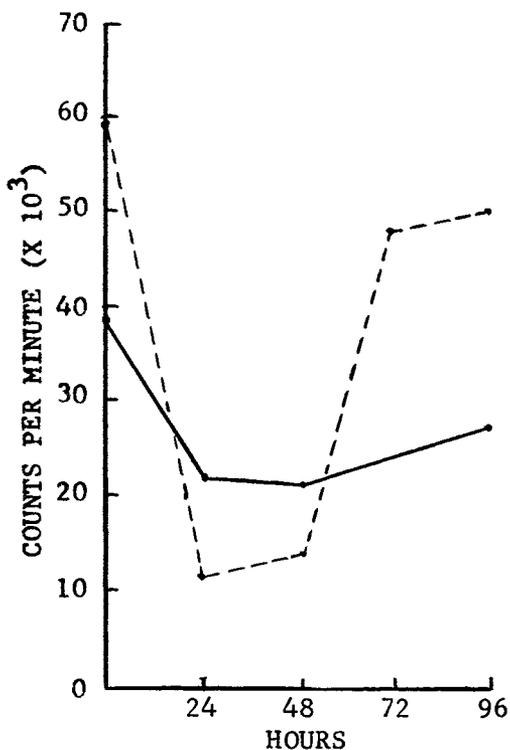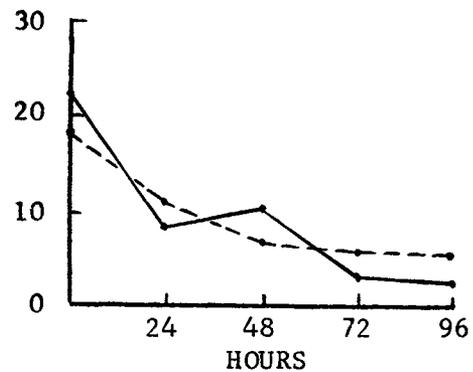Figure 10. Culture 35

