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THE BIOCHEMISTRY OF LYMPHOCYTES FROM PATIENTS

WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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CHAPTER I

INTRODUCTION

Chronic Lymphocytic Leukemia

The clinical manifestations of leukemia have been known since the time of Hippocrates, although the full description of the disease was not published until 1845. In that year, Bennett (1) and Virchow (2), working independently, reported that patients who, at autopsy, were found to have marked changes in the blood also had unusually large livers and spleens. Bennett described the blood as having "great numbers of pus and lymph cells", whereas Virchow recognized an inversion of the usual numbers of red and white cells. Thus, Virchow's concept of the predominance of the white blood cells rather than "pus" cells has become the hallmark of diagnosis, and his proposed name for the disease "Weisses Blut or Leukamie" has been adopted.

After their definitive work, it became apparent that many earlier reports in the literature were descriptive of this same process (3,4). Within the next 45 years many additional cases were reported in the literature, but it was not until the year 1891 when Ehrlich introduced specific staining techniques to differentiate cell types that a histological basis

for classification and accurate diagnosis could be made.

Classification was based upon the following: a) whether the disease was acute or chronic, and b) what type of cell was predominant in the peripheral blood. The disease process explored in this study was chronic lymphocytic leukemia (CLL) and was recognized as a protracted disease with an accumulation of small, mature lymphocytes in the peripheral blood and lymphoid tissues. Within this broad definition, several types of CLL have been recognized (5), a benign form in which the patient has little distress, an aggressive form in which the lymphocytes rapidly infiltrate the peripheral lymphoid tissues and bone marrow, and an unusual form of chronic dermatologic disturbance. The patients in this study had either the benign or aggressive form of disease; the dermatologic form was not studied.

Epidemiological studies of CLL indicated that the ages of greatest occurrence were between 45 and 54 years in America and England, and the disease occurred more frequently in males (74%) than in females (26%) (6,7). Studies of the geographical distribution of the disease indicated widespread occurrence with an increased incidence in Israel, the United States, Denmark and among the Jews in New York City (8). In contrast, there was virtually no incidence of CLL in Japan (9) and significantly reduced incidence among Chinese (10) and New Zealand Maoris (11).

Many etiologic agents have been proposed at one time or another as the primary causative agent of CLL; and although trauma, parasites, bacteria, and viruses have been suggested, there were no unequivocal data supporting any one agent. The earliest report of an etiologic agent suspected the small bacillus (12), this was followed by Osterwald's report of the micrococcus (13) and Jousset's report of the coccobacillus

(14). Since these initial reports, staphylococcus (15), streptococcus (16), diphtheroid bacillus (17), tubercle bacillus (18), Bacillus typhosus (19), Bacillus paratyphosus B (20), Pneumococcus (21) and Bacillus proteus (22) have all be suspected of causing CLL, however Forkner (23) concluded 30 years ago that these agents were not the primary causes of leukemia.

A second possible etiologic agent suggested was trauma, specifically fractures, which was reported by Norikoff in 1933 (24), but, subsequently, this has not been found to be of significance in other studies. In 1933 Luisada suggested that the etiologic agent was malaria (25), after Loesch (26) had noted the development of CLL in a patient who had been treated for syphilis with an inoculation of the malaria-causing parasite. This was later discounted because of the failure to substantiate it in other cases of CLL or malaria. Studies which were designed to assess the effects of bacterial, parasitic and viral agents should be evaluated carefully due to the severe complications of tuberculous, viral, fungal or acute bacterial infections which were commonly found in patients with all forms of leukemia, including CLL.

Radioactive substances have also been suggested as the etiologic agent in CLL. Jagic et al. (27) reported, in 1911, the occurrence of "lymphogeneous leukemia" in a patient who had worked with roentgen rays for eight years. In 1945, two atomic bombs were dropped on Hiroshima and Nagasaki, thus providing the largest experiment to assess the effects of one dose of ionizing radiation on a human population. A significant increase in leukemia was evident three years later, especially in persons within 2,000 meters of the site of detonation (hypocenter) (28). However, chronic granulocytic leukemia was the most common type found, acute lymphoblastic the second most common type and CLL was found in merely

negligible quantities in the exposed population. In a recent report of the 13 cases of CLL found in the Japanese population since 1945 (29), it was shown that only five of these patients had been within the Nagasaki city limits at the time of detonation. These five had been at a distance greater than 3,000 meters from hypocenter, and the calculated dose of radiation was less than one roentgen. These data and those of Cronkite (30) strongly suggested that ionizing radiation was not the primary etiologic agent in CLL.

The most promising areas of reasearch in elucidating the primary etiologic agent in CLL were virology and genetics. Virus particles have been found as the mode of transmission of the acute leukemias of fowl (31) and mice (32), and subsequently two models of genetic transmission have been suggested. The first of these was vertical transmission from one generation to the next of an altered genome or the potential for an altered genome carried in the germ cells (33). The second was the horizontal transmission of the viral genome from an infected host to a "potential" host with the altered genome transmitted to the somatic cell progeny.

In both models it was essential that the viral genome become incorporated into the host deoxyribonucleic acid (DNA) in order for it to be stably transmitted to the progeny cells. For DNA viruses, incorporation into host DNA was facilitated by cellular DNA polymerases; in contrast, ribonucleic acid (RNA) viruses posed many conceptual problems as to the mode of genetic transmission. Recently, however, an RNA-dependent DNA polymerase has been found which used RNA as a template to transcribe a complementary copy of DNA (34,35) which was then converted to double-stranded DNA, cleaved to 7 S pieces and integrated into the host DNA during replication of the host genome. It was believed that this

provided for the stable transmission of the viral genome, either fully or in part, to progeny cells. This RNA-dependent DNA polymerase appeared to be associated with oncogenic viruses (36), non-oncogenic viruses (37) and a wide range of host cells including lymphocytes (38).

The role of genetic predisposition in CLL has been suggested by many authors (39), and two lines of evidence supported this hypothesis. First, there was an increased tendency for leukemia to occur in kinships, especially sibships (40). Parental consanguinity with an increased incidence of CLL has been observed (41). In one family studied, there was an increase in immune defects in those sibs which did not have leukemia (42). The second line of evidence was from the epidemiological studies of the geographical distribution of CLL. These studies indicated that the almost negligible incidence of CLL in Japan was quite striking since in Japan 4 to 5% of the marriages were between first cousins (43), and there was a lower frequency of CLL in Japanese who were living in the United States than in the non-Japanese population (44). This suggested that an undetermined alteration of metabolism was controlled by predisposing genetic factors, and that the lack of genetic mixing has had profound effects on the incidence of the disease.

Cell Culture

Non-Stimulated Lymphocytes

Biochemistry. Leukemia cells were first cultured in 1914 (45), and in 1929 Timofejewsky and Benewolenskaja (46) showed that CLL lymphocytes could be maintained in cell culture. However, due to the many cell types present in their culture system, it was impossible to draw any conclusions about the "physiologic or diagnostic value of the system" (23). Since

that time with the isolation of pure populations of lymphocytes and the introduction of radioactive labelling techniques, it has been possible to study some of the biochemical events which occur in cultured lymphocytes and to make comparisons between normal and CLL lymphocytes.

Brody et al. (47) have shown in CLL lymphocytes the occurrence of significantly reduced amounts of radioactive carbon dioxide evolved from glucose and suggested that CLL lymphocytes metabolized less glucose through the hexose monophosphate shunt than did normal lymphocytes. These studies were consistent with other reports indicating decreased glucose-6-phosphate dehydrogenase (48) and 6-phosphogluconate dehydrogenase (49) activities in leukemic lymphocytes which would result in decreased substrate availability for the hexose monophosphate shunt.

Stjernholm et al. (50) have reported slightly decreased utilization of glucose in the glycolytic pathway under aerobic conditions in CLL lymphocytes, but the precise enzymic alteration in glucose metabolism was not hypothesized.

Torelli et al. (51) have shown that non-stimulated normal and CLL lymphocytes synthesized RNA primarily of a high sedimentation rate (30 to 80 S) with very little, if any, subsequently converted to 18 S and 28 S ribosomal RNA.

Increased activities of di- and tripeptidases, and a reduction in β-glucuronidase and phosphatases (acid and alkaline) have been reported (52) in CLL lymphocytes. The lysosomal enzymes, the peptidases and β-glucuronidase,were reportedly associated with pathological and physiological activities in cellular metabolism, but the importance of either increased or decreased activities was unknown. The acid and alkaline phosphatases were involved in the cleavage of phosphate esters, but again

the precise significance in cellular metabolism was unknown. Therefore, the meaning of decreased activity in the CLL lymphocyte was unclear.

Several studies have characterized events which occurred in normal cultured lymphocytes. Cooper et al. (53) found that only 1% of the non-stimulated lymphocytes incorporated labelled thymidine after 72 hours in culture. McIntyre and Ebaugh (54) using labelled phosphate found negligible amounts of radioactivity in the DNA which was subsequently isolated. Thus, it was concluded that lymphocytes in vitro, in the absence of a mitogen, did not undergo DNA synthesis.

Weisberger et al. (55) have shown that the in vitro uptake of cystine and methionine was an active process, and it has also been reported (56) that protein synthesis did occur in non-stimulated lymphocytes to a limited extent.

RNA synthesis also occurred in non-stimulated lymphocyte cultures, and it was of interest that about half of the 18 S ribosomal RNA was degraded immediately after synthesis (57). The significance of this will be discussed later. Cooper and Fitzgerald (58) have shown that normal lymphocytes produced little lactic acid under aerobic conditions. Also, Balogh and Cohen (59) have reported that normal lymphocytes contained β-hydroxybutyric dehydrogenase and were capable of oxidizing fatty acids to form acetate for use in the Kreb's cycle.

Thus, the small, circulating lymphocyte from either a normal donor or a CLL patient, when placed in cell culture, underwent little RNA, protein or carbohydrate synthesis and in the absence of a stimulant did not divide (60).

Morphology. Morphological studies to elucidate differences between normal and leukemic lymphocytes using supravital and Romanowsky

stains, with phase contrast or light microscopy, have failed to show significant differences (61). Wislocki et al. (62) in 1949 reported that cytoplasmic particles which stained with periodic acid-Schiff reagent were present in CLL lymphocytes but not in normal lymphocytes. Using these histochemical techniques with examination of the cells with light microscopy, Astaldi and Verga (63) observed that the glycogen contents of lymphocytes from normal donors, patients with acute lymphocytic leukemia and patients with CLL differed significantly. They found little or no glycogen in normal and acute lymphocytic leukemic lymphocytes but greatly increased amounts in the cytoplasm of CLL lymphocytes. The glycogen content of normal lymphocytes has been quantitated and found to be 4.1 ±0.5 mg/ 10^{10} cells. In CLL lymphocytes the glycogen content has been found to be 7.0 ±0.7 mg/ 10^{10} cells (64).

Chromosomal studies in lymphocytes from CLL patients have not been as fruitful as those with chronic myelocytic leukemic cells in which an abnormal chromosome, the Philadelphia-1, has been found (65). Goh (66) reported, however, a pseudodiploid number of chromosomes in CLL lymphocytes, but no conclusions have been drawn from these studies.

PHA-Stimulated Lymphocytes

Biochemistry. In 1960 Nowell (67) reported that an extract of the red kidney bean, Phaseolus vulgaris, termed phytohemagglutinin (PHA) when added to cell cultures caused leukocytes to divide. Since that report, it has been shown that in the presence of PHA it was the small, circulating lymphocyte which changed from a resting cell to a "blastogenic" or "transformed" cell with increased RNA, protein, carbohydrate and DNA synthesis followed by cell division. The temporal relationships

of these synthetic events have been expressed within the concept of the "cell cycle", as discussed recently by Abell (68). The resting or non-stimulated cell resided in a Gap 1 phase. When a mitogen such as PHA was added, a specific series of changes occurred.

One of the first changes occurred within the first few minutes and was an increase in cyclic adenosine 3', 5'-phosphate (cyclic AMP) (69). There are, however, conflicting reports in the literature concerning this increase in cyclic AMP. Novogrodsky and Katchalski (70) reported that PHA did not increase cyclic AMP levels nor did it stimulate adenyl cyclase in rat lymph node lymphocytes. In contrast, Smith et al. (71) reported 25 to 300% increases in cyclic AMP which were observed 1 to 2 minutes after PHA addition. Furthermore, during the next 6 hours, there was a decrease in cyclic AMP, and at 24 hours it was below control (non-stimulated) values. It is possible that the early measurements were performed in populations of lymphocytes different from circulating lymphocytes in their mode of responsiveness to PHA. Smith et al. (71) suggested that cyclic AMP was a secondary messenger of PHA activity, however, cyclic AMP addition to normal lymphocytes did not either increase the appearance of labelled phosphate into lymphocyte phospholipids or stimulate DNA synthesis, as did PHA. Based upon the former data, Smith et al. (71) have concluded that increased cyclic AMP was an early but not necessarily the initial change, in the process of lymphocyte transformation.

Within the first 10 minutes of PHA addition, Pogo et al. (72) have observed a marked uptake of radioactive acetate in the arginine-rich fractions of nuclear histones. They also suggested that this reflected increased histone acetylation rather than increased histone synthesis because puromycin, an inhibitor of protein synthesis, did not affect the

increased labelling. When tritiated uridine was added to the PHA stimulated lymphocytes, it was shown that the acetylation of histones preceded the increased rate of nuclear RNA synthesis. These authors suggested that these early changes brought about alterations in DNA and histone interactions which signaled later RNA synthesis initiation.

It has also been suggested that modifications in DNA and histone interaction could be produced by regions of high negative charge density resulting from the phosphorylation of proteins in the cell nucleus. It has been reported (73) that immediately after PHA stimulation there was an increase in phosphorylation of serine and threonine residues associated with nuclear histones, and within 1 hour a 2-fold increase was found. This was also consistent with a shift from "condensed" chromatin to a more "diffuse" state associated with greater gene activation reflected as chromatin availability for RNA synthesis.

Fisher and Mueller (74) have shown that during the first 10 minutes after PHA addition, there was a 10-fold increased incorporation of labelled phosphate into phosphatidyl inositol, and by 30 minutes there was a 20-fold increase. The other 2 phospholipid fractions, 1) phosphatidyl choline and 2) phosphatidyl serine and phosphatidyl ethanolamine, showed only 0.08-fold and 0.61-fold increases in radioactive phosphate incorporation. This increased phosphatidyl inositol was independent of protein synthesis which suggested that existing enzymes were activated. These authors suggested that PHA-induced activation of phosphatidyl inositol metabolism and thus protein transport mechanisms could bring about the removal of a gamma-globulin from the cell. This removal would then allow the cellular genetic information to be expressed.

Torelli *et al.* (51) have reported that the RNA labelled within 1

hour of PHA addition had a high molecular weight but within 4 hours most of the label was associated with ribosomal and transfer RNA of a smaller molecular weight. In contrast, the RNA labelled in non-stimulated lymphocytes was not a ribosomal RNA precursor. They suggested that this increase in ribosomal RNA allowed for greater translation of messenger RNA which was synthesized prior to stimulation. Cooper (75) has shown that prior to PHA-stimulation there was a marked wastage of 18 S ribosomal RNA. After PHA addition, this wastage was appreciably reduced, however, within 20 minutes ribosomal RNA wastage commenced again and by 40 hours was about the same as non-stimulated cells. He concluded that 2 models were consistent with his data, either (a) a fixed number of ribosomal RNA molecules must survive, or (b) a constant proportion of the ribosomal RNA molecules must survive. The implications of genetic control with either mechanism could not be clearly proposed.

Within a few hours after PHA addition, marked increases in protein synthesis occurred (76). In studies on glycoprotein synthesis, Hayden et al. (77) found that within 3 hours of PHA addition there was a doubling of the amount of labelled glucose into glucosamine and by the fourth day of culture a 10 to 20-fold increase was evident. Eighty-four percent of the labelled glucosamine appeared in the uridine diphosphate-N-acetylglucosamine pool. Incorporation into membrane fractions was found to be inhibited by puromycin. Proteolytic enzymes preferentially released 3 classes of glycopeptides from the plasma membrane fractions. These authors also noted an increased release of radioactivity from the cells without trypsin treatment, suggesting again that intracellular, soluble proteins or perhaps membrane autolysis was involved in early events of PHA-stimulation.

The DNA synthetic phase of the cell cycle commenced at 24 hours

and continued for approximately a 6 hour period in which the cellular DNA was replicated. At the end of this 6 hour period, there was an increase in RNA and protein synthesis (the Gap 2 phase), followed by mitosis which lasted about 2 hours. Subsequently, the human peripheral blood lymphocyte in culture completed 2 to 4 rounds of replication.

The primary or initial event in PHA-stimulation is still unknown, partly because of the heterogeneity of the compound. It has been suggested that an interaction at the cell membrane causing either an agglutination of leukocytes (78), antigen to leukocyte attachement (79), precipitation of an alpha-globulin in the plasma (80), or an alteration of the cell membrane which facilitated entry of another factor was involved (67). It should be noted that although many investigators have examined early changes in PHA-stimulated lymphocytes to ascertain what this "initiator event" might be, this event is still unknown.

PHA was not the only compound to cause the small, circulating lymphocyte to divide in cell culture, although the apparent nonspecific or general nature of the stimulation has been an important feature of its usefulness. Other agents have been found including specific antigens to which the cells have been pre-sensitized (81) and such nonspecific conditions as cultures composed of lymphocytes from two donors (82). Recently inorganic and organic mercury (83), neuraminidase (84) and periodate (85) have been reported to initiate blastogenesis, however, the plant agglutinin PHA has been studied most extensively.

Although PHA had been used since 1916 (86) to hemagglutinate erythrocytes in the preparation of anti-hog cholera serum, and since 1949 to separate leukocytes from erythrocytes (87), its usefulness in stimulating lymphocytes to undergo events culminating in mitosis and cell division

marked the beginning of extensive studies on the biochemistry of both normal and leukemic lymphocytes. PHA, a heterogeneous compound, can be separated into 17 components by acrylamide gel electrophoresis (88). Goldberg et al. (89) have recently shown that the DNA-stimulating activity was due to neither protein, RNA, DNA nor approximately 98% of the carbohydrate constituent. Rigas and Head (90) have isolated a glycoprotein of 138,000 molecular weight which was shown to be composed of subunits with either erythroagglutinating or lymphocyte-simulating activities.

