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HUMAN LYMPHOCYTE IMMUNOREACTIVITY
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OF ^{3}H-URIDINE

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Robert Glenn Ulrich

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By

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ABSTRACT

HUMAN LYMPHOCYTE IMMUNOREACTIVITY ASSESSED
BY EARLY INCORPORATION OF ^{3}H-URIDINE

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The detection of an early increase in the rate of RNA synthesis by phytohemagglutinin stimulated human peripheral blood lymphocytes has been investigated as a rapid in vitro means of assessing cellular immunoreactivity. ^{3}H-uridine uptake and incorporation into RNA precedes the increase of S phase thymidine incorporation providing for quantitation of lymphocyte stimulation occuring before DNA synthesis. This assay offers advantages over the commonly used thymidine incorporation method primarily in conveniance and potential accuracy of results. By measuring events occurring during the first stages of blastogenesis this technique may also circumvent the effects of helper and suppressor functions present in culture. Heparinized blood samples were obtained from healthy adult volunteers by venipuncture. The culture conditions that were optimum for producing the highest increments of stimulation were determined. 1×10^{5} lymphocytes isolated by Ficoll-Hypaque density centrifugation and a phytohemagglutinin concentration of 50 ug

per ml led to a level of ^{3}H-uridine incorporation at 20 hours of culture that was over 5 times the rate for unstimulated controls. At 12 hours of culture significant uptake of label was obtained, and by 36 hours over 10 times greater accumulation of ^{3}H-uridine had occurred in stimulated cells above controls. Beyond 36 hours the stimulation index declined mainly because of a plateau of label uptake in stimulated cells accompanied by a rise in uptake by unstimulated cells. The enhanced ^{3}H-uridine incorporation in response to phytohemagglutinin was inhibited by actinomycin D and was precipitable by trichloroacetic acid and thus occurs as a result of stimulated early RNA synthesis. Significant RNA synthesis stimulation was observed when lymphocytes were cultured in medium without serum thus providing a chemically defined system for assessing immunoreactivity. T and B cell fractions enriched by rosetting T cells with aminoethylisothiouronium bromide sheep red blood cells and characterized by surface markers were incubated with phytohemagglutinin. RNA synthesis was stimulated only for T cells in response to this T cell mitogen. Significant early RNA synthesis was always detected but the magnitude of stimulation for the individual's lymphocytes varied between days of observation. ^{3}H-uridine incorportion was exhibited by a specifically stimulated lymphocyte subpopulation, occurred at an early stage of the cell cycle, and thus provides a practical assay for evaluating cellular immune competence.

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INTRODUCTION

A number of techniques have been established for analysis of the immune history and potential immune responsiveness of the individual. Detection of antigen-specific antibodies is widely relied on for diagnosis of infectious disease states. Cell-mediated immune events are generally assessed clinically by cutaneous delayed hypersensitivity reactions which correlate with prior exposure to specific antigens. *In vitro* methods have also been developed to assess cellular antigen-specific responsiveness, referred to as immunoreactivity, most commonly by lymphocyte stimulation (119, 140). Positive lymphocyte immunoreactivity has been found in individuals that do not respond to cutaneous hypersensitivity tests but were vaccinated (145, 147) or were in an active disease state (72, 84, 133). Similarly lymphocyte immunoreactivity has been found in cases without humoral immunity present (203). *In vitro* techniques are particularly useful in testing for specific hypersensitivity in cases of contact dermatitis, sensitivity tests for multiple antigens, and for patients anergic by cutaneous methods.

Specific antigen stimulation of lymphocytes *in vitro* has been studied in a variety of systems including challenge with antigens of virus (143, 144, 155), bacteria (146, 147),

fungi (148), protozoa (149-151), helminths (152), mycoplasma (154), allergens (141-142), and tumor antigens (153). The typical blastogenic response of lymphocytes cultured with specific anamnestic antigens or nonspecific mitogens is generally quantitated by radio-labeled thymidine incorporation. Using thymidine incorporation as an index of immunoreactivity, however, imposes a few major restrictions on interpreting results and method performance. The extended culture time necessary (at least 72 hours) is inconvenient and introduces nonspecific proliferation from serum factors, and the possibility of <u>in vitro</u> sensitization of lymphocytes (178-179). In addition measuring stimulation only in terms of the relatively late event of DNA synthesis complicates the interpretation by introducing the effects of helper and suppressor factors present in culture which influence the G_1 to S phase shift in the cell cycle of stimulated lymphocytes (122-125). Thus an analysis of early events in the cell cycle may prove more useful as an assay for cellular immunoreactivity.