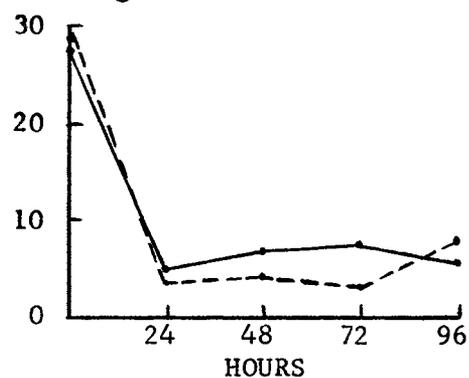
Figure 12. Culture 37

Figure 13. Culture 38

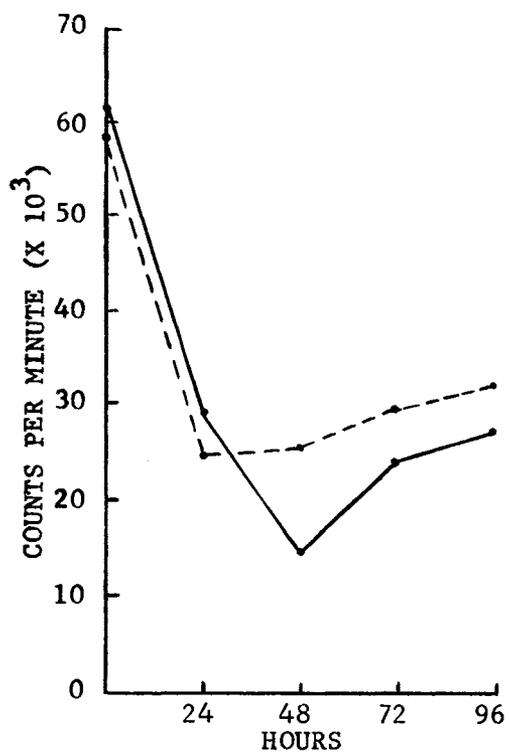Figure 14. Culture 39

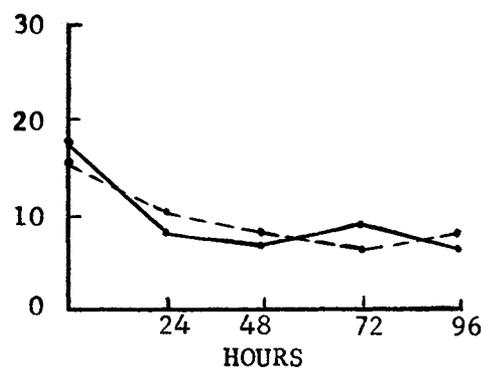Figure 15. Culture 40

Figure 16. Culture 41
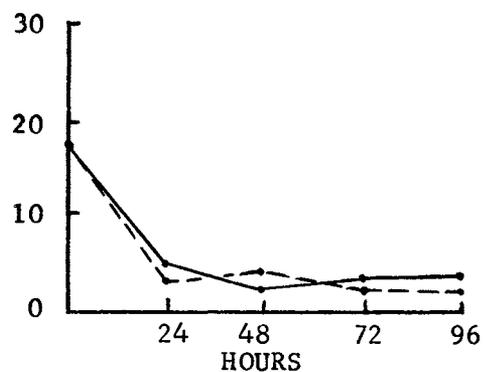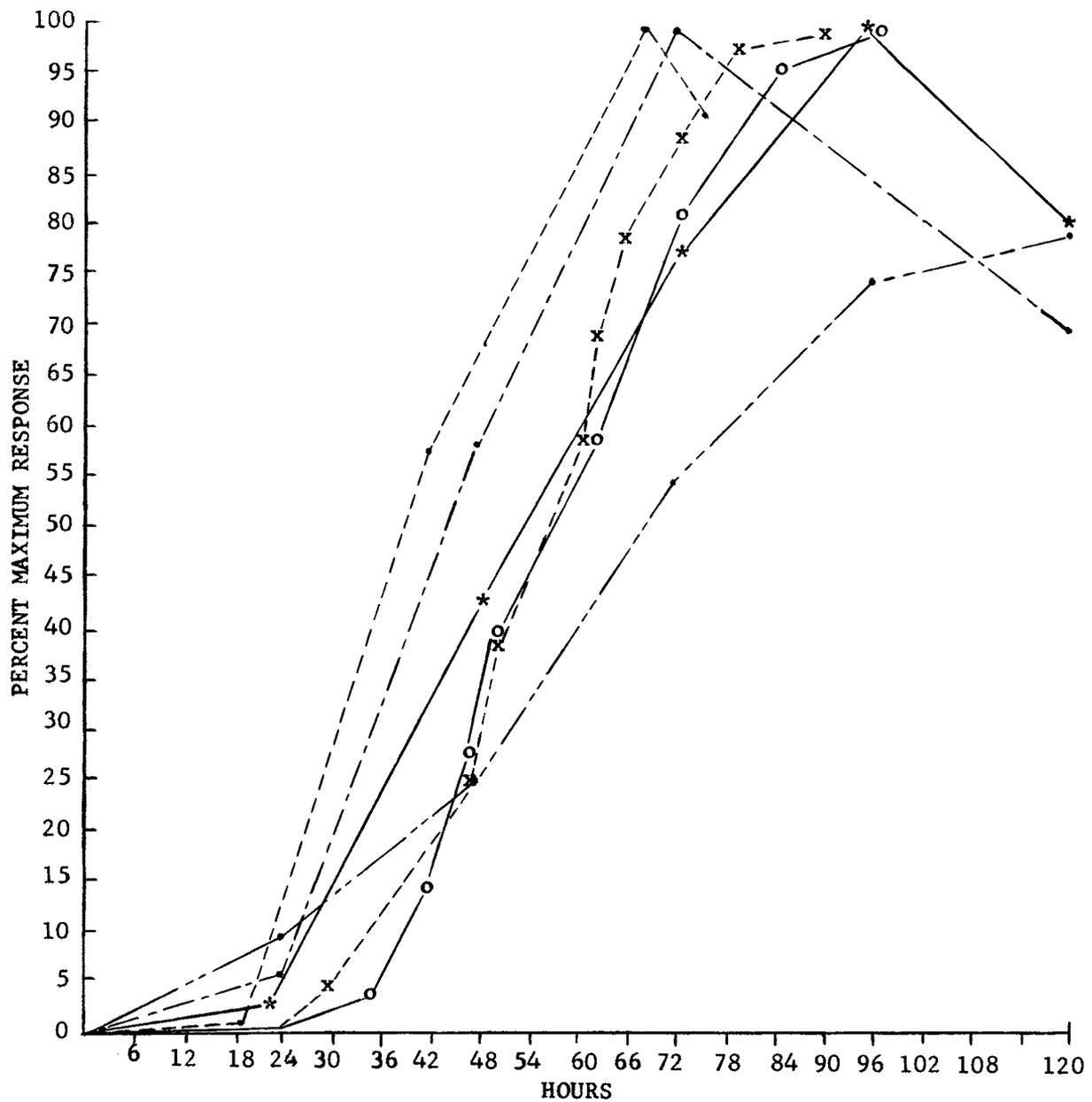

Figure 17. Culture 42

Figure 18. Culture 43

Figure 19. Mean percent maximum respone curves

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●---●    Borjeson et al. (9)
●---●    Havemann et al. (27)
*———*    Caron et al. (11)
X---X    Results utilizing tritiated deoxycytidine
            (See text)
o———o    MacKinney et al. (34)
●--—●    McIntyre et al. (37)
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