The events following PHA-stimulation of CLL lymphocytes have not been studied as extensively as normal lymphocytes. Many reports in the literature stated that the CLL lymphocyte was a differentiated, fully mature cell without proliferative capabilities (91,92). However, after this study began, it was reported that CLL lymphocytes did respond to a limited extent to PHA, but the response was delayed until days 5 to 7 after PHA addition.

Morphology. The morphological changes occurring after PHA addition have been studied in normal and CLL lymphocytes. The small, non-stimulated lymphocyte contained rather dense chromatin, no nucleolar structures, few free ribosomes and a regular double-nuclear membrane (61). Following stimulation with PHA, the chromatin appeared loosely packed, nucleolar structures were present, many ribosomes appeared in the cytoplasm and the nuclear membrane was irregular with large pores. Using periodic acid-Schiff staining, it was evident that glycogen synthesis commenced 18 hours after PHA addition, reached a peak at 24 hours and remained constant up to 48 hours (93). With the exception of the periodic acid-Schiff work, morphological differences between the normal and CLL lymphocytes during blastogenesis have not been noted (94). Histochemical studies of glycogen

in CLL lymphocytes during cell culture have not been performed.

Glycogen Metabolism

Glycogen Regulation with Isoproterenol

As stated earlier, a temporal relationship was found between the synthesis of glycogen and the onset of DNA synthesis in normal, peripheral blood lymphocytes in cell culture. A similar temporal relationship between glycogen content and DNA synthesis has been found in salivary gland cells of mice (95). In these studies, isoproterenol, a catecholamine, was injected intraperitoneally, and there was a marked increase in the number of salivary gland cells. Also, after isoproterenol injection, there was a slight decrease in glycogen content, followed by a marked increase which reached a maximum shortly before the onset of DNA synthesis. Furthermore, these studies by Malamud and Baserga (95) showed that high doses of isoproterenol resulted in decreased incorporation of tritiated thymidine into DNA, and this decrease was correlated with decreased cellular glycogen. Malamud (96) has shown a three-fold increase in adenyl cyclase two to three minutes after isoproterenol injection, thus suggesting a role for cyclic AMP in the catecholamine-associated activities.

Catecholamines and Cyclic AMP

Cyclic AMP was first observed and reported by Rall and Sutherland (97) to be increased after the addition of epinephrine to liver homogenages. Since the report in 1958, cyclic AMP has been investigated in many tissues and has been found to cause either increases or decreases in many diverse and seemingly unrelated cellular activities. The concept of a two-messenger system has evolved from these studies (98). The first-messenger was a hormone or hormone-like substance which was released from its site of

synthesis or storage and traveled to a "target-cell" where it interacted at the membrane to bring about the adenyl cyclase-mediated conversion of adenosine triphosphate (ATP) to cyclic AMP which then acted as the intracellular or secondary messenger. Increased cyclic AMP has been related to various cellular processes, in response to epinephrine, norepinephrine, isoproterenol, prostaglandins and theophylline additions. Several reports indicated that alterations in enzyme activities brought about major changes in many biochemical processes within the cells, such as glycogenolysis, lipolysis, steroidogenesis and protein synthesis (99).

Cyclic AMP and Glycogen Regulation

In muscle and liver it has been shown that cyclic AMP regulates both the synthesis (100,101) and degradation (102,103) of glycogen. It has been known for many years that separate pathways were involved in the synthesis and degradation of glycogen, and it was recently shown (104) that cyclic AMP interacted with a protein kinase with enzymic specificities for both processes. This activated protein kinase phosphorylated the serine residues of phosphorylase b to convert it to the active form which then removed by phosphorolysis one glucose molecule at a time from the intact glycogen structure (105), until it reached a point approximately seven residues from an α-1,6 linkage, the branch point. At that point, amylo-1,6-glucosidase and transferase acted specifically to transfer the remaining six glucose units and cleave the α-1,6 -bond, thus removing the last glucose residue. These enzymes could completely degrade the glycogen structure to glucose-1-phosphate and glucose. The second function of the protein kinase was to convert the enzyme of glycogen synthesis, glycogen synthetase, to a form which was dependent upon high intracellular amounts

of glucose-6-phosphate for maximal activity. Glycogen synthetase transferred glucose residues from uridine diphosphoglucose forming α-1,4 bonds with the non-reducing ends of the growing glycogen molecule. Thus increased intracellular cyclic AMP could cause both the phosphorolysis of the stored glycogen and decreased synthesis, unless high concentrations of glucose-6-phosphate were present.

Specific Aims

This study was designed to ascertain biochemical differences between normal and CLL lymphocytes. First, however, it was necessary to ask if the CLL lymphocyte could be maintained in cell culture and undergo extensive amounts of DNA synthesis. Second, how could lymphocytes from CLL patients be characterized in terms of maximal DNA synthesis and the progression of this disease? Third, what was the mode of action of the catecholamine isoproterenol in the regulation of the CLL lymphocyte? Fourth, since catecholamines regulate intracellular cyclic AMP in other cells, was this cyclic nucleotide an important regulator in CLL lymphocyte metabolism? Fifth, what were the characteristics of glycogen metabolism in CLL lymphocytes from CLL patients?

At the outset, it was anticipated that the answers to these questions would increase our understanding of the basic alterations in the leukemic process and also broaden our knowledge of the events occurring during cellular proliferation.

CHAPTER II

MATERIALS AND METHODS

Materials

Donors

Peripheral blood was obtained by venipuncture from fasting, female employees of the University of Oklahoma Medical Center, for studies with normal lymphocytes. For studies with CLL lymphocytes, peripheral blood was obtained from CLL patients who were either untreated or not treated within six months prior to the study. Table 1 indicates the white blood cell counts and percent lymphocytes for several typical CLL patients, and the normal ranges for non-leukemic female donors.

Cell Culture

Lymphocytes were separated on Leukopak filters purchased from Fenwal Laboratories, Morton Grove, Ill., or on glass wool purchased from Owens Corning, Corning, N. Y. Sodium heparin was purchased from the Upjohn Company, Kalamazoo, Mich. For isolating lymphocytes, Eagle's Minimum Essential Medium (MEM) was used; and for cell culture, McCoy's Modified 5A Medium without glutamine, antibiotics, and serum was used. At the time of incubation, the medium was supplemented with L-glutamine and either autologous serum or fetal calf serum. To reduce culture contamination by air-borne organisms, a laminar-flow hood purchased from

TABLE 1

CORRELATION OF IN VITRO DATA WITH INCREASING PERIPHERAL LEUKOCYTE COUNTS

Donor	WBC ($10^3/mm^3$)	% Lymphocytes	Maximal DNA Synthesis (Day)	(Extent)
CLL				
1	15.5	92	5	41,500
1	67.2	93	7	56,000
2	19.6	77	5	92,500
3	26.0	88	5	23,500
3	34.4	90	6	47,000
4	46.5	97	6	68,000
5	60.0	92	5	34,000
6	108.5	91	7	37,000
7	152.5	92	7	12,500
Normal Range	7-10	20-30	3-4	37,500 (average)

Pure Aire Corporation, Van Nuys, Calif. was used for all cell transfers and manipulations. Cell cultures were incubated in a National water-jacketed CO_2 incubator purchased from National Appliance Co., Cherry Hill, N. J. All media, media supplements and trypsin solutions (0.25%) were purchased from Grand Island Biological Company, Grand Island, N. Y. Tritiated methyl-thymidine was purchased from New England Nuclear, Boston, Mass. Phytohemagglutinin-P, a protein extract of the red kidney bean, Phaseolus vulgaris, was purchased from Difco Laboratories, Detroit, Mich.; isopropyl norepinephrine (isoproterenol) from K and K Laboratories, Inc., Plainview, N. Y.; cyclic AMP and AMP-5' from Sigma Chemical Co., St. Louis, Mo.; AMP-3' from Calbiochem, Los Angeles, Calif.; N^6-2'-O-dibutyryl cyclic AMP from Boehringer Mannheim Corp., Mannheim, Germany; and theophylline from Eastman Chemicals, Rochester, N. Y.

Histochemistry

Electron microscopy was carried out by Dr. Robert E. Nordquist with an Hitachi HU-11B electron microscope distributed by Perkin Elmer Corp., Norwalk, Conn. The embedded material was sectioned with a Porter Blum Ultramicrotome from Ivan Sorvall, Inc., Norwalk, Conn. The cells were embedded in Cargille's Epoxy Resin (Araldite 6005) purchased from Cargille Laboratories, Cedar Grove, N. J.

Light microscopy was performed with a microscope purchased from Carl Zeiss, Inc., New York, N. Y.

Glycogen Determinations

Phosphorylase a, imidazole buffer (grade III), B-D-glucose, O-dianisidine-HCl and glycogen (types III and VI) were purchased from

Sigma Chemical Co., St. Louis, Mo.; glucose-1-phosphate, glucose-6-phosphate and phosphoglucomutase from Calbiochem, Los Angeles, Calif.; glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP) and hexokinase from Boehringer Mannheim, Corp., Mannheim, Germany; ATP from Schwarz Bioresearch, Inc., Orangebury, N. Y.; glucose oxidase (from Aspergillus niger) and peroxidase (horseradish) from Mann Research Laboratories, New York, N. Y.; phenol from Analar, The British Drug Houses, Ltd., Poole, England; and optical grade cesium chloride from Harshaw Chemical Co., Solon, Ohio.

Other Experimentation

All common chemical reagents were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J. Quantitative studies on DNA, glycogen and glucose and all enzymic assays were performed with a Gilford Spectrophotometer, Gilford Instrument Laboratories, Inc., Oberlin, Ohio. Refractive indices were read on an Abbe-3L-refractometer, purchased from Bausch and Lomb, Inc., Rochester, N. Y. Ultracentrifugation was performed with a Model L preparative ultracentrifuge purchased from Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.

Methods

Cell Culture

Isolation and culture procedures. Human peripheral blood lymphocytes from both normal donors and patients with chronic lymphocytic leukemia (CLL) were readily obtained and easily separated from whole, venous blood by the procedure of Abell et al. (106), a modified procedure of Cooper and Rubin (107). One unit of heparinized (20,000 units) blood was

obtained by venipuncture from normal donors, aseptically dispensed into sterile 50 milliliter (ml) centrifuge tubes with lids and allowed to incubate for one hour at 37°. The plasma supernatant which formed by gravity sedimentation of the erythrocytes was aseptically withdrawn and mixed with an equal volume of Eagle's Minimum Essential Medium (MEM), pre-warmed to 37°. Since 20 to 30% of the leukocytes are lymphocytes, it was deemed necessary to separate the lymphocytes from the polymorphonuclear cells. This was feasible because of the selective binding of polymorphonuclear cells to glass wool or Leukopak fibers (108).

Columns packed with one of these materials were prepared in advance by washing the fiber with distilled water to remove toxic materials and sterilizing them at 120° for 20 minutes. These sterile, fiber-packed columns were used to separate leukocytes from only one unit of blood. The plasma supernatant and MEM mixture were percolated through the fiber column at 37°. Wright stain smears of the effluent cell population indicated 97 to 99% lymphocytes. These lymphocytes in the effluent medium were pelleted by centrifugation for 30 minutes at 280 x g.

Only 50 ml of venous blood were required from patients with CLL because of the increased peripheral leukocyte count and the increased lymphocyte component in the leukocyte population. Separation of lymphocytes from polymorphonuclear ceels on fiber columns was unnecessary due to the predominance of lymphocytes (85 to 99%). Immediately after venipuncture, therefore, the blood was placed in a sterile, heparinized (2,000 units) centrifuge tube and allowed to incubate for one hour at 37°. The plasma supernatant was then removed aseptically, mixed with the pre-warmed MEM and centrifuged, as above, to pellet the lymphocytes.

The lymphocytes resolved from the peripheral blood of both normal donors and CLL patients were counted with a standard hemacytometer after removal of contaminating erythrocytes by treatment with 1% acetic acid for one minute. The lymphocytes were appropriately diluted to a concentration of 10^7 cells per 5 mls McCoy's 5A Medium which was supplemented to 2 mM L-glutamine and 10% serum. For normal lymphocyte cultures autologous serum was used and was obtained by centrifuging the erythrocyte-plasma component which remained in the tubes after removal of the leukocyte-rich plasma supernatant. The serum was aseptically withdrawn after centrifugation for 30 minutes at 280 x g. For CLL lymphocyte cultures, fetal calf serum was added. In control experiments, it was shown that fetal calf serum or autologous serum could be used without altering either the pattern or the extent of DNA synthesis, and therefore the problem of obtaining sufficient quantities of autologous serum from such a small amount of blood from CLL patients was circumvented.

The cells and media were placed in sterile, two-ounce prescription bottles and incubated at 37° in a humid atmosphere of 5% CO_2 in balanced air.

Experimentation.

Design. The addition of 40 ug of PHA to each culture bottle converted the cells from the resting to dividing state and was considered zero time for each experiment. As shown in Figure 1, all pretreatments and posttreatments of the cells were relative to this point. Figure 1 is a schematic representation of the temporal relationships of the manipulations occurring during cell culture.

Medium change. When it it was desirable to add a drug for a

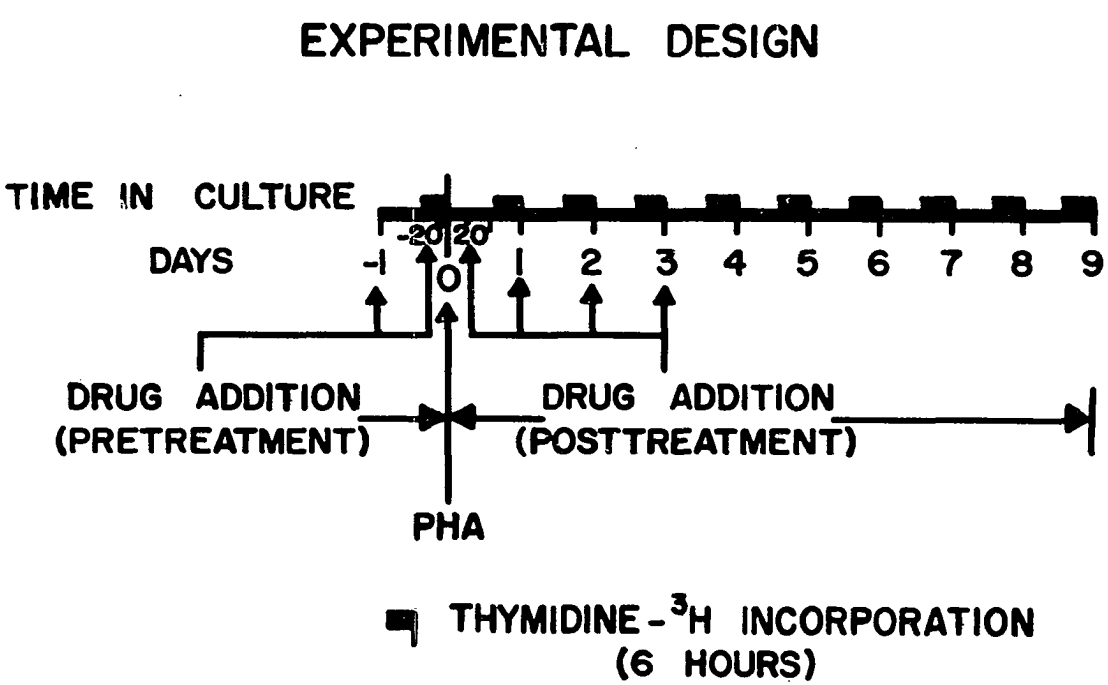


Figure 1 - A Schematic Outline of Culture Events

limited period of time and remove it without significantly altering the cells, the following procedure was used. The cells were removed from the culture bottles by carefully pouring cells and media into sterile centrifuge tubes at the end of the drug incubation and centrifuging at 37^{o} for 10 minutes at 280 x g. The medium was decanted, fresh medium was readded quickly, and the cells were pipetted back into the warm prescription bottles. At this point another drug or PHA could be added, and the cells were replaced in the incubator. Control experiments were performed with each experiment to assess the effects of non-specific alterations on subsequent DNA synthesis, after such manipulations.

Assessment of DNA synthesis. DNA synthesis was estimated by adding tritiated methyl-thymidine (2.0 C/mm, 10 uC/10^{7} cells) to the cultures for a 6 hour period. Radioactive determinations were performed with liquid scintillation spectrometer methods, and calculations gave specific activities, as labelled precursor incoporated per microgram DNA. This measurement, therefore, reflected the proliferative or DNA synthetic phase of the cell cycle following stimulation. Data within the same experiment could also be used to evaluate quantitatively differences due to manipulations, such as drug additions or other medium alterations.

Isolation and measurement of DNA. At the termination of the six hour period of tritiated methyl-thymidine incorporation, the cells and media were poured into chilled centrifuge tubes. The CLL lymphocytes, in contrast to normal lymphocytes, adhered somewhat to the glass bottles after PHA addition and, therefore, had to be removed with a trypsin solution (1 ml) which was incubated at 37^{o} for 5 minutes. The

chilled cells and media were centrifuged for 10 minutes at 4^{o} at 1,000 x g, and the supernatant was decanted. To the pellet homogenizing solution (0.4 mM potassium phosphate, pH 6.7 and 2 mM magnesium chloride) (1 ml) was added, the pellet was resuspended and fast-frozen. Subsequent procedures to remove unwanted cellular constituents and unincorporated radioactive material were carried out at 4^{o}.

Briefly, the procedure was as follows: the cells were freeze-thawed 7 times in an acetone-dry ice solution and 1.6 N perchloric acid (PCA) and 2.0 mg carrier-RNA were added. The cells were then chilled for 30 minutes. The standard centrifugation between each addition of solvent was 10 minutes at 1000 x g, after which the supernatant was decanted, the next solution added, and the resuspended pellets were dispersed with glass stirring rods. The sequence of solution additions in 3 ml aliquots were 0.5 N PCA (3 times), 95% ethanol, chloroform:ethanol: ether (2:2:1) and acetone. The pellet obtained after the acetone treatment was dryed at 57^{o} for 30 minutes. Five-tenths ml of 0.5 N PCA was added, and the pellet was hydrolyzed at 90^{o} for 30 minutes.