This study developed a semimicro technique adapted for human peripheral blood lymphocytes which is based on quantitation of RNA synthesis stimulated by the plant lectin phytohemagglutinin (PHA-P), (187). RNA synthesis as determined by incorporation of ^{3}H-uridine precedes DNA synthesis of stimulated lymphocytes. Significant stimulation indices are routinely obtained by 12 hours of culture, with assay sensitivity increasing to 36 hours. The incorporation of uridine by lymphocytes cultured with

the T cell mitogen PHA is enhanced exclusively for T cells, mediators of secondary cellular immune responses. The optimum culture conditions have been established and consistant results were obtained both in the presence and absence of serum supplements.

REVIEW OF THE LITERATURE

I. Lymphocyte Membrane-Ligand Interactions

A complex cascade of physical-biochemical events is initiated in lymphocytes stimulated *in vitro*. Agents that induce stimulation or transformation of lymphocytes may be loosely categorized as mitogenic when they produce nonspecific stimulation or antigenic when immunological priming of the organism is required and specific memory cells respond. Allogeneic cells are a special category of antigens that stimulate other lymphocytes based on differences in histocompatability antigens between individuals. Most biological mitogens are also potentially antigenic as they readily produce antisera in immunized animals (1). The most widely studied class of biological mitogens are the plant lectins which are composed of glycoprotein or protein and possess varying capacities to bind saccharides.

The plant lectins conconavalin A (Con A) and phytohemagglutinin (PHA) are frequently used in developing models of immunological lymphocyte stimulation. When present in soluble form both Con A and PHA are polyclonal T cell activators. The addition of α-D-methyl mannoside competitively inhibits the binding of the Con A molecule to membrane receptors (3).

A certain threshold time of incubation with mitogen

is necessary for activation to occur (27) and an optimal cell and mitogen concentration are also observed (4, 12). Lectin mitogenicity is dependent on Ca^{++}concentration, temperature and pH (12, 13).

Stimulation progressing to blastogenesis as measured by radioactive thymidine uptake is maximal when 16-25% of the Con A binding sites are filled and 3% of the PHA sites (6). Although only T cells are activated by Con A and PHA the lectins bind equally to B, and T cells (199). PHA association with the lymphocyte membrane has a $t_{\frac{1}{2}}$ of of 3 to 5 minutes at 22° C reaching equilibrium at 30 minutes (12).

It is generally agreed that an effective lectin must be at least bivalent for mitogenicity (7) although a required tetravalency (8) and mitogenic monovalent antibodies (9) have been reported. The dimeric succinylated form of Con A does not produce a falling limb on the dose response curve while the native tetramer does (93) when the proliferative response is quantitated by thymidine incorporation. At higher doses of mitogen there is a decline in stimulation resulting from competition for binding sites that reduces the amount of a presumed necessary cross-linking of surface receptors or a toxic effect produced at higher lectin concentrations (198). At lectin concentrations greater than the optimal dose when lymphocyte viability decreases, lymphokine production, which is DNA synthesis independent, may actually increase (9).

The nature of antigen interaction with T cell membrane receptors has remained obscure. The surface membrane immunoglobulins IgM and IgD of B cells are clearly implicated in antigenic stimulus of B cells (16). Marchalonis has presented evidence (10) in support of an immunoglobulin-like T cell receptor that is an integral membrane constituent like IgD of B cells that is not freely secreted into serum. Ig-like molecules have been reported associated with T cells. Szenberg (19) identified molecules isolated from murine thymocytes and thymoma lines with k chain specific fowl anti-IgG $(Fab)_2$ antisera that upon reduction showed k light chain electrophoretic mobility and a unique heavy chain mobility.