After hydrolysis, 0.2 ml of the hydrolysate was assayed for total DNA content with the diphenylamine reagent according to the method of Burton (109), and another 0.2 ml was added to 15 ml of Bray's solution (110), for the counting of radioactivity. DNA content was based upon a standard calf thymus preparation. Precursor incorporation was determined by calculating the disintegrations per minute (DPMs) of each sample. DPMs were calculated by dividing the counts per minute (CPMs) by the efficiency of the counting system. This efficiency was determined by adding a known amount of tritiated water to several of the samples, after

the initial counting, thus obtaining the number of additional CPMs and determining the percent of the additional radioactivity recovered. All data shown represent the average of duplicate cultures, and each experiment was performed at least twice.

Study of Glycogen

Histochemistry.

Light Microscopy. Normal and CLL lymphocytes were examined using several histochemical techniques to identify cellular glycogen. The cells were removed from cell culture medium by centrifugation, and the pelleted material was placed on clean microscope slides to dry. The cells were then stained with periodic acid-Schiff (PAS) reagent and with a hemotoxin counterstain (111). Briefly, the method was as follows: the dried cells were fixed with methanol, placed in 0.5% periodic acid for 10 to 15 minutes, washed with distilled water, placed in the Schiff reagent until color developed, and finally rinsed with tap water. The cells were then counterstained by placing the slides in hemotoxin solution for 1 to 2 minutes and removing the excess stain with sequential rinses in tap water, acid alcohol (1:100, concentrated sulfuric acid: 70% ethanol), tap water, lithium carbonate solution, tap water, graded ethanol solutions and finally in xylene.

Diastase, an α, β -amylase preparation with specificity for α-1,4 glucosidic bonds, was used in control studies to show the presence of cellular glycogen, the mammalian polysaccharide. Glycogen was digested during a 2 hour pretreatment with 0.1% diastase solution in 0.02 M phosphate buffer (pH 6.0) suspended in 0.8% sodium chloride at 37°. Following this incubation, the cells were stained with PAS and hemotoxin.

Electron microscopy. Two methods were used to stain for glycogen at the electron microscopic level of resolution. The first method utilized uranyl acetate and lead citrate (112) in the following procedure. The lymphocytes were removed from the cell culture medium by centrifugation at 1000 x g for 10 minutes and placed in 2% glutaraldehyde in cacodylate buffer (0.1% sodium cacodylate, pH 7.2) for 2 hours at 4^{o}, centrifuged at 1000 x g for 10 minutes and placed in cacodylate buffer for 1 hour. The cells were centrifuged at 1000 x g for 10 minutes and placed in neutral buffer-formalin (10% formaldehyde in 0.03 M phosphate buffer) for 30 minutes, followed by treatment with a series of graded ethanol solutions (25, 50, 75 and 95%) for 15 minutes each, and twice in 100% ethanol solution for 30 minutes. At room temperature, the cells were placed sequentially in propylene oxide, then araldite and propylene oxide solutions (25:75, 50:50 and 75:25) for 15 minutes, and then were left in 100% araldite overnight at 4^{o}. The cells were embedded the next day at 37^{o} for 16 to 18 hours with fresh araldite (100%), cut, and placed on the grids. After fixing, the cells were stained with saturated uranyl acetate (in distilled water) for 15 to 60 minutes and then in Reynold's lead citrate for 30 to 60 minutes.

The second method used for staining cellular glycogen was the periodic acid-silver methenamine (PAS-M) method (113), which was as follows: the cells were fixed in araldite, as described above, treated 12 minutes with 2% hydrogen peroxide, rinsed with distilled water, placed in 1% periodic acid for 10 minutes, washed in distilled water, and then placed in silver methenamine (0.6% hexomethylenemine, 0.2% silver nitrate, 2% sodium tetraborax in distilled water) for 15 minutes at 60^{o}. The

cells were washed 2 minutes in tap water, 1 minute in 3% sodium thiosulfate and 5 minutes in tap water.

In both staining procedures, diastase control slides were carried along to determine the digestibility of the stained particles.

A rapid method for examining glycogen particles isolated during the cesium chloride centrifugation utilized a negative stain. One drop of sample and one drop of phosphotungstic acid were placed together on a collodion grid and allowed to dry. When the material was dry, the grids were examined with the electron microscope.

Isolation. It was thought that the most suitable method for the isolation of glycogen from CLL lymphocytes was the cold water technique of Bueding and Orrell (114). In this procedure, all steps were performed at 4^{o}. The pelleted cells were homogenized with a tight-fitting glass homogenizer in 4 volumes of glycine buffer (0.2 M, pH 10.5) and 2 volumes of water-saturated chloroform. The homogenate was centrifuged at 200 x g for 5 minutes, the aqueous phase carefully removed and stored at -20^{o}. The lower phase and the interphase were rehomogenized with 2 volumes of glycine buffer, centrifuged, as previously described, and the aspirated aqueous phase was pooled with the frozen material. This re-extraction procedure was repeated 4 times, and after the 5th re-extraction the material was thawed and centrifuged at 200 x g for 5 minutes. The resultant supernatant was centrifuged for 16 hours at 70,000 x g in a Model L ultracentrifuge. The gelatinous pellet which was obtained was homogenized in glycine buffer, centrifuged at 200 x g for 5 minutes, and enough water was added to the supernatant to adjust the final concentration of glycogen to approximately 0.5%. The solution

was then shaken three to five minutes with one-third its volume of chloroform and 1-octanol (3:1). Following centrifugation for 5 minutes at 200 x g, the aqueous phase was removed with the interphase, and the procedure was repeated with successively longer organic solvent treatments (for 1, 2, 4, and 9 hours) or until no protein interphase was evident.

Glycogen density. After isolation of glycogen from CLL lymphocytes by the cold water technique of Bueding and Orrell (114), the material was placed over cesium chloride (saturated at 0°), then centrifuged for 3 hours at 54,000 x g in a number 40 fixed-angle rotor (115). Fractions were collected from the bottom of the tube by displacing 0.5 ml of the gradient with 0.5 ml of light paraffin oil which was added at the top of the tube. The density of the cesium chloride gradient formed was determined by reading the refractive index at 25° on a refractometer, and converting the number obtained to density with a standard table (116). The phenol-sulfuric acid method was used to determine the amount of carbohydrate present, and an aliquot was stained with phosphotungstic acid and examined with the electron microscope.

Measurement. Three different methods were selected for use in determining the amount of glycogen present in the cytoplasm of normal and CLL lymphocytes. After isolation and characterization in cesium chloride density gradients, the high salt concentrations present necessitated the use of a non-enzymatic method. The phenol-sulfuric acid method was chosen due to its convenience and rapidity. The glucose oxidase method was useful in determining the amount of D-glucose present in cytoplasmic, acid-hydrolyzed homogenates, whereas glycogen quantitation performed without hydrolysis of whole cell homogenates was possible

with the phosphorylase a system due to its increased specificity and sensitivity.

Non-enzymic method. The phenol-sulfuric acid method (117) was rapid and was used to determine hexoses, di-, oligo-, and polysaccharides, including the methylated derivatives which possessed a free or potentially free reducing group. This system was advantageous when sugars could not be separated easily from proteins or salts. However, its major disadvantage was its lack of specificity which was especially detrimental when nucleic acids were present. The reagent used was phenol (80%) in distilled water. The procedure was as follows: to the phenol reagent (0.05 ml) an aqueous solution (2 mls) containing 10 to 70 ug of the sugar was added; the tube was shaken, and concentrated sulfuric acid (5 mls) was added immediately. After 30 minutes incubation at room temperature, the optical density was determined at 490 nanometers (nm) and compared with a glucose standard.

Glucose oxidase method. Glycogen (α-1,4,α-1,6-linked glucose) when hydrolyzed yields only glucose residues. Barton (118) described a method for measuring D-glucose by utilizing glucose oxidase (E.C. 1.1.3.4), horseradish peroxidase (E.C. 1.11.1.7) and O-dianisidine (DH_2), as a hydrogen donor. The reaction was as follows:

$$\begin{array}{l} \text{D-glucose} \\ + \\ \text{glucose oxidase-FAD} \end{array} \longrightarrow \begin{array}{l} \text{gluconic acid} \\ + \\ \text{glucose oxidase-FADH}_2 \end{array}$$

$$\text{glucose oxidase-FADH}_2 + O_2 \longrightarrow \text{glucose oxidase-FAD} + H_2O_2$$

$$H_2O_2 + DH_2 \longrightarrow 2H_2O + D$$

Glucose oxidase had major specificity for β-D-glucose with only minimal activity toward 2-deoxy-D-glucose, mannose, xylose and galactose and therefore provided a rather sensitive method for the measurement of

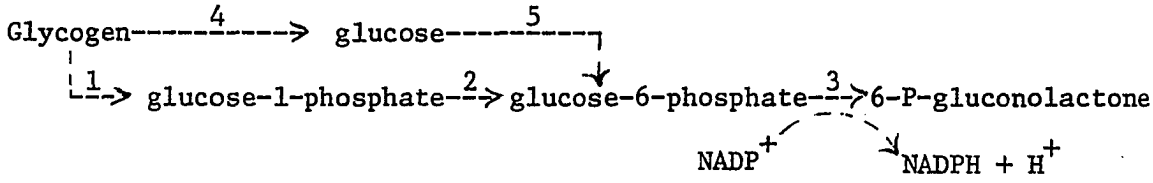
glucose in the presence of the common cellular sugars. Glucose oxidase, isolated from Aspergillus niger, contained two FAD molecules per molecule of enzyme and catalyzed the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide when the system was well aerated (119). It had been reported (120) that hydrogen peroxide inactivated reduced glucose oxidase at acidic pH by oxidizing the methionine residues to methionine sulfoxide. Horseradish peroxidase circumvented this inactivation of glucose oxidase by converting the hydrogen peroxide to water, and O-dianisidine functioned as a hydrogen donor in the reaction.

A modified method of Black and Anglin (121) was adopted because of the limited number of lymphocytes available, and the greater requirement for sensitivity. The major changes included incubation at pH 7.1, to decrease maltase activity, and extension of incubation time to 60 minutes. The reaction mixture contained horseradish peroxidase (0.135 mg), O-dianisidine (0.135 mg) and glucose oxidase (0.135 mg) in phosphate buffer (0.5 M, pH 7.1). The sample (0.2 ml) and reaction mixture (2.7 mls) were incubated at 37° for 60 minutes. The reaction was stopped with hydrochloric acid (1.3 mls, 2 N), and the optical density determined at 450 nm.

Cells were prepared as follows: lymphocytes were removed from the medium by centrifugation at 1,000 x g for 5 minutes at 4°. Because the medium contained three grams of glucose per liter, the cells were rinsed three times in an isotonic solution, pelleted by centrifugation, as described above, and the supernatants were assayed for glucose and for any cellular glycogen which might have been leached from the cells during the rinses. The cells were then homogenized and hydrolyzed at 100° for 30 minutes in hydrochloric acid (2.5 N), centrifuged at 1,000 x g for 5 minutes

at 4°, and the supernatant pH neutralized with sodium hydroxide.

Phosphorylase and hexokinase method. Passonneau et al. (122) described an enzymatic method which was useful in measuring as little as 0.5 microgram of glycogen spectrophotometrically in the presence of all cellular structures. The specificity of phosphorylase a (E.C. 2.4.1.1), phosphoglucomutase (E.C. 2.7.5.1) and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) provided a system to measure the breakdown of glycogen to glucose-1-phosphate. The product of the amylo-1,6-glucosidase cleavage at the 1,6 branch points, glucose, was measured in a second system with hexokinase (E.C. 2.7.1.1). A schematic representation of the entire reaction is shown below:



Enzymes: 1. phosphorylase a; 2. phosphoglucomutase; 3. glucose-6-phosphate dehydrogenase; 4. amylo-1,6-glucosidase; and 5. hexokinase.

The incubation mixture consisted of imidazole buffer (0.05 M, pH 7.0) containing magnesium acetate (0.5 mM), $NADP^+$ (1 mM), AMP-5' (0.1 mM), K_2HPO_4 (5 mM), (ethylenedinitrilo)tetraacetic acid (1 mM), bovine plasma albumin (0.02%), activated phosphorylase a (10 ug), phosphoglucomutase (10 ug) and glucose-6-phosphate dehydrogenase (10 ug). The second system consisted of ATP (1 mM), magnesium chloride (1 mM) and hexokinase (10 ug) which was added to the phosphorylase system, after the complete degradation of glycogen to glucose-1-phosphate and glucose. The glycogen which was degraded to glucose in the second system was measured and found to be 9%

of the total glucose present in the standard glycogen preparation.

Control experiments indicated that the commercially prepared glycogen contained no phosphorylase a, glucose-6-phosphate dehydrogenase, phosphoglucomutase or nonspecific glucosidase activity and was free of contaminating glucose.

CHAPTER III

RESULTS

Cell Culture

The first studies were designed to ascertain the culture conditions necessary for maximal cellular proliferation to occur. In this experimental system, DNA synthesis was assessed and was reflective of the stability and proliferative capabilities of the lymphocytes in culture. The schematic representation of culture events is shown in Figure 1. In Figure 2, the mitogen PHA was added to the normal lymphocyte cultures to determine the concentration required for maximal DNA synthetic activity. An aqueous solution of PHA was added to the culture bottles at time zero at concentrations from 3 to 600 ug per culture bottle, and DNA synthesis and DNA content were determined at day 3. The non-stimulated control cultures, without PHA, contained 15 ug DNA per culture at this time compared with the PHA-stimulated cultures which contained 28 to 32 ug DNA per culture when 30 to 60 ug PHA were added. This two-fold increase in DNA content, indicative of a doubling of cells, along with the occurrence of maximal DNA synthetic activity indicated that 30 to 60 ug of PHA was optimal for these cultures. Other laboratories had reported that PHA was cytotoxic at higher concentrations (123), and our results confirmed those findings.

Using this information and other data (106) concerning the

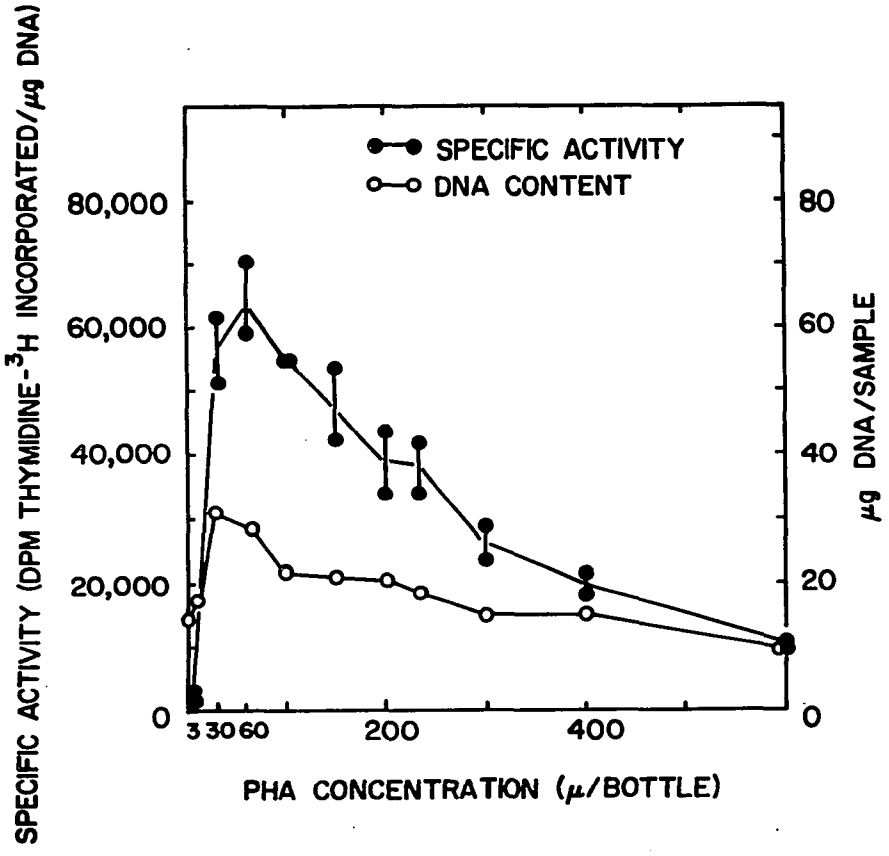


Figure 2 - PHA Concentration Range for Maximal DNA Synthesis in Normal Lymphocyte Cultures.

optimal concentration of cells, a typical PHA-response curve, as shown in Figure 3, was obtained. In this study, normal lymphocytes were cultured in the presence of 40 ug of PHA, and DNA synthesis was found to begin between days 1 and 2 with maximal synthesis occurring at day 3 or 4. In most experiments, however, by day 6 DNA synthesis was only approximately 10% of the maximal amount observed at day 3.

Earlier reports indicated that CLL lymphocytes were immunologically defective and when placed in cell culture were either unable to divide (124) or did so to a limited extent and at a delayed time (125,126). This investigation indicated, however, that CLL lymphocytes did synthesize DNA to an extent comparable to normal lymphocytes, and the time of the maximal response was delayed. Maximal DNA synthesis occurred 5 to 7 days after PHA addition which was 1 to 4 days later than the maximal response of normal lymphocytes. It was found in these studies that patients could be arbitrarily grouped according to the progression of the disease and the day of maximal DNA synthesis. As shown in Figure 4, maximal DNA synthesis in a patient with a relatively low peripheral leukocyte count or white blood cell count (WBC) of less than 30,000/ mm^3 occurred at day 5. In contrast, a patient with a WBC count of 30,000 to 100,000/ mm^3 exhibited maximal DNA synthesis at day 6, as shown in Figure 5, and a patient with a WBC count of greater than 100,000/ mm^3 demonstrated maximal DNA synthesis at day 7, as shown in Figure 6.