Rosenthal (11) using bovine insulin as an antigen model found that guinea pig T cells recognize only a minor portion of the antigen primary structure (a single amino acid residue) while B cells are able to respond to many different conformational determinants. The "carrier effect" presents another argument against an immunoglubulin T cell receptor. This phenomenon is observed when an animal is immunized with a hapten (chemical modification) conjugated carrier antigen molecule and challenged with a booster of hapten-carrier conjugate or hapten conjugated to a new carrier. Maximum anti-hapten antibody production is obtained only when the original carrier-hapten is used to challenge. Carrier recognition is T-dependent (40). When an antigen is reacted with antisera from an immunized animal and used to challenge another immune animal

only delayed type hypersensitivity, which is T cell dependent, is exhibited (41).

Stimulation of lymphocytes to mitosis requires the continual presence of both antigen and mitogen (14, 15). Macrophages are required accessory cells for antigenic and most mitogenic stimulation (94-96). Additionally the macrophage and lymphocyte must be histocompatability matched for maximum stimulation to occur (17, 18). Pulsing macrophages with antigen before lymphocyte addition produces the same results as continuous presence of antigen. Rosenthal has suggested a differential route for antigen presentation as opposed to degradation by macrophages (11). The vast majority of antigen is internalized and either fuses with golgi apparatus membrane and is recycled to the cell surface (inhibited by cytochalasin) or fuses with lysosomes and is degraded (ablated by metabolic inhibitors). When the fate of ferritin coupled PHA is followed in human blood lymphocytes,within a few minutes at 37° C the majority of label is found intracellularly and after several hours appears to coalesce and condense within the cytoplasm (25). In 40% of blast cells the labeled PHA was exocytosed to the cell surface. The majority of mitogen is found intact in T cell blasts (26).

Robinson et al (20) found that murine lymphocytes which ordinarily possess few receptors for the lectin wheat germ agglutinin (WGA) were markedly labeled by fluorescent WGA conjugates when cultured in the presence of B or T cell mitogens. Increased WGA binding was also observed with

lymph node cells of infected mice (21). Petris and Raff (22) using immuno-ferritin electron microscopy found the mouse lymphocyte membrane antigens theta, TL, H-2 allo-antigens and Con A receptor sites randomly distributed unless cross-linked by multivalent antibodies or ligands. There is a random distribution of Con A receptors and immunoglobulins when Con A is present in excess (23) but a non-random patch and cap formation when α-D-methyl mannoside or the microtubule assembly inhibitors colchicine, colcemid, vinblastine and vincristine are added. When the native tetrameric Con A is succinylated to a dimeric form capping and patching of receptors do not occur but mitogenic stimulation still takes place. Glucocorticoid receptors increase during mitogen stimulation, and there is an increase in membrane fluidity detectable within the first 30 minutes of lectin association by spin labeling techniques (24).

II. Intracellular Mediators of Lymphocyte Stimulation

Quastel and Kaplan (28) first reported the ouabain sensitivity of human lymphocytes activated by PHA. The ouabain inhibition of synthesis of RNA, DNA, and protein as well as blast and mitotic progression could be reversed by adding potassium to cultures. K^+ is taken up by PHA stimulated cultures (29) thus suggesting a role for $Na^{\pm}$, K^+-ATPase in stimulation events (30).

By medium depletion studies or through addition of cationic sequesterants Ca^{++} was shown to be necessary for mitogenic stimulation, intracellular accumulation

occuring within the first hour of PHA stimulation (31). Ca^{++}uptake is independent of ATP and membrane depolarization, modulated by cyclic nucleotides,and inhibited by mitochondrial uncouplers, colchicine and vinblastine. (32). Ca^{++}accumulation appears to be biphasic with a rapid uptake occurring within 5-30 minutes of mitogen addition, a stable intracellular concentration for 4-6 hours and a gradual efflux over the next 12-20 hours. Although PHA responsiveness is depressed by cation chelation using EGTA a significant response still occurs (33). Measurable intracellular calcium accumulation seems to result only at supraoptimal mitogen doses (34). Calcium ion fluxes have been associated with secretion in other mammalian cells e.g. insulin secretion by pancreatic islet cells (167) and the possibility remains that Ca^{++} accumulation is required for lymphocyte mediator secretion but not as an obligation for progression through the cell cycle.