The correlation between the WBC count and the day of maximal DNA synthesis has been found in almost all of the more than 30 CLL patients which have been studied in our laboratory (106). Two patients were followed during the course of the disease, and the increasing WBC counts were also

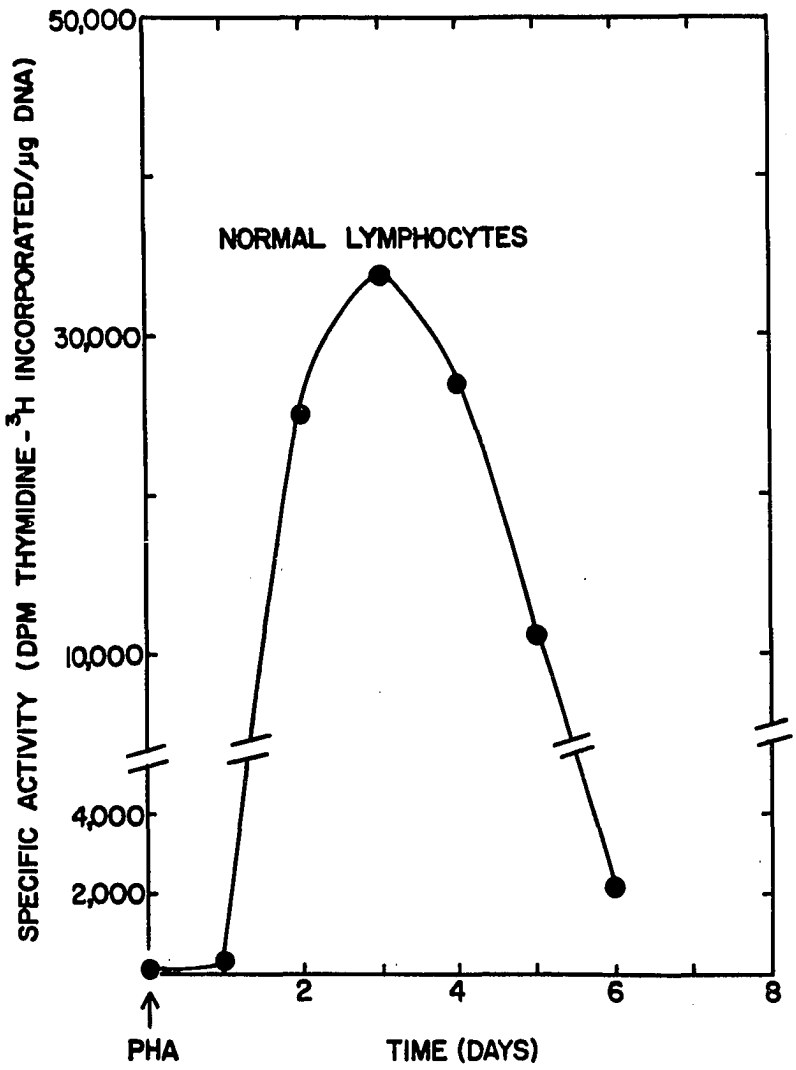


Figure 3 - DNA Synthesis Estimated in a Normal Lymphocyte Culture following PHA Stimulation

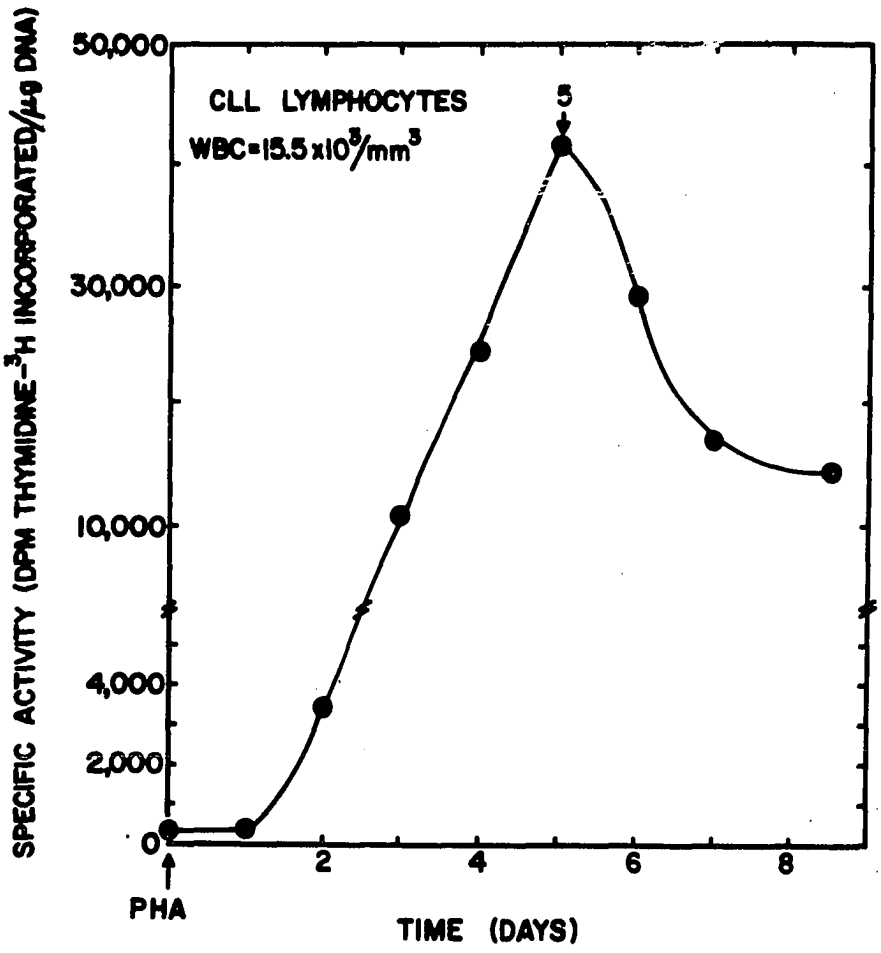


Figure 4 - DNA Synthesis Estimated following PHA Stimulation of Lymphocytes from a CLL Patient with a Low Peripheral Leukocyte Count

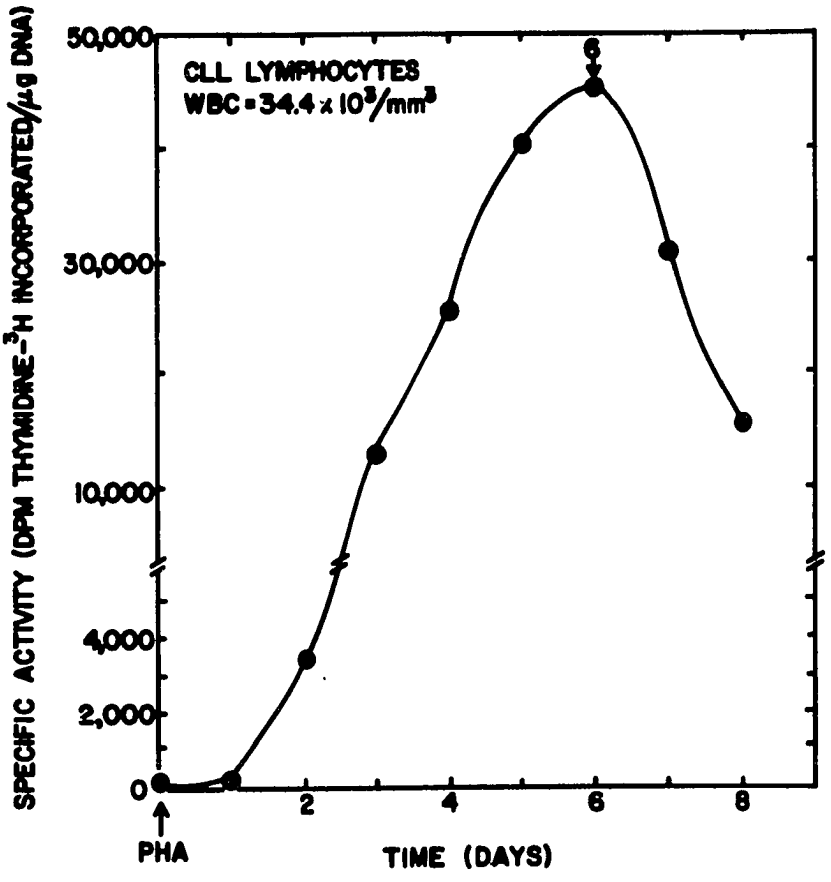


Figure 5 - DNA Synthesis Estimated following PHA Stimulation of Lymphocytes from a CLL Patient with a Medium Peripheral Leukocyte Count

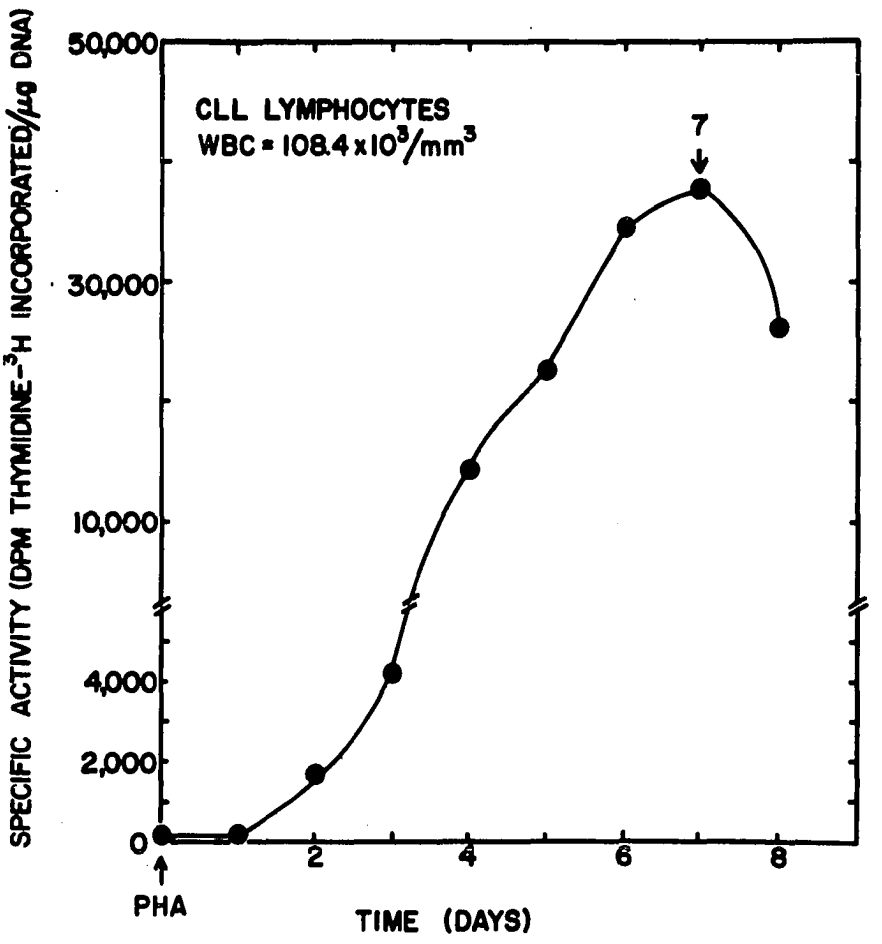


Figure 6 - DNA Synthesis Estimated following PHA Stimulation of Lymphocytes from a CLL Patient with a High Peripheral Leukocyte Count

correlated with a subsequent delay in the time of maximal DNA synthesis. Table 1 indicates the WBC count, the percent lymphocytes, the day of maximal DNA synthesis and the extent of DNA synthesis for each of the seven typical CLL patients listed. Also, in these studies none of the CLL lymphocyte cultures was unresponsive to PHA additions.

The PHA requirements for cultured normal and CLL lymphocytes are shown in Tables 2 and 3. In the experiments described in Table 2, the mitogen PHA was added to the lymphocyte cultures at time zero, and after 24 hours the medium was changed. In the control cultures, PHA was immediately readded (40 ug/culture), and the specific activities thus obtained were expressed at 100% activity. In other cultures, PHA was either not readded or readded at days 2, 3 or 4 for the CLL cultures and at day 2 for the normal lymphocyte cultures. The results indicated that if PHA was not readded 46 to 51% of the control activity was found in either the CLL or normal cultures at day 3. In contrast, at days 5 to 7 in the CLL lymphocyte cultures only 12 to 15% activity was observed, and in normal lymphocyte cultures 37 to 64% of the control values were obtained at days 4 and 5, respectively. The readdition of PHA to CLL cultures was followed by a marked increase in DNA synthesis, while the readdition of PHA to normal cultures had only slight effects when compared with control values.

In the studies with CLL lymphocytes, it was apparent that the initiation of proliferative events began between 48 and 72 hours after PHA addition, rather than within a few minutes of mitogen addition, as was evident in normal cultures, and CLL lymphocytes required greater amounts of PHA than normals. A second feature of the CLL lymphocyte culture was that when PHA was readded at either day 2 or 3, maximal DNA synthesis was delayed until day 6, and this synthesis was increased 2.6 to 3.2-fold.

TABLE 2

PHA REQUIREMENT OF CLL LYMPHOCYTES

Conditions	Day in Culture 3	5	6	7
Control: Media change at day 1 Readd PHA immediately (% of control)[a]	100	100	100	100
Experimental: (% of control)				
No PHA readded	51	12	15	13
Readd PHA at day 2	100	155	263	181
Readd PHA at day 3		46	321	197
Readd PHA at day 4		19	136	207

PHA REQUIREMENT OF NORMAL LYMPHOCYTES

Conditions	Day in Culture 3	4	5
Control: Media change at day 1 Readd PHA immediately (% of control)[b]	100	100	100
Experimental: (% of control)			
No PHA readded	46	37	64
Readd PHA at day 2	44	43	151

Control Specific Activities (DPMs/ ug DNA)

[a]CLL lymphocytes: day 3 (4,615), day 5 (48,807), day 6 (41,199) day 7 (37,744).

[b]Normal lymphocytes: day 3 (30,842), day 4 (15,099), day 5 (2,787).

TABLE 3

PHA REQUIREMENT OF CLL LYMPHOCYTES

Conditions:	Day in Culture: 5	6	7
Control: Media change at day 3 Readd PHA immediately (% of control)[a]	100	100	100
Experimental: (% of control)			
No PHA readded	71	65	53
Readd PHA at day 5		107	115

[a]Control Specific Activities (DPMs/ ug DNA) CLL lymphocytes: day 5 (43,559), day 6 (61,385), day 7 (45,883).

Although there was a 1.5-fold stimulation of DNA synthesis at day 5 in the normal lymphocyte cultures, the day of maximal synthesis was still day 3.

Table 3 shows the PHA requirements of CLL lymphocyte cultures following medium change at day 3, with and without the readdition of PHA to the cultures. When PHA was not readded, it was evident that the DNA synthesis observed at days 5 to 7 differed significantly from the synthesis observed after media change at day 1, without PHA readdition. Therefore, 50 to 70% of the potential DNA synthetic activity had already been initiated, and readdition of PHA at day 5 increased this to the control values. This strongly suggested that PHA was required in the medium at specific times in order to initiate events culminating in DNA synthesis and cellular division.

Table 4 shows the changes in maximal DNA synthesis noted when CLL lymphocytes were preincubated 24, 48 and 72 hours before PHA was added to the medium. Preincubation for 24 or 48 hours did not significantly alter the amount of DNA synthesis noted at days 1 through 5 in cell culture, with a 48 hour incubation, however, synthesis was slightly increased at day 7. In contrast, when the cells were preincubated for 72 hours, there was a 2-fold increase in DNA synthesis at day 5, although the time of maximal synthesis was not altered.

Marked alterations in both the extent and time of maximal DNA synthesis were observed when the CLL lymphocytes were pretreated for 24 hours with the catecholamine isoproterenol (2×10^{-5} M) before the addition of PHA, as shown in Figure 7. In this experiment there was a marked reduction in maximal DNA synthesis which occurred at day 4, when DNA synthesis was maximal in some normal lymphocyte cultures. When

TABLE 4

EFFECT OF PREINCUBATION ON SUBSEQUENT DNA SYNTHESIS IN CLL LYMPHOCYTE CULTURES

Conditions:	Day in Culture: 3	5
Control:		
No preincubation (% of control)[a]	100	100
Experimental: (% of control)		
24 hour preincubation	132	100
48 hour preincubation		100
72 hour preincubation	41	204

Control Specific Activities (DPMs/ ug DNA)
[a]CLL lymphocytes: day 3 (6,200), day 5 (18,800).

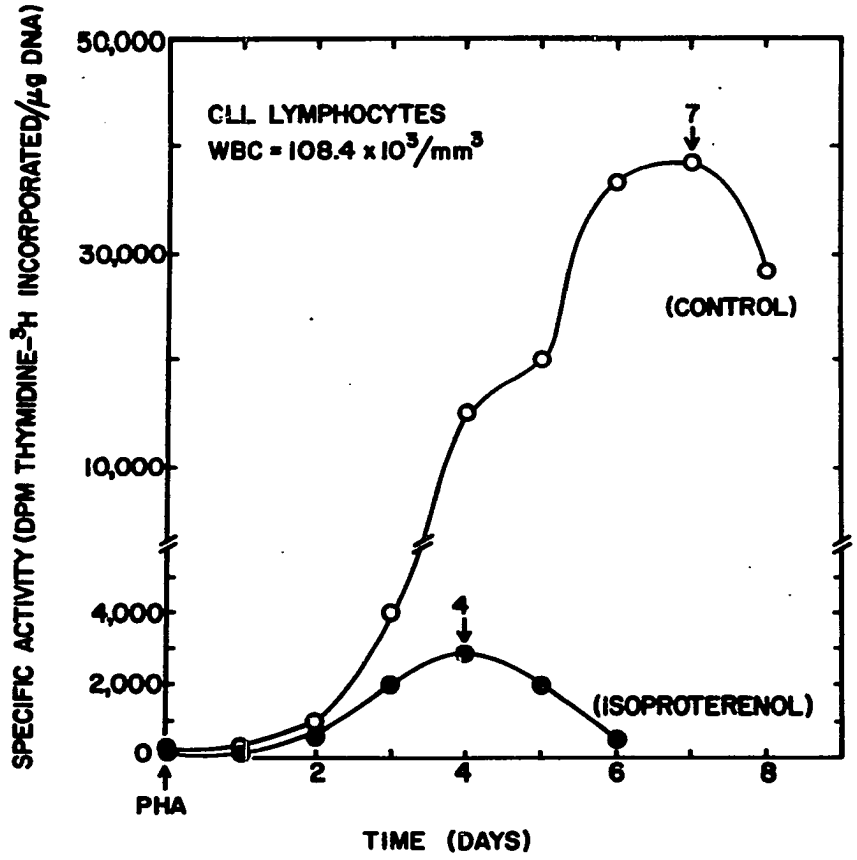


Figure 7 - DNA Synthesis Occurring in a CLL Lymphocyte Culture after 24 Hour Preincubation with and without Isoproterenol

normal lymphocytes were treated with the same concentration of isoproterenol, no inhibition of DNA synthesis was noted (106).