Whitney and Sutherland (35) were able to modulate lymphocyte activation in a mixed lymphocyte culture by varying the concentration of and time of exposure to cAMP (cyclic adenosine 3', 5'-monophosphate). cAMP functions antagonistically to cGMP (cyclic guanosine 3', 5'-monophosphate) in its effect upon mitogen stimulation. Elevated levels of cGMP appear to be associated with enhancement of cell proliferation while increase in cAMP depresses proliferation (35, 36). cAMP phosphodiesterase activity is enhanced in Con A stimulation or by addition of

cGMP (38). Although significant type I and II cAMP dependent protein kinase levels are present in resting human peripheral blood lymphocytes Con A stimulation activates only type I (39). Dibutyryl cAMP at concentrations that block RNA and DNA synthesis results in activation of both enzymes. Their regulation within the lymphocyte may be a consequence of cAMP/cGMP ratios.

III. Membrane Transport

A rapid increase in membrane transport of many metabolites after mitogenic stimulation of lymphocytes occurs. 5 fold increase above controls in transport of the non-utilizable glucose analog 3-O-methyl-glucose is obtained (43) reaching a maximum 30 minutes after PHA addition. There was no change in Km for the glucose analog transport and the bidirectional transport probably was mediated by facilitated diffusion. There is increased transport for the amino acid analog α-aminoisobutyric acid (AIB) with an increased Vmax and constant Km (200), after a lag of 30 minutes from addition of PHA. AIB transport enhancement is inhibited by metabolic and protein synthesis inhibitors, is independent of RNA or DNA synthesis (45, 46) but parallels the increase in DNA synthesis (44). The relative mitogenicity of different lectins as determined by thymidine uptake by lymphocytes correlates with stimulation of proline transport (42).

Hanrahan et al (47) propose four phases of thymidine transport that are independent of the stimulant dose or nature. First there is a period equal to control uptake;

a rapid rise in uptake; exponential increase of uptake; and a declining phase. Uridine transport is increased within minutes and thymidine by 24 hours both as a result of higher respective Vmax but stable Km (48). Uridine and thymidine transport are both believed to operate by facilitated diffusion.

IV. Other Biochemical and Metabolic Events of Stimulation

In an eloquent series of studies (49-51) Loos and Roos examined carbohydrate metabolism in PHA and antigenically stimulated human peripheral blood lymphocytes. With mitogenic stimulation changes in metabolism were noted within a few hours of culture but antigenic (tuberculin purified protein derivative=PPD) and allogeneic cell stimulation required 2-3 days for detection and the magnitude of change was less. They calculated that 15% of the cellular ATP originated from glycolysis, while 85% formed from oxidative phosphorylation. It was concluded that either glycolysis or oxphos could support the early stage of lymphocyte stimulation but that both were required for extended maintenance of stimulation.

Heat evolution in antigen stimulated horse lymphocyte cultures parallels thymidine incorporation, increasing in 48 hours of culture and peaking at 5-6 days. With Con A heat liberation begins at 6 hours while inhibition of RNA and protein synthesis blocked generation of heat for both mitogen and antigen cultures, but DNA synthesis inhibition had no effect (52).

PHA induces a 10 fold increase of selective incorporation

of inorganic phosphate (Pi) into phosphatidyl inositol of the plasma membrane within 10 minutes of culture and enhances Pi turnover (53, 54). Most mitogens accelerate Pi incorporation specifically into phosphatidyl inositol within 30 minutes with the exception of pokeweed mitogen which requires hours of incubation (55). Pi is also incorporated into phosphatidyl choline and phosphatidyl ethanolamine by tetanus toxoid mitogenic stimulation (56). Labeled acetate is rapidly found in long chain fatty acids (57), sterols and phospholipids (58).

PHA induces a doubling in the rate of glucosamine incorporation into UDP-N-acetylglucosamine (59). T cell blasts take up GDP-mannose into cell surface molecules possibly linked to glycoprotein formation (60).

Changes in nucleoproteins also accompany immunological stimulation of lymphocytes. Protein synthesis-independent histone acetylation is observed within the first minutes of PHA interaction (63, 168). Increased rate of phosphate turnover occurs during the initial period of RNA synthesis but there is no net increase in histone phosphate content (169). Nucleoprotein methylations have also been reported (170).

Protein synthesis rates are sensitive to the effects of mitogens. PHA activation leads to a rapid recovery of labeled amino acids in cell proteins (61 - 63). Actinomycin D which intercalates between the dG-dC base paired dinucleotide sequence of double stranded DNA (64, 190) has been a useful probe for separating DNA-dependent from

DNA independent phenomena. By varying the concentration different RNA species may be inhibited from synthesis to proportionate degrees. When rRNA synthesis is inhibited by 0.01 micrograms/ml of actinomycin D the characteristic 18 S and 28 S peaks on RNA sedimentation profiles are diminished (65). The PHA enhanced amino acid label incorporation still occurs but the rate of uptake ceases to rise after 24 hours of culture, suggesting that the primary increase in protein synthesis rate is independent of transcription. Using RNA-DNA hybridization with RNA from stimulated human lymphocytes and DNA from resting lymphocytes, and polyacrylamide gel electrophoresis of proteins,results of Neiman and MacDonnell (66) suggest that protein synthesis of early stimulation is performed with preexisting RNA templates and the synthesized proteins are amplifications of proteins expressed in resting cells.

PHA stimulated lymphocytes after about 72 hours of culture contain 60-70% polysomes compared to about 25% for controls (67). The polysomal profile and amount of ribosomes sedimenting at 80 S seem to correspond quantitatively with protein synthesis (68). The majority of ribosomes in resting lymphocytes exist in an inactive 80 S complex (69). Within the first 20 hours of stimulation most of the ribosomal units are polysomal or dissociated into subunits, but after 48 hours there again is an increase in the inactive 80 S ribosomes. It would appear that the rate of dissociation of the inactive 80 S to the

60 S and 40 S subunits of the ribosome prior to initiation of translation limits protein synthesis activity. Kay et al (68) concluded that control of the increase in protein synthesis lies in control of the formation of the protein synthesis-active 80 S complex by initiation factors. Cytoplasmic factors support an increased capacity of ribosomes from PHA stimulated lymphocytes to synthesize proteins in a cell-free system (67).

When lymphocytes are stimulated with PHA and concurrently inhibited by actinomycin D there is an immediate decrease in leucine incorporation along with the decreased uridine uptake (71). A brief lag period before the decline in leucine uptake when sufficient quantity of actinomycin D is present would be expected if the RNA synthesis was merely a non-limiting corequirement for protein synthesis. There is actually a decrease in the amount of mRNA bound to ribosomes during the first few hours of PHA stimulation (70).

V. Ribonucleic Acid Synthesis

Precursors for RNA synthesis in mammalian cells are derived from de novo synthesis or from salvage pathways for degraded nucleic acids. The parent pyrimidine molecule uridine 5'-monophosphate (UMP) originates from the starting compounds aspartic acid and carbamoyl phosphate (64). The intermediate orotic acid is joined by a ribose-5-phosphate group derived from 5'-phosphoribosyl-1-pyrophosphate (PRPP) and decarboxylated to form UMP. UMP is phosphorylated to uridine-5'-triphosphate

through the action of kinases. Cytosine is synthesized from UTP through a specific synthetase. The triphosphate pyrimidine derivative is the form incorporated into the RNA polymer.

The parent purine ribonucleotide inosine-5'-monophosphate (IMP) originates from an amino group transfer from glutamine to PRPP. Subsequent steps involve combining with glycine, two formylations involving tetrahydro folate intermediates, another amino group transfer from glutamine, carboxylation and aspartate addition. Amination of IMP produces adenosine-5'-monophosphate (AMP) and amination of xanthosine-5'-monophosphate gives GMP.