Figure 8 shows a CLL lymphocyte culture in which isoproterenol (1.6×10^{-4} M) was added either before or after the addition of PHA. Curve A represents the PHA-stimulated control cultures, without drug addition, with maximal synthesis observed at day 5. Curve B shows the results of 24 hour pretreatment with isoproterenol, followed by the addition of PHA at time zero. Maximal DNA synthesis was still seen at day 5, but synthesis was inhibited 70%. Likewise, extensive inhibition was obtained when isoproterenol was added after PHA (curves C, D, E and F). Isoproterenol addition 20 minutes after PHA (curve C) effectively inhibited any further DNA synthesis (99%), whereas isoproterenol addition at day 1 (curve D) had little effect on the synthesis observed at day 2, but subsequent synthesis was completely inhibited. When isoproterenol was added at day 2, (curve E), the DNA synthesis at day 3 was similar to the day 2 control. At later days, however, a decrease in synthesis was noted. The addition of isoproterenol at day 3 (curve F) was followed by an increase in synthesis at day 4, and a decrease was again found at later times, and this decrease observed was parallel with the decreased DNA synthesis of control cultures.

Figure 9 shows the pattern of inhibition of isoproterenol in CLL cultures over a 100-fold concentration range. In these studies, maximal DNA synthesis was observed 7 to 8 days after PHA addition to the cultures at time zero. Isoproterenol was added to the cultures 20 minutes after PHA at 10^{-5} to 10^{-3} M concentrations. Curve A reflects the DNA synthesis observed with 10^{-5} M isoproterenol, curve B reflects 10^{-4} M

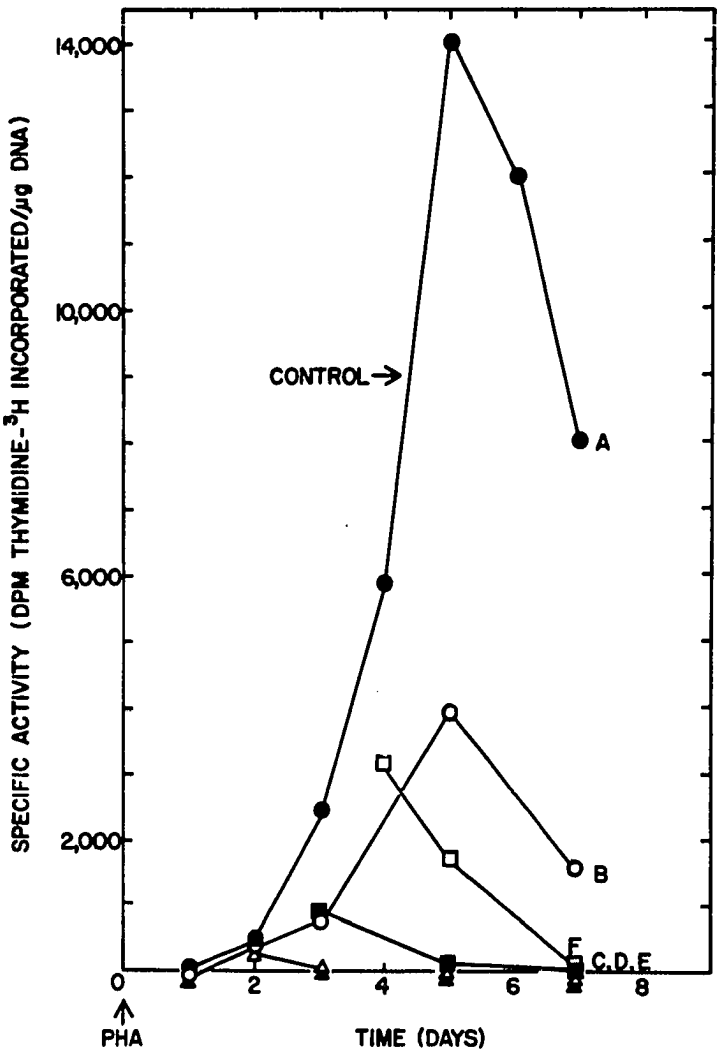


Figure 8 - Isoproterenol Treatment of CLL Lymphocytes before and after PHA Stimulation and Assessement of Subsequent DNA Synthesis

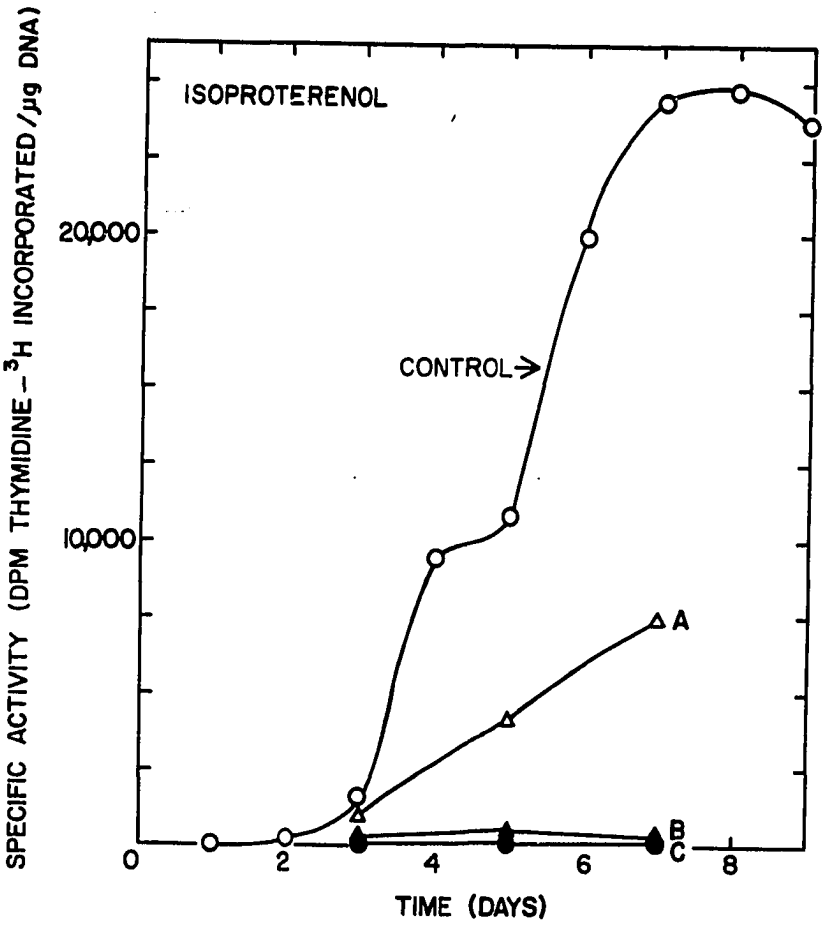


Figure 9 - DNA Synthesis in CLL Lymphocyte Cultures with and without Isoproterenol Additions at 10^{-5} to 10^{-3} M Concentrations after PHA Stimulation

isoproterenol and curve C 10^{-3} M isoproterenol additions. At culture day 7, DNA synthesis was inhibited 70, 99 and 100% at 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively.

Since there were many reports in the literature (127,128) which indicated that catecholamines in general and isoproterenol in particular inhibited various cellular processes by effecting intracellular increases in cyclic AMP, it was important to ascertain the effects of this cyclic nucleotide on DNA synthesis in PHA-stimulated lymphocytes. Figure 10 shows the results of cyclic AMP additions of 10^{-5} M to 10^{-3} M, curves A to C, to CLL lymphocytes at 20 minutes after PHA addition. Although the observed inhibition of DNA synthesis was not as great as that observed with isoproterenol, the effects were dose-dependent at day 7 with 12, 48 and 72% inhibition at 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. This was not unexpected, due to the reports in the literature (128) which suggested that the dibutyryl derivative of cyclic AMP was more effective in bringing about cellular alterations than cyclic AMP.

The results obtained following dibutyryl cyclic AMP addition are shown in Figure 11. As stated previously, the additions were made at 20 minutes after PHA at 10^{-5} M to 10^{-3} M, curves A to C, and at day 7, the inhibition observed was dose-dependent and found to be 65, 91 and 99% for 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively.

Figure 12 illustrates that dibutyryl cyclic AMP and isoproterenol produced essentially the same inhibition of DNA synthesis, expressed as percent inhibition, over the entire 100-fold concentration range, 10^{-5} to 10^{-3} M, therefore, strongly suggesting that isoproterenol and dibutyryl cyclic AMP were producing similar changes within the CLL lymphocytes.

In other experiments, the noncyclic adenosine monophosphates were

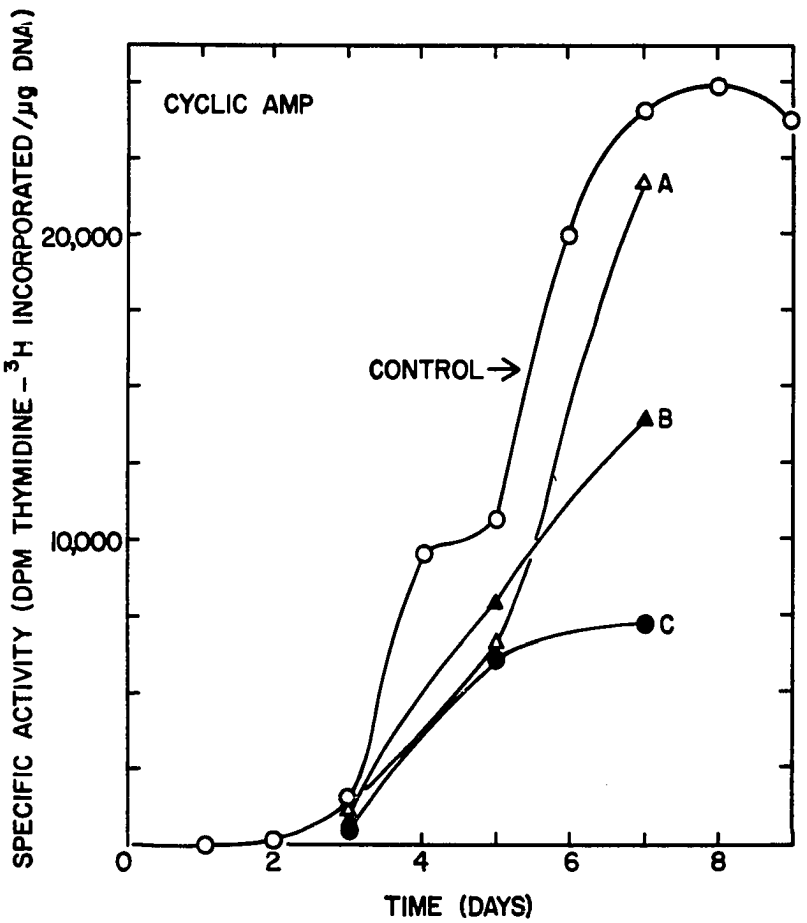


Figure 10 - DNA Synthesis in CLL Lymphocyte Cultures with and without Cyclic AMP Additions at 10^{-5} to 10^{-3} M Concentrations after PHA Stimulation

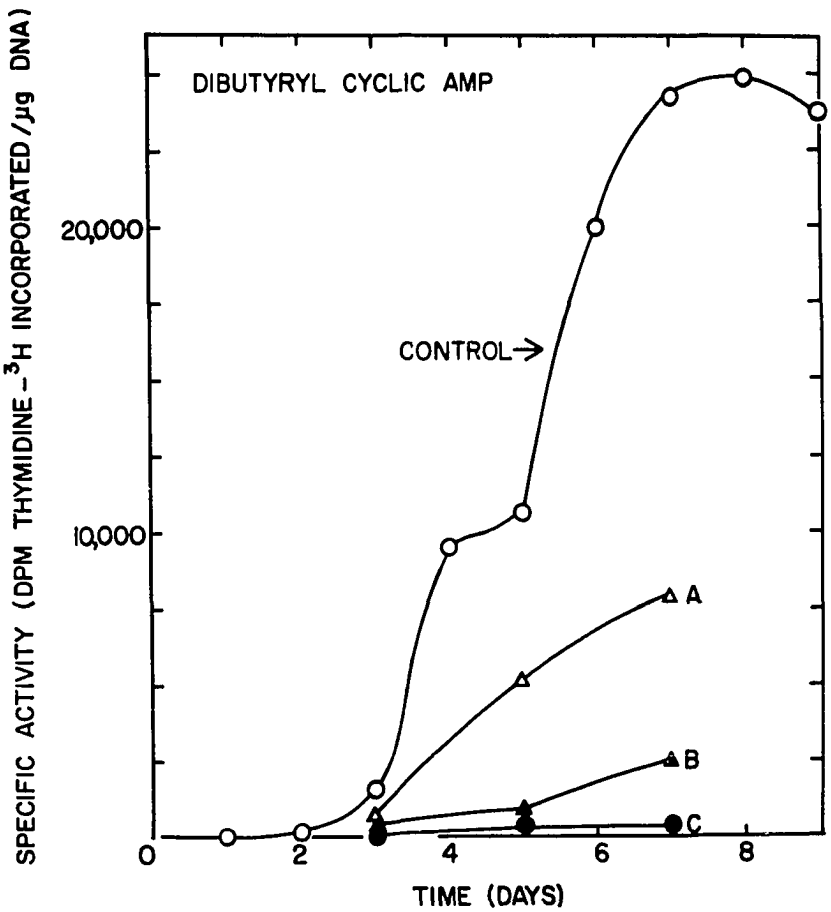


Figure 11 - DNA Synthesis in CLL Lymphocyte Cultures with and without Dibutyryl Cyclic AMP Additions at 10^{-5} to 10^{-3} M Concentrations after PHA Stimulation

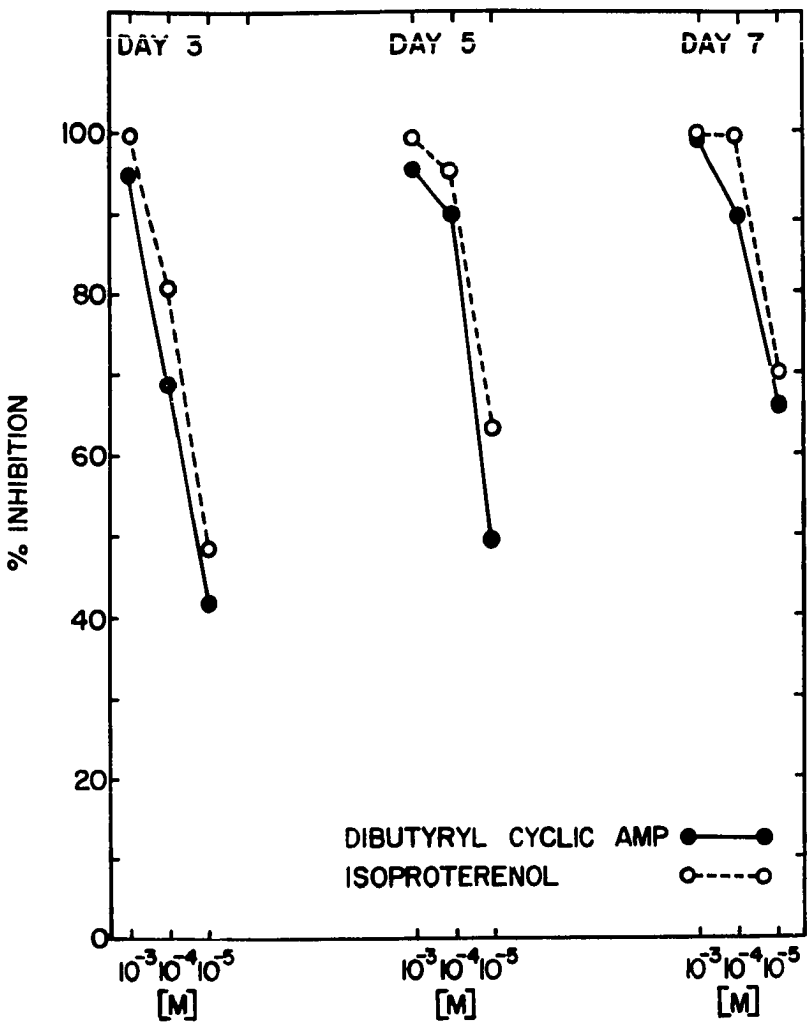


Figure 12 - The Percent Inhibition of DNA Synthesis in CLL Lymphocyte Cultures after Dibutyryl Cyclic AMP and Isoproterenol Additions

tested. Additions of AMP-3' produced slight inhibition at 10^{-5} M and 50% inhibition of DNA synthesis at high concentrations (10^{-3} M). The addition of AMP-5' also caused 50% inhibition at high concentrations (10^{-3} M), but neither monocucleotide produced dose-dependent effects.

Isoproterenol and dibutyryl cyclic AMP produced dose-dependent inhibition and at 10^{-4} M essentially inhibited DNA synthesis completely. Therefore, it was highly probable that AMP-3' and AMP-5' at high concentrations were involved in nonspecific reactions which were independent of adenyl cyclase-mediated activity. Another control experiment showed that each of these adenosine mononucleotide additions did not change the pH of the medium more than 0.1 pH unit, and therefore this inhibition of DNA synthesis could not have been due to cellular alterations effected by extreme pH shifts.

Part of the enhanced effectiveness of dibutyryl cyclic AMP was its insensitivity to cleavage by phosphodiesterase (129) to the less active AMP-5' with concurrent loss of cyclic-AMP mediated effectiveness. It was suggested that the methyl xanthine theophylline inhibited this cleavage and thereby potentiated or mimicked the effect observed after isoproterenol addition by either increasing or sustaining the levels of intracellular cyclic AMP. Figure 13 illustrates the results obtained after isoproterenol (4×10^{-5} M) and theophylline (2×10^{-4} M) were added to the cultured CLL lymphocytes either 24 hours before or at various times after PHA-stimulation. The results, expressed as percent inhibition of DNA synthesis, indicate that the greatest effect occurred when the drugs were added after PHA either at 20 minutes (curve B) or 24 hours (curve C). When the drugs were added 24 hours before PHA (curve A), 91% inhibition occurred at day 5, and with drug addition at day 2 65% inhibition was found at day 5 (curve D).

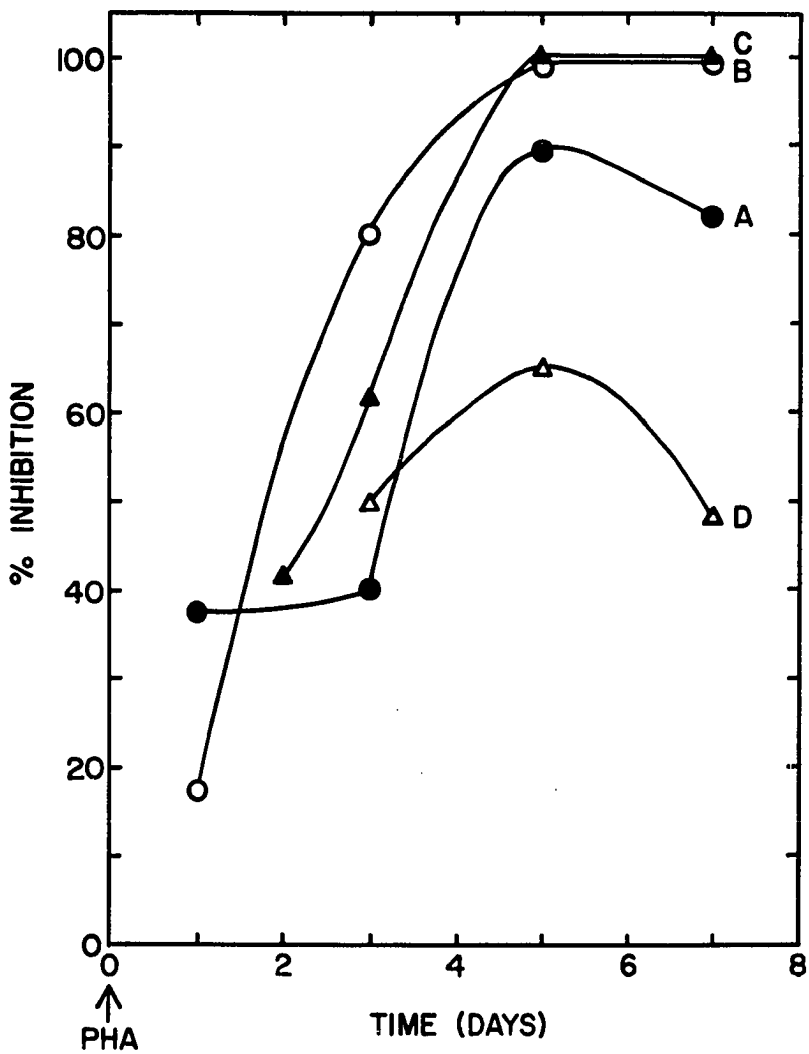


Figure 13 - The Percent Inhibition of DNA Synthesis in CLL Lymphocyte Cultures after the Addition of Both Isoproterenol and Theophylline Either before or after PHA Simulation

As shown in Table 5, the addition of theophylline (2×10^{-4} M), alone, had no effect on the cultures of CLL lymphocytes either 24 hours before or 20 minutes after PHA addition. Isoproterenol (4×10^{-5} M) addition resulted in 45 to 55% inhibition. In contrast, when isoproterenol and theophylline were added to the cultures at the same time, 91 and 99% inhibition was observed. These studies, therefore strongly suggested that theophylline was potentiating the intracellular effectiveness of isoproterenol in the CLL lymphocyte.

In other experiments additions of theophylline at concentrations from 10^{-6} M to 2×10^{-4} M to normal and CLL lymphocytes at 20 minutes after PHA resulted in no significant changes in either the expected time or extent of DNA synthesis. The additions of isoproterenol at 4×10^{-5} M, theophylline at 2×10^{-4} M, or both compounds to PHA-stimulated normal lymphocyte cultures resulted in no inhibition of DNA synthesis. Furthermore, neither cyclic AMP nor dibutyryl cyclic AMP at 10^{-4} M concentration demonstrated any significant effect on DNA synthesis in normal lymphocytes.

There were several reports in the literature concerning the stimulation of human lymphocytes with various agents, including cyclic AMP (69), and trypsin solution (130). As shown in Table 6, preliminary experiments with normal, non-stimulated lymphocytes indicated that cyclic AMP at concentrations of 10^{-8} to 10^{-12} M did not stimulate the cells to undergo DNA synthesis. Also, theophylline at 10^{-6} M neither mimicked nor potentiated any cyclic AMP activity. Trypsin incubation (0.25%) for 10 minutes at 37^{o} was without effect on normal lymphocyte cultures when PHA was not present.

TABLE 5

EFFECTS OF THEOPHYLLINE AND ISOPROTERENOL ON DNA SYNTHESIS

Time of Addition	Additions	Specific Activity (DPM/ug DNA)	% Inhibition
24 hours before PHA:	None	11,390	0
	Theophylline	11,320	0
	Isoproterenol	5,150	55
	Theophylline and Isoproterenol	990	91
20 minutes after PHA:	None	11,400	0
	Theophylline	12,820	0
	Isoproterenol	6,290	45
	Theophylline and Isoproterenol	290	99

TABLE 6

EFFECTS OF CYCLIC AMP, DIBUTYRYL CYCLIC AMP, THEOPHYLLINE AND TRYPSIN ON NON-STIMULATED, NORMAL LYMPHOCYTES

Agent	% of Control DNA Synthesis	Concentration
Cyclic AMP	0.1	10^{-12} M
	0.1	10^{-10} M
	0.1	10^{-8} M
Dibutyryl Cyclic AMP	0.2	10^{-8} M
Theophylline	0.1	10^{-6} M
Trypsin	0.1	(0.25%)

Characterization of Glycogen

The previous studies have shown that the CLL lymphocyte was uniquely sensitive to both isoproterenol and dibutyryl cyclic AMP, and, therefore, further experimentation was undertaken to elucidate the cellular mechanism which brought about these changes after drug additions. Earlier studies (62) had clearly shown that CLL lymphocytes contained large amounts of stored glycogen, and therefore one possible role for cyclic AMP in the CLL lymphocyte was the phosphorolysis of glycogen to glucose-1-phosphate which had been reported to occur in muscle and liver (102,103).

Figure 14 is an electron micrograph of a normal lymphocyte, typical of the many cells examined. In contrast to the ultrastructure observed for the normal lymphocyte, the CLL lymphocyte contained a globular particle in the cytoplasm, as shown in Figure 15. This particle was identified as glycogen on the basis of digestion with diastase and stain retention with periodic acid-silver methenamine. The membrane-bound particle of glycogen varied in size from 0.2 to 0.5 microns which corresponded in size with the periodic acid-Schiff positive material observed with light microscopy. Most of the particles were round suggesting a spherical shape, and one or two were found in each CLL lymphocyte. The inset, of Figure 15, shows a higher magnification of the particle (x120,000). In size and appearance this particle of glycogen was unlike the classical beta (individual) or alpha (aggregated) particles which have been observed in liver and other tissues (112), therefore suggesting that the stored glycogen in the CLL lymphocyte had either an unusual structure or had been packaged within the cell in a rather unique form.

A second method used to characterize the ultrastructure of the CLL lymphocyte glycogen was the periodic acid-silver methenamine, as shown

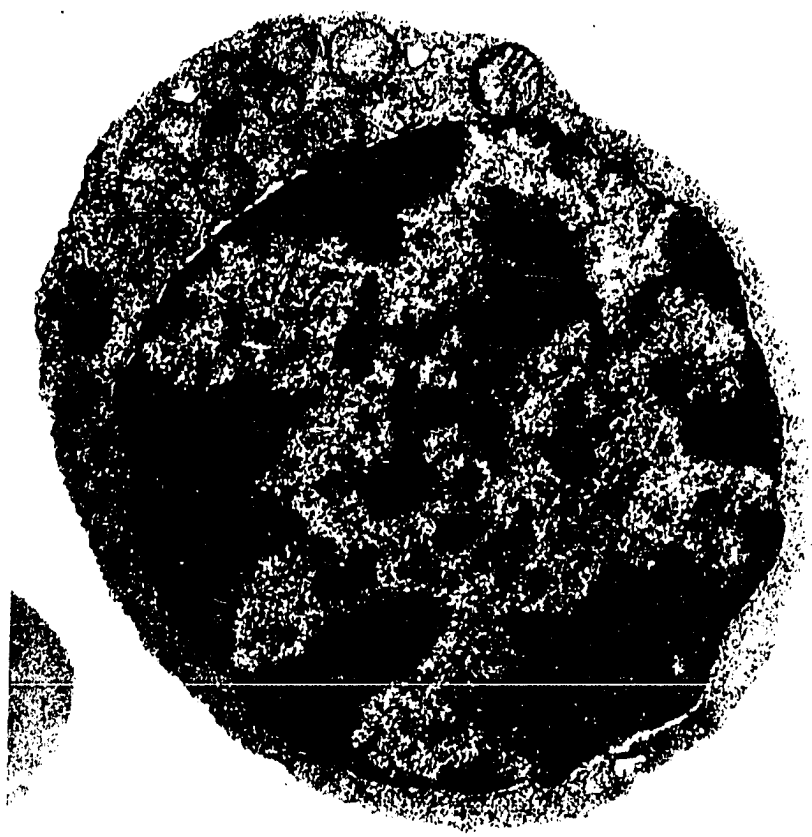


Figure 14 - An Electron Micrograph of a Normal Peripheral Blood Lymphocyte (Magnification: x38,000)

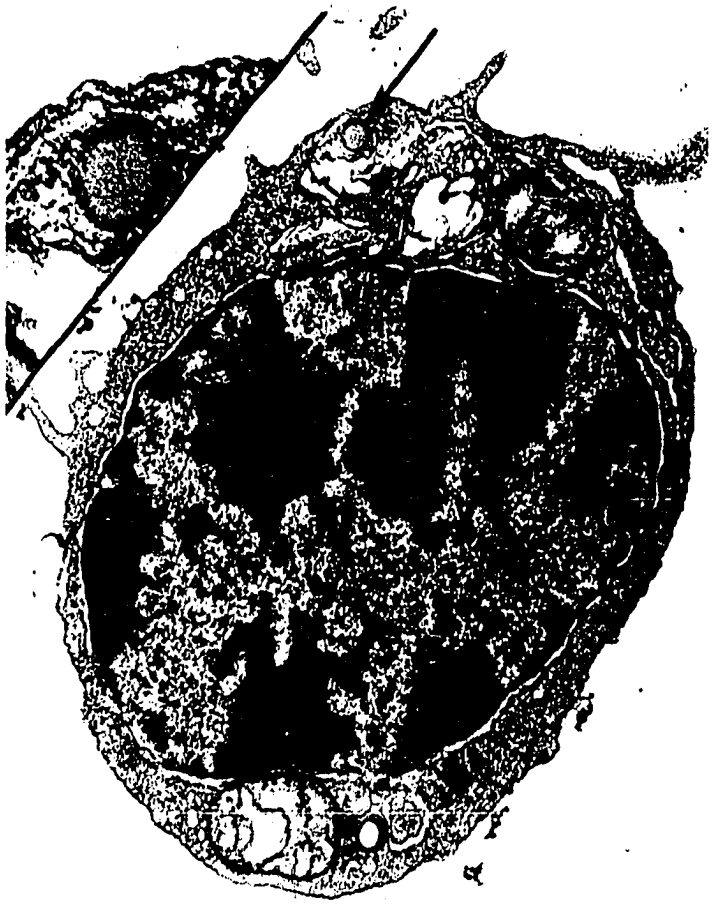


Figure 15 - An Electron Micrograph of a CLL Peripheral Blood Lymphocyte (Magnification: x35,000), with Higher Magnification (x120,000) of the Glycogen Particle as Inset

Figure 16 - An Electron Micrograph of a CLL Peripheral Blood Lymphocyte Stained with Periodic Acid-Silver Methenamine (Magnification: x240,000)

in Figure 16. Once again the particle of cytoplasmic glycogen was observed, and, although not shown here, was identified by digestion with diastase.

Examination of periodic acid-Schiff (PAS) stained cells under the light microscope also indicated the presence of large amounts of glycogen in CLL lymphocytes, as shown in Figure 17. These brightly-stained red particles were abundant in the cytoplasm of the non-stimulated CLL lymphocyte, and approximately 10-fold greater amounts of stored glycogen were observed in the CLL lymphocyte than in normal lymphocytes. This PAS-stained material was comparable in size to the particles of glycogen observed with the electron microscopy, and the ability to stain with PAS was completely abolished with diastase treatment.

Preliminary studies with PAS staining of PHA-stimulated CLL lymphocytes, during the course of cell culture, have shown that the addition of isoproterenol to the cells was followed by the complete loss of stored glycogen within 24 hours, as shown in Figure 18. PHA-stimulated control cultures, without the addition of isoproterenol, did not show this specific loss of glycogen.

Having visualized the intact glycogen particles at the electron microscopic and light microscopic levels, the next step involved the isolation of the cytoplasmic material to unequivocally identify it as glycogen. The material, extracted by the cold-water technique, was insoluble in cold ethanol and was resistant to ribonuclease, deoxyribonuclease and pronase treatments but was degraded by α-amylase.

The isolated glycogen banded in a cesium chloride gradient with the major component at a density of 1.64, as shown in Figure 19. As reported for rat liver (115), a minor component of membrane-bound glycogen banded at 1.33 (g/cc). A sample from the 1.64 peak was removed for

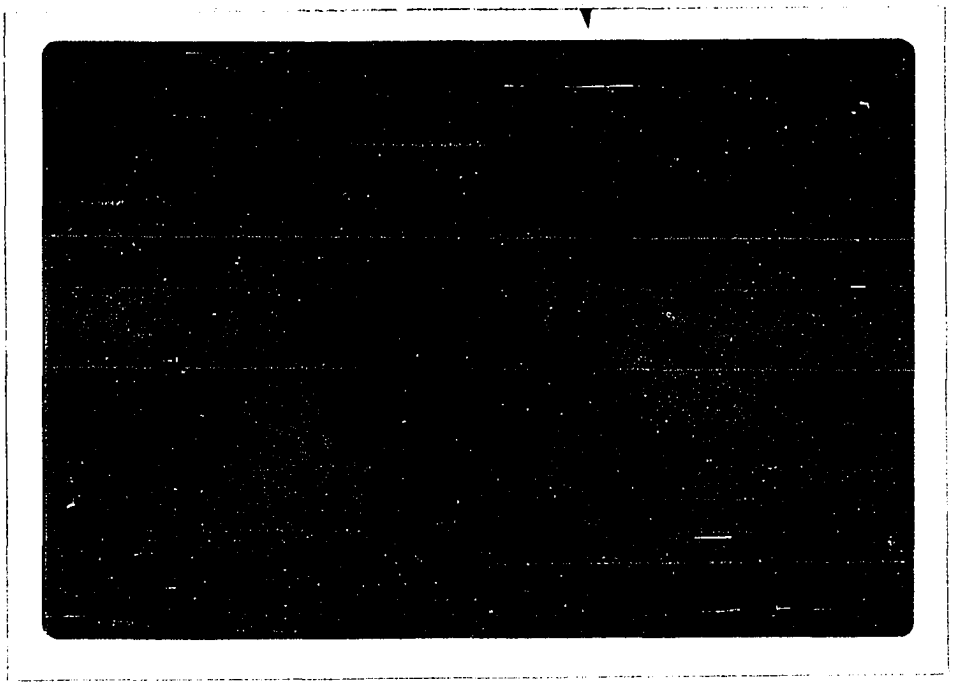


Figure 17 - A Light Photomicrograph of CLL Lymphocytes Stained with Periodic Acid-Schiff Containing Large Glycogen Particles

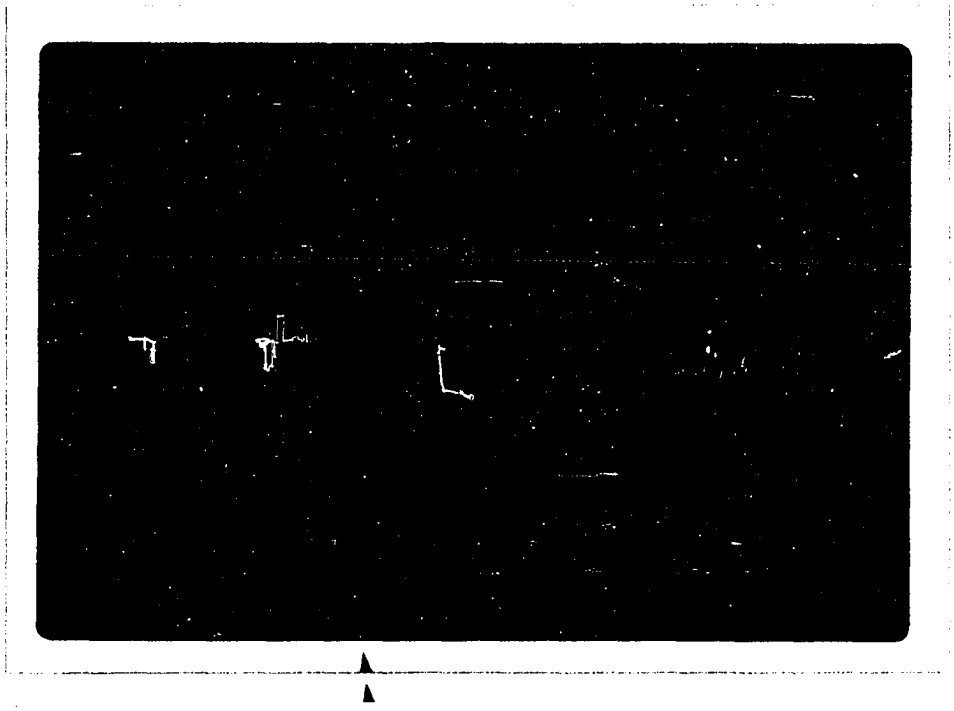


Figure 18 - A Light Photomicrograph of a CLL Lymphocyte Stained with Periodic Acid-Schiff after 24 Hour Treatment with Isoproterenol in Culture