Mammalian cells also have the capacity to convert free bases via PRPP and nucleosides derived from nucleic acid catabolism to nucleotides for reuse in synthesis of RNA and DNA. The relative importance of the salvage vs de novo pathways in lymphocyte stimulation has been elucidated by investigations of certain purine metabolism disorders in humans. Patients with Lesch-Nyhan syndrome have normal blood lymphocyte counts and their lymphocytes respond well to PHA, pokeweed mitogen, and allogeneic cell stimulation (73) even though they are deficient in the major salvage pathway enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Analogs of de novo purine synthesis precursors such as azaserine cause complete inhibition of PHA response in these lymphocytes while the purine analog 6-mercaptopurine has no effect. In normal PHA stimulated lymphocytes azaserine has only

a slight effect at the same concentration while 6-mercaptopurine strongly inhibits the response. Deficiencies in adenosine deaminase (74) which deaminates adenosine to inosine and purine nucleoside phosphorylase (75) for conversion of inosine to hypoxanthine have been associated with immune disorders.

Human lymphocytes incorporate significant amounts of glysine into purine nucleotides within two hours of exposure to PHA and have an increased capacity to take up adenine and adenosine into adenine nucleotides (76). Levels of PRPP are also enhanced in PHA stimulated cells so that both pathways of nucleotide synthesis appear to be functioning in normal lymphocytes. Pyrimidine salvage and de novo synthesis are also stimulated by PHA (77), although uracil does not seem to be utilized (1). Adenosine has been shown to inhibit lymphocyte blast cell growth by blocking de novo pyrimidine synthesis (78, 79). Uridine addition to PHA stimulated human lymphocytes shifts the cell from de novo synthesis to the salvage pathways for direct incorporation into RNA (80).

The plasma membrane-bound enzyme 5'-nucleotidase that catalyzes dephosphorylation of nucleoside-5'-monophosphates has increased activity on mitogen stimulated murine lymphocytes (81) and appears to be higher in concentration on T cells (83).

Net RNA increases over the course of stimulation (82) with short lived 4 S and polydisperse species appearing first in the sedimentation profile. Next the ribosomal

18 S, 28 S, and 45 S peaks appear. PHA activation reverses the rapid degradation of 28 S and 18 S species that normally occurs in the nucleus of the resting lymphocyte (85), although there is no apparent change in cytoplasmic ribonuclease activity (86). Extractable levels of RNA polymerase I, II, and III increase upon cell activation (87). The activity of soluble deoxyribonucleoprotein-bound RNA polymerase increased after a detectable rise in uridine uptake (88).

Heterogenous nuclear RNA (hnRNA) is specifically labeled by short pulses of radioactive nucleotides in the presence of quantities of actinomycin D sufficient to block rRNA synthesis (89). Polyadenylation may be related to stability and processing of mRNA (89). In the human lymphocyte PHA stimulation appears to increase rates of RNA maturation and transport from nucleus to cytoplasm (90). Processed tRNA seems to appear in cytoplasm before rRNA is detected. Polyadenylation of RNA and the appearance of polyadenylated RNA in the cytoplasm does not occur until several hours of lymphocyte exposure to the lectin (91, 92).

VI. Lymphocyte Cell Cycle

The human lymphocyte is fastidious in culture with a steady loss of viable cells within 48 hours (99). Media"conditioned" by stimulated cells (5) or lectin, antigen stimulation is necessary to maintain a continuously growing culture. Generally less than one percent of the cells respond to antigens (97, 98), and

approximately 50% respond to mitogens (99).

In the normal individual 90% of the peripheral blood lymphocytes are in a resting state possessing few visible organelles, generally scattered ribosomes, large mitochondria, and undeveloped Golgi apparatus (171). Large blast cells appear in culture at least by 48 hours of stimulation and 50% or more of the cells are blasts at 72 hours of lectin activation (100). These changes are accompanied by increasing nuclear volume (101) and cytoplasmic basophilia (102). Nucleoli undergo morphological transformations that follow the phase of the cell cycle (103).

Understanding of the lymphocyte cell cycle has increased from data of flow cytometry studies. The instrumentation has become available (at considerable cost) allowing rapid 50,000 cell/minute multiple parameter analysis of cell populations. The flow microfluorometer measures the fluorescent intensities of tagged cells passing rapidly single-file through a beam of laser light which activates the fluorescent labels. Data is collated by computer interfacing. Using the stoichiometric DNA stain mithramycin, Cassidy et. al. (104) were able to detect blastogenic responses of human lymphocytes to PHA, PPD and viral antigens. DNA-specific propidium iodide (105) and the metachromatic stain acridine orange (106) have also been used. Stacking of acridine orange to RNA and intercalation in DNA produces different fluorescence peaks (107, 108). Cell transition from cell cycle phase G_o to

G_1 reflected by an increase in DNA fluorescence and decrease in RNA peak occurring in absence of DNA synthesis is observable after 6 hours in cultures stimulated by PHA or allogeneic cells (201, 202). After two days of culture the number of cells in transition decreases and the population of cells in G_o and G_1 becomes distinct. There was observed a heterogenicity in duration of the S phase, with cells having the highest RNA concentration completing S phase at the fastest rate. A shift in the average length of G_1 in cultured cells is usually the way a change in cell generation time is mediated (112).

The quiescent lymphocyte exists in G_o phase until antigenic or mitogenic stimulus pushes the responsive cell into G_1 growth phase. The majority of blast cells appear to be derived from repeated mitoses of the same cell population (97). For some mammalian cells G_1 may be bypassed during a period of rapid cell cycling (113, 114).

DNA synthesis occurs in S phase up to the boundary of G_2, the tetraploid stage before mitosis (115). The S phase is the point frequently assessed by thymidine incorporation. The activity of thymidine kinase is increased 200 fold (116) and thymidine catabolizing enzymes have a much decreased activity during S phase (117) of lymphocytes. Low molecular weight DNA is excreted into the culture medium (118). Cells restimulated after a decline in DNA synthesis require less time for onset of

blastogenesis and DNA synthesis is more rapid and greater in activity than unprimed cells (120. 131).

VII. Cell-Cell Interactions

The term lymphokine (109) was first suggested to characterize biological activities expressed by cell-free soluble factors of lymphocyte origin. "Migration inhibition factor" was the first reported (110, 111) as a factor obtained from culture media of stimulated lymphocytes that inhibited the random migration of macrophages. Currently there is an extensive list of lymphokines that affect many functions of lymphocytes (2, 121) and any assay of cell mediated immunity must take into account their actions.

There is evidence that PHA induces only a shift to the G_1 activated state in responsive lymphocytes and lymphocyte activating factor (LAF or Interleukin I) produced by macrophages is necessary for induction of S phase (2, 174). cGMP mimicks the affects of LAF when present during G_1. Another factor produces a rise in cAMP in late G_1 and inhibits DNA synthesis and mitosis in T, B cells and fibroblasts. Pretreatment of lymphocytes with Con A suppresses subsequent response of thymidine incorporation to antigenic or mitogenic stimulants (122). With allergic rhinitis patients desensitized to ragweed antigen E antigen-specific suppressor cells could be detected in vitro when generated by incubation with antigen (123). Both "suppressor" or "helper" functions have been described as defective in certain immunological disorders (124, 125).

A defect in helper activity causing immunoglobulin deficiency is suspected when co-cultured X-irradiated normal T cells augment in vitro immunoglobulin synthesis or blastogenesis of patient's lymphocytes. Suppressor function is assessed in a similar manner. 1-2 KR of radiation ablates suppressor activity, while leaving helper intact. A cycloheximide sensitive, actinomycin D and colchicine insensitive B cell suppressor of T cell blastogenesis has been reported (126). There are also T helper cells that induce another T cell subset to exert suppressor activity (134, 135).

The macrophage plays another role besides antigen processing and presentation. Addition of normal peritoneal or splenic macrophages to murine splenic T lymphocytes at a level beyond 5-10% suppresses rather than enhances T cell proliferation (127). "Activated" macrophages exhibit an even greater suppressor activity (128). Lymphokines such as macrophage activating factor (129) activate the macrophage, and suppression may be mediated by prostaglandins and hydrogen peroxide (128).

A soluble factor produced by polymorphonuclear leukocytes has been found to enhance thymidine incorporation in stimulated lymphocytes (130).

Parasitic infections have been linked to suppression of the cellular and humoral immune response mounted against the parasite (132). A recent study (133) found that patients with patent microfilaremia were unresponsive to in vitro lymphocyte stimulation by filiarial

antigens although cell-mediated responses to other non-parasitic antigens such as PPD were normal.

VIII. Effector Cells

Immunological stimulation