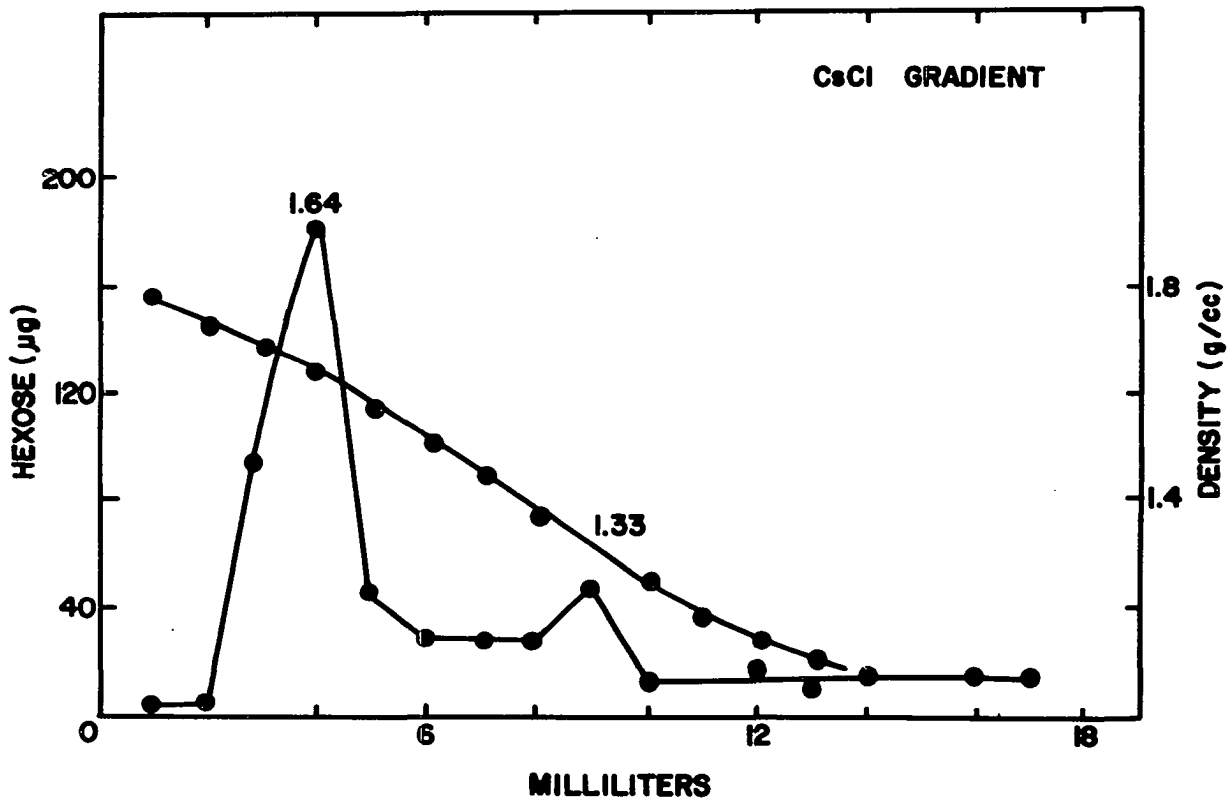


Figure 19 - Glycogen Isolated from CLL Lymphocytes Banded in a Cesium Chloride Density Gradient at 1.64 and 1.33

negative staining with phosphotungstic acid. Figure 20 is an electron micrograph of the isolated glycogen particles. The particles were found to range from 50 to 57 millimicrons in size which corresponded with the upper theoretical limits of B-particle size, as shown in Table 7 (115).

Preliminary studies were undertaken to correlate the pattern of DNA synthesis with the pattern of glycogen synthesis in both the normal and CLL lymphocyte. Figure 21 illustrates that DNA synthesis in normal lymphocytes commenced about 24 hours after PHA addition and reached a maximum at day 4. In the same experiment, glycogen synthesis commenced shortly after the addition of PHA and reached a maximum at day 2. The amount of glycogen remained approximately constant until day 3 and thereafter declined slowly.

Figure 22 shows the relationship of glycogen synthesis to DNA synthesis in lymphocytes from a CLL patient. In this culture, maximal DNA synthesis occurred at day 5. In contrast to the normal lymphocytes, the amount of glycogen present at zero time of culture gradually decreased during the first 2 days of culture, but glycogen was later resynthesized. This 2-fold increase, compared to the initial amounts of glycogen, was comparable to the increase noted in normal lymphocytes. From days 3 to 5, the amount of glycogen remained relatively constant, during the time of maximal DNA synthesis, and dropped significantly in a manner parallel with the decreased DNA synthesis.

The glycogen contents of freshly isolated lymphocytes from two normal donors and two patients with CLL were also determined using either glucose oxidase or phosphorylase a assays. In lymphocytes from normal donors the glycogen concentration was found to be 0.060 ug/ ug DNA and 0.130 ug/ ug DNA, and in CLL lymphocytes the glycogen content was found to

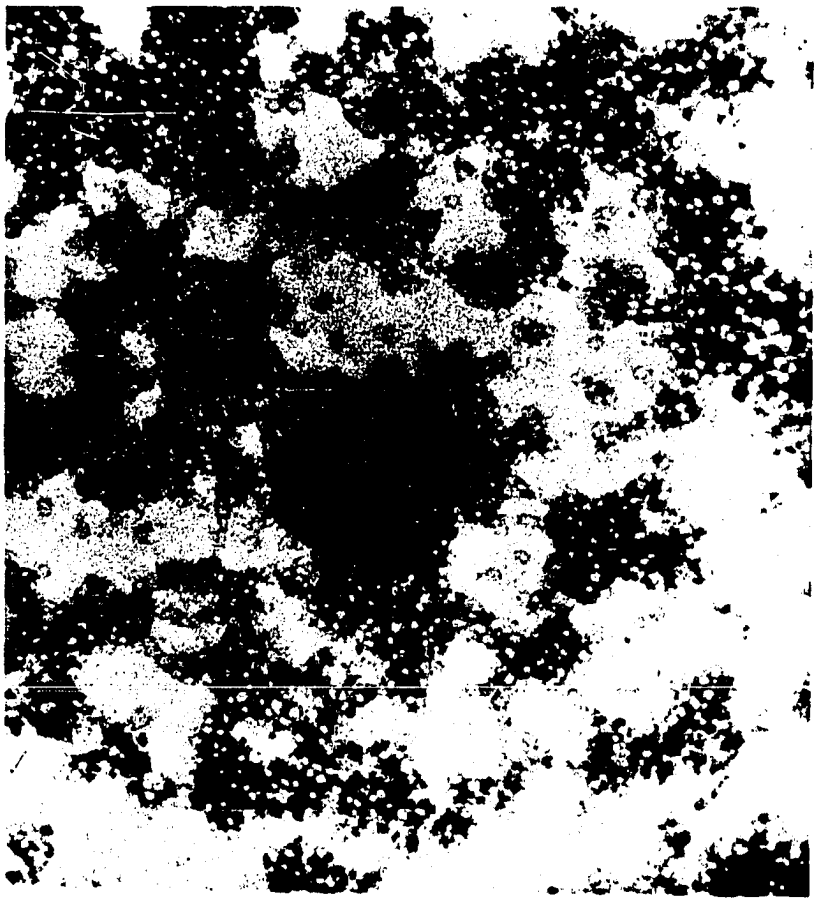


Figure 20 - An Electron Micrograph of the Glycogen Particles Banded at 1.64 in the Cesium Chloride Gradient with a Phosphotungstic Acid Stain.

TABLE 7

GLYCOGEN PARTICLE SIZES

Particle	Millimicrons	Å
Alpha (α)	<200	<2000
Beta (β)	15-55	150-550
Gamma (γ)	2.5-3.5	25-35

from Barber, A. A., Harris,W. W. and Anderson, N. G. 1966
National Cancer Institute Monograph 21: 285.

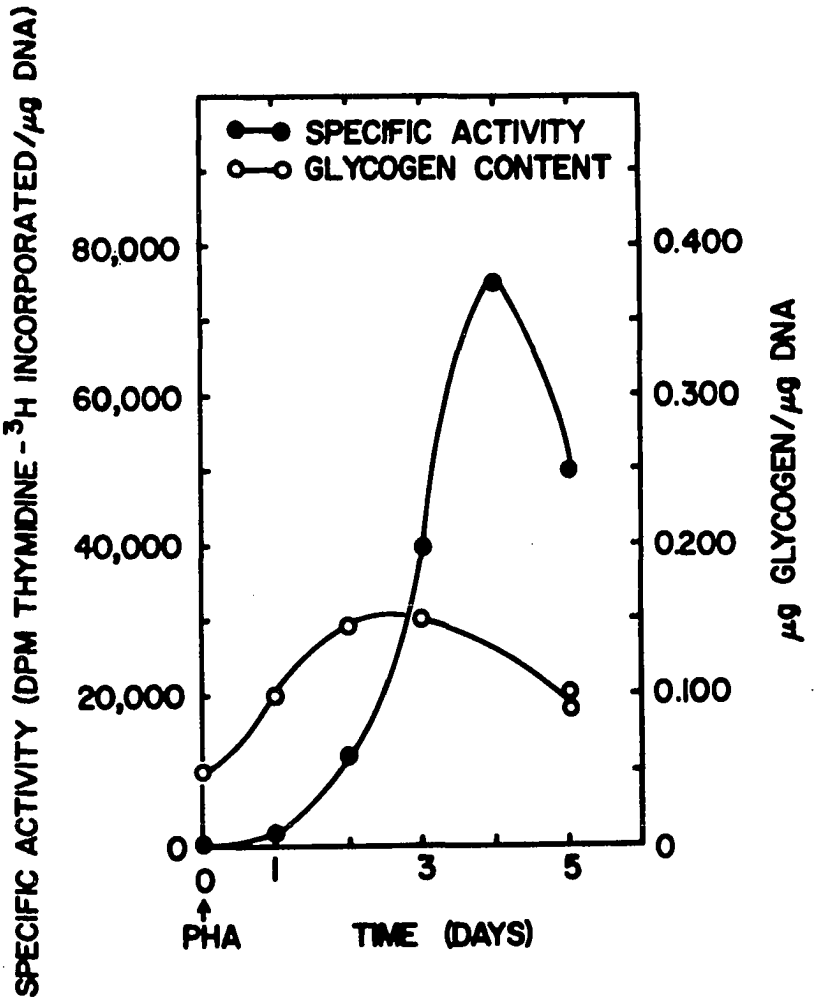


Figure 21 - Glycogen Content and DNA Synthesis Assessed in a Normal Lymphocyte Culture after PHA Addition with Glucose Oxidase Measurement of Glycogen

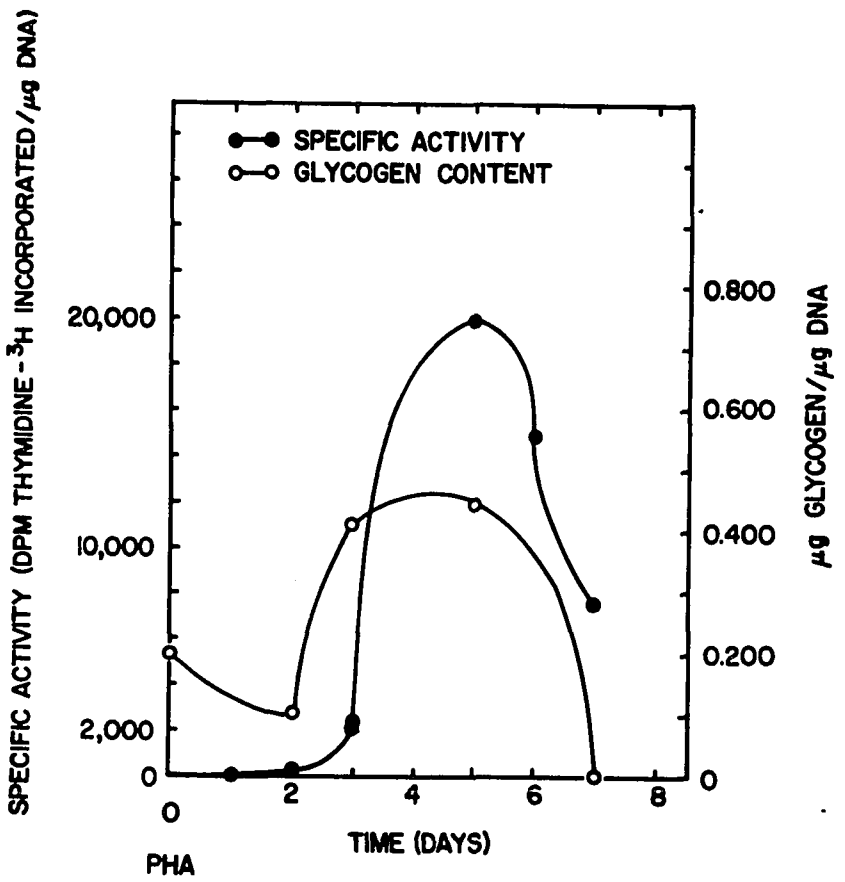


Figure 22 - Glycogen Content and DNA Synthesis Assessed in a CLL Lymphocyte Culture after PHA Stimulation with Phosphorylase a Measurement of Glycogen

be 0.209 ug/ ug DNA in a patient with a low WBC count of 25,000/ mm^3 and 0.475 ug/ ug DNA in a patient with a WBC count of 77,100/ mm^3.

Because of the contamination of cultured lymphocytes with erythrocytes (RBCs) and therefore a possible effect on the glucose oxidase assay, it was necessary to assess any possible effects, both positive and negative. It was conceivable that RBCs could contribute to the glycogen determinations by providing either glucose from the membranes or heme-containing compounds and reduce the values by interference with either uric acid (131) or lysosomal enzymes released after cell death (132). Consequently, normal blood (0.02 ml) was removed from culture and assayed with and without exogenously provided glycogen (type III) at the culture days listed (Table 8). All samples except the last one were hydrolyzed for 30 minutes at 100°. From this study, it was evident that even great numbers of RBCs did not contribute significantly to the total glucose determinations. RBCs <u>per se</u> contributed slightly positive effects at zero time and slightly reduced the values at later times in cell culture.

TABLE 8

EFFECTS OF ERYTHROCYTE CONTAMINATION
ON GLUCOSE OXIDASE ASSAY

Sample	Day	Addition	ug Glycogen
1	0	RBC	6; --
2	0	RBC + 25 ug Glycogen	27; 28
3	1	RBC	5; 8
4	1	RBC + 25 ug Glycogen	22; 22
5	3	RBC	4; 5
6	3	RBC + 25 ug Glycogen	24; 23
7	4	RBC	7; 4
8	4	RBC + 25 ug Glycogen	22; 24
9	7	RBC	3; --
10	7	RBC + 25 ug Glycogen	21; --
11	8	RBC	4; 4
12	8	RBC + 25 ug Glycogen	20; 21
13	1	RBC + 25 ug Glycogen	28; --

CHAPTER IV

DISCUSSION

Several reports in the literature have correlated the PHA-responsiveness of CLL lymphocytes, observed as blastogenesis or transformation, and the immunocompetency of the leukemic patient (124). Similarly, other studies have shown that lymphocytes from patients with Down's syndrome (133) responded to PHA very poorly, and the authors suggested an impairment of immune functions. The studies presented here, however, have indicated that CLL lymphocytes did respond to PHA and exhibited levels of maximal DNA synthesis which were comparable to those observed in normal lymphocytes, although the response was delayed. Possibly, the reduced response observed in the earlier studies with CLL lymphocytes was due to either a nutritionally inadequate medium or non-ideal cell concentrations.

In considering the first possibility, it was noted in a study (125) that tissue culture medium 199 was used, and in another study (126) Eagle's basal medium was used. Modified McCoy's 5A medium was used in our study, and the major differences between these two media and Modified McCoy's 5A medium were the presence of Bacto-peptone (600 mg/liter), asparagine (45 mg/liter) and cysteine (31.5 mg/liter) which were present in Modified McCoy's 5A medium. From these differences, it was apparent that the leukemic cells required greater amounts of free nitrogen or amino acids than normal lymphocytes for the events of blastogenesis and cell maintenance.

A second possibility for the differing results was the cell concentrations used in lymphocyte cultures. In one study (125), the cells were cultured at 10^6 per ml medium, in another study (126) at 5 x 10^5 per ml medium, and in our studies at 2 x 10^6 per ml medium (106). From previous experiments (106) it has been ascertained that these higher concentrations of cells were required in order to observe maximal DNA synthesis. It is possible that for CLL lymphocytes in culture this higher concentration is necessary for the cells to establish optimal cell contact in order to initiate or continue the events required for cell division. In support of this concept, it is of interest to note that membrane alterations have been found in virus-transformed cells (134). These altered growth characteristics were reversed when the cells were treated with peptide fragments of concanavalin A (135), a compound which probably altered the membrane configuration.

The studies presented here demonstrated that by utilizing the optimal culture conditions CLL lymphocytes responded to the mitogen PHA to an extent comparable with normal lymphocytes, but the time of maximal synthesis was delayed. Normal lymphocytes demonstrated maximal DNA synthesis at day 3 or 4, whereas CLL lymphocytes demonstrated maximal DNA synthesis 1 to 4 days later. Lymphocytes from CLL patients with relatively low, medium and high WBC counts demonstrated maximal DNA synthesis at days 5, 6 and 7, respectively. It was also of interest to note that our studies showed that the delay in DNA synthesis with the progression of the disease was also correlated with increased glycogen content. These findings were consistent with Vereschagina's studies on glycogen content in CLL (136).

Although CLL lymphocyte cultures demonstrated maximal DNA synthesis 5 to 7 days after PHA addition to the culture, there was some DNA

synthesis occurring at days 3 and 4. This moderate amount of DNA synthesis occurred at a time when lymphocytes from normal donors demonstrated maximal DNA synthesis, and therefore suggested that two or more populations of cells existed in the peripheral blood of patients with CLL. Theoretically there are at least two possible approaches, physical and biochemical, for differentiating potentially different lymphocyte populations. Recently, Sykes et al. (137) reported that Ficoll gradients could be used to separate cell types which were of different densities, but such differences are not known to exist between normal and CLL lymphocytes. Therefore, in this study, methods were employed for distinguishing the "leukemic" component of the CLL cultures using manipulations such as media changes and drug additions. From the results of these studies, it was apparent that the DNA synthesis observed at day 3, in CLL cultures, represented a more normal-like component of lymphocytes which coexisted with the "leukemic" lymphocytes in the peripheral leukocyte population of the CLL patient. Two lines of experimental evidence supported this hypothesis.

First, the patterns of DNA synthesis were different in normal and CLL lymphocyte cultures after medium changes both with and without the re-addition of PHA. Previously published papers were quite confusing in their characterizations of the PHA requirements of normal lymphocytes. Cooper and Rubin (138) stated that PHA was required continuously in culture in order to observe the increased synthesis of nonribosomal RNA. Furthermore, if PHA was removed by washing, the RNA synthetic pattern changed back to the 45 to 50 S species which were synthesized prior to PHA addition. Polgar et al. (139) published that the events leading to the formation of the blastoid cell were identical in cultures continuously exposed to PHA and those exposed to PHA for only 24 hours. At days 8 to 12 in cultures

exposed to PHA for only 24 hours, however, DNA synthesis was significantly reduced in comparison to those normal lymphocyte cultures in which PHA was continuously present. Experiments undertaken in this laboratory to determine the PHA requirements for normal lymphocyte cultures demonstrated that when PHA was added to normal lymphocyte cultures at time zero and removed at 24 hours 46% of the control synthetic activity was observed at day 3, and significant amounts of synthesis were noted at later culture days, 4 and 5. These findings are more consistent with those of Polgar et al.(139).

When CLL lymphocytes were exposed to PHA during the first 24 hours of culture, with media change and no subsequent readdition of PHA, 51% of the control DNA synthetic activity was observed at day 3, and subsequently only 12 to 15% of the control specific activities were observed at days 4 to 7. This 51% activity observed at day 3 was the same percent (within error of the assay) found in normal lymphocyte cultures, using the same experimental conditions. When PHA was removed from the medium, 12 to 15% of the control DNA synthetic activity was observed, whereas in normal lymphocyte cultures, 37 to 64% was observed at days 4 and 5. Therefore, the possibility that the lymphocytes in the CLL population which were responding at days 5 to 7 were normal lymphocytes was quite unlikely.

Furthermore, in studies with CLL lymphocytes, removal of PHA at day 3, without its subsequent readdition, resulted in 71% of the control activity at day 5. These studies suggested that the cells which were synthesizing maximal amounts of DNA at day 5 were a portion of the population stimulated by PHA between days 2 and 3 and were not the lymphocytes which were stimulated during the first 24 hours, as was characteristic of normal lymphocytes in cell culture.

The three-fold enhancement of DNA synthesis observed when PHA

was added back at either days 3 or 4 could be due to a) exposure to PHA of a new population of more normal-like daughter cells, b) optimal concentrations of PHA added after autolysis of polymorphonuclear cells, or c) optimal concentrations of PHA present at a time when the CLL cell membrane or cellular activities or both have been conditioned to the medium and are metabolically stabilized. The first suggestion (a) is unlikely because the normal-like component probably represents only 10 to 20% of the total lymphocyte population and could not contribute 3-fold increases in total synthesis. The second suggestion (b) is also unlikely because preincubation of normal cells, before PHA addition, has not produced such marked increases in DNA synthesis. The last suggestion (c) is supported by some experimental evidence. Studies in which CLL lymphocytes were maintained in culture for 3 days before adding PHA showed maximal DNA synthesis increased 2.5-fold and occurred at day 5. The control cultures, without preincubation, exhibited maximal DNA synthesis at day 5. This significant increase in DNA synthesis was not observed when cells were preincubated for 24 or 48 hours. The precise events occurring during this 72 hour period are not known, although it is possible that the lymphocyte undergoes some metabolic changes, as discussed later.

The second line of evidence to support the hypothesis of a "leukemic" component of lymphocytes comes from the experiments showing the differential effects of isoproterenol on normal and CLL lymphocyte cultures. In the initial experiments, it was shown that 24 hour pretreatment with isoproterenol resulted in significant reductions in DNA synthesis with maximal synthesis occurring at day 4, whereas the non-isoproterenol treated control CLL cultures exhibited maximal DNA synthesis at days 5 to 7 after PHA addition. Control experiments with normal lymphocytes showed little or

no effects when isoproterenol at the same concentrations which markedly affected DNA synthesis in CLL lymphocytes was added to the medium either before or after addition of PHA.

The marked inhibition of DNA synthesis observed in these late-responding lymphocytes in CLL cultures, following treatment with the catecholamine isoproterenol, suggests that a metabolic alteration exists in this "leukemic" component of CLL lymphocytes. Further experimentation was undertaken to determine if there was a certain culture time or cell-state which was more sensitive to isoproterenol and the role of this catecholamine in the metabolic activities of the CLL lymphocyte. It was shown in these studies that the CLL lymphocyte was most sensitive to isoproterenol during the initial 24 hours after PHA addition; furthermore, both 24 hours before and particularly after this initial 24 hour period (at 48 and 72 hours) the effectiveness of the drug to inhibit DNA synthesis was reduced. This could be due to the PHA-induced changes in the membrane which occur soon after the addition of the mitogen (73). Possibly, the intracellular product of the catecholamine induced activation of adenyl cyclase, cyclic AMP, is present in either too great an amount or at an inopportune time and brings about a series of events which are lethal to the cell. These damaged cells then undergo autolysis, as observed by a loss of DNA, and any more normal-like cells which are present are destroyed due to the leaking lysosomal enzymes (132).

The intracellular, secondary messenger of catecholamine-induced activity, cyclic AMP, can be added exogenously to the lymphocyte cultures and should circumvent the adenyl cyclase-mediated production of cyclic AMP from its nucleoside triphosphate precursor, ATP. However, following cyclic AMP addition, less inhibition of DNA synthesis was obtained than with

dibutyryl cyclic AMP. This is consistent with two findings in the literature: first, the recent report (140) that calf serum contained phosphodiesterase activity cleaving cyclic AMP to adenosine monophosphate; secondly, a report suggesting that the membrane provided a barrier for the charged nucleotide (140). Both of these limitations for cyclic AMP were inconsequential when dibutyryl cyclic AMP was added to the cultured lymphocytes. From earlier studies it was asserted that the dibutyryl derivative was better-adapted for transport across the membrane from the extracellular to the intracellular spaces and was also more resistant to enzymic cleavage by phosphodiesterase (141). Our studies indicated that dibutyryl cyclic AMP produced the same inhibition, as observed with isoproterenol over a 100-fold concentration range. This strongly supports the concept that isoproterenol increases intracellular cyclic AMP in CLL lymphocytes.

Cleavage of cyclic AMP to AMP-5' by phosphodiesterase results in none of the original effectiveness in regulating many diverse cellular events. Phosphodiesterases are known from studies in brain (142) and liver (143) tissues to be found associated with the membrane or in the cytoplasm of the cells, as well as in calf serum (140). The methyl xanthines, specifically theophylline, inhibit one or more of these enzymes. When added to cultured CLL lymphocytes within 20 minutes after PHA addition, theophylline had no inhibitory effect upon subsequent replication, however, when added in conjunction with isoproterenol, DNA synthesis was completely inhibited (99%). In these studies isoproterenol alone inhibited DNA synthesis in CLL cultures 45%, whereas normal lymphocytes did not respond to isoproterenol, theophylline, or isoproterenol and theophylline additions, at these same concentrations. In agreement with the restriction found on timing, as found in experiments with isoproterenol treatment alone, the

addition of isoproterenol with theophylline at day 2 was less damaging (50%) than either pretreatment for 24 hours or posttreatment within the first 24 hours after PHA addition. Also, pretreatment with both compounds did not affect the synthesis at day 3 as greatly as treatment within the first 24 hours. These studies strongly suggest that theophylline is potentiating the activity of isoproterenol in maintaining increased intracellular concentrations of cyclic AMP which are detremental to cellular activities. The differences in sensitivities of lymphocytes before and after PHA addition, also, suggest the occurrence of metabolic or membrane alterations during the course of cell culture.

One role of cyclic AMP in other tissues (101,102) is the regulation of glycogen synthesis and degradation in order to meet cellular demands during periods of stress (103) or cell division (96). In the presence of elevated amounts of intracellular cyclic AMP, glycogen synthetase undergoes phosphorylation to a form which is dependent upon increased glucose-6-phosphate (101) within the cell to be active, therefore increases in cyclic AMP depress the synthesis of glycogen. At the same time, increases in intracellular cyclic AMP bring about the conversion of phosphorylase b to the active form, phosphorylase a, which occurs by the specific activation of a protein kinase by cyclic AMP (104). It is conceivable that the acute sensitivity of the CLL lymphocyte to isoproterenol is due to the phosphorolysis of stored glycogen, an event from which the leukemic lymphocyte may not be able to recover in order to continue the processes necessary for DNA synthesis and cell division.

The early work of many groups of investigators (62,63,144) had clearly shown by semi-quantitative, histochemical methods that the non-stimulated CLL lymphocyte contained greater amounts of cytoplasmic glycogen than

the normal lymphocyte. Further studies, reported by Seitz (64), using enzymic quantitation showed that normal lymphocytes contained 4.1 ±0.5 mg glycogen/ 10^{10} cells, and CLL lymphocytes contained 7.0 ±0.7 mg glycogen/ 10^{10} cells. In the study presented here, glycogen was also quantitated and found to be 0.060 ug glycogen/ ug DNA (4.20 mg glycogen/ 10^{10} cells) to 0.130 ug glycogen/ ug DNA (9.10 mg glycogen/ 10^{10} cells) in normal lymphocytes, whereas the CLL lymphocytes were found to contain from 0.209 ug glycogen/ ug DNA (14.63 mg/10^{10} cells) to 0.475 ug glycogen/ ug DNA (33.25 mg/10^{10} cells). The lower value for the amount of stored glycogen agreed with that found by Seitz (64), since a 2 to 7-fold increase was found in the CLL lymphocytes. There are several feasible reasons for the higher values found in the studies presented here. First, the methods of lymphocyte isolation and incubation could result in cellular trauma with loss of stored glycogen, thus resulting in lower values. Second, the amount of stored glycogen could be dependent upon the progression of the disease. This interpretation is supported by the findings of Vereschagina (136) which indicated that as the disease progressed, noted by increasing WBC counts, an increase in the cellular content of glycogen in the freshly isolated lymphocytes occurred.

The stored glycogen which was found in the CLL lymphocyte therefore provides a specific site for isoproterenol-induced cyclic AMP activity. In the CLL lymphocytes, it was found that within 24 hours of isoproterenol addition to PHA-stimulated lymphocytes, there was a specific loss in glycogen, as shown by the loss of PAS-stained material in the light photomicrographs. The phenomenon of isoproterenol-mediated loss of stored, cellular glycogen is further supported by Malamud (96) who demonstrated that administration of isoproterenol to mice initially increased the adenyl

cyclase activity in salivary gland cells and subsequently decreased glycogen content (95). In contrast to the CLL lymphocyte, however, the salivary gland cells resynthesized glycogen, replicated their cellular DNA and then divided.

Preliminary experiments, in this study, were undertaken to enzymically quantitate glycogen in both normal and CLL lymphocytes after PHA-stimulation. In CLL lymphocytes there was a loss of glycogen during the first 48 hours after PHA addition, and it was only after the resynthesis of glycogen that DNA synthesis commenced. In contrast, however, the amount of glycogen in normal lymphocytes increased significantly during the initial 48 hours after PHA-stimulation, followed by a plateau at days 2 and 3 and decreased thereafter.

The significance of the unusual accumulation of glycogen in the cytoplasm of the CLL lymphocyte is not fully understood at this time. It is possible to postulate that this storage of glycogen is either a primary or secondary defect which is associated with the disease. If the glycogen storage is the primary or fundamental defect in the leukemic process, one would expect that one of the enzymes either in the synthesis or the degradation of glycogen is defective, and the glucose residues are inaccessable during events of cellular stress. In support of an alteration in the degradative pathway, Abell and Kamp (145) have found that phosphorylase a was not activated in CLL lymphocytes after PHA-stimulation, whereas in normal lymphocyte cultures phosphorylase a activity was increased markedly after PHA-stimulation. This type of enzymic defect has been clearly demonstrated in six of the nine types of glycogen storage diseases. In these six forms of disease (146,147,148,149,150,151) an enzymic defect was demonstrated in one or more of the enzymes involved in the degradation of

glycogen, and in the other three types of glycogen storage diseases enzymatic defects were demonstrated in the synthesis (152) of glycogen or the transport (153) and metabolism (154) of glucose. These diseases have as the hallmark of diagnosis increased amounts of glycogen in either liver or muscle cells, and of special interest were reports which indicated that the same enzymic defects found in the liver cells were also found in the leukocytes (105) of the affected patients. Although specific enzymic defects are known to occur in glycogen storage diseases, the ramifications of these defects in cellular metabolism are not fully understood, especially in suggesting a role for the altered metabolism in the affected leukocytes.

It is also possible that in CLL the accumulation of glycogen is a secondary defect, and therefore one could hypothesize that an alteration occurs at some metabolic point quite distant from the synthesis or degradation of glycogen. The accumulation of glycogen could occur if a product of altered metabolism were either accumulated or excessively utilized and thus bring about an alteration in the control of glycogen metabolism. It would be the "distant metabolic alteration" which would be the deleterious event in the leukemic process with the unusual storage of glycogen as a "symptom" of this defect.

The studies presented here strongly suggest that the relationships of increased glycogen content and delayed responsiveness to PHA with the progression of the disease are important features to be considered in elucidating the precise alteration in CLL lymphocytes. Further experimentation should be undertaken to measure the enzymes associated with the synthesis of glycogen, and also, in addition to phosphorylase a, the enzymes involved in the degradation of cytoplasmic glycogen. In differentiating primary from secondary mechanisms, it would be appropriate to study the

enzymes which are active at control points in metabolic processes.

Another significant finding of this study is the acute sensitivity of the CLL lymphocyte to isoproterenol, the bronchodilator, which is extensively used in medical practice. It is conceivable that a structurally-related compound which does not have the adverse cardiac effects would be quite useful in the treatment of CLL.

In conclusion, this study has answered the questions which were proposed at the outset. First, a cell culture system has been developed which supported maximal proliferation of the CLL lymphocyte, and therefore a study of the biochemical activities associated with proliferation was feasible. Second, it was found that while lymphocytes from CLL patients did respond to PHA with maximal DNA synthesis occurring to an extent comparable with normal lymphocytes, the response was delayed one to four days. Also, this delay in maximal DNA synthesis was correlated with the progression of the disease distinguished clinically as increasing peripheral leukocyte counts. Third, results of studies on the mode of action of the catecholamine isoproterenol strongly suggested that intracellular cyclic AMP was involved in the inhibition of DNA synthesis in CLL lymphocytes. Fourth, it was shown that cyclic AMP presumably caused the phosphorolysis of the accumulated glycogen in the CLL lymphocytes. Fifth, the unusual glycogen of the CLL lymphocytes was examined by electron microscopy, isolated and characterized chemically and quantitated in both the normal and CLL lymphocytes in the resting and PHA-stimulated states.

CHAPTER V

SUMMARY

Human peripheral blood lymphocytes from both normal donors and patients with chronic lymphocytic leukemia (CLL) were maintained in a cell culture system and studied in both the resting and mitogen-stimulated states. These CLL lymphocytes responded to PHA, a mitogenic extract of Phaseolus vulgaris, with maximal DNA synthesis occurring 5 to 7 days after PHA addition to the cultures. This is 1 to 4 days later than the time of maximal response in normal lymphocytes, at day 3 or 4.

It was also found that the portion of cells which responded at days 3 and 4 in the CLL lymphocyte cultures reflected a more normal-like population of lymphocytes, biochemically different from the cells demonstrating maximal DNA synthesis at days 5 to 7. The "leukemic" component of the CLL lymphocyte population was uniquely sensitive to additions of isoproterenol, presumably through an adenyl cyclase-mediated increase in intracellular cyclic AMP. This interpretation was supported by two lines of evidence. First, the same extent of inhibition produced by isoproterenol was demonstrated with the N^6-2'-O-dibutyryl derivative of cyclic AMP, over a 100-fold concentration range. Second, theophylline, a methyl xanthine reported to inhibit the phosphodiesterase catalyzed cleavage of cyclic AMP to AMP-5', potentiated the effectiveness of low concentrations of isoproterenol in inhibiting DNA synthesis.

One effect of isoproterenol in CLL lymphocytes was shown by histochemical techniques to involve the phosphorolysis of stored glycogen. The ultrastructure of this glycogen, not observed in normal lymphocytes, was examined by electron microscopy and was unusual in appearance. These particles were large, 0.2 to 0.5 microns in diameter, and membrane-bound. The glycogen from them was isolated and found to sediment at a density of 1.64, and electron micrographs of the isolated material (1.64) indicated that these were beta-particles.

Thus, these studies have elucidated several biochemical differences between CLL and normal lymphocytes. The development of a culture system and the elucidation of the interrelationships of altered nucleic acid synthesis, glycogen metabolism, and the role of cyclic AMP indicate new directions of investigation to broaden the knowledge of basic cellular mechanisms and to suggest potential new methods of treatment for this disease.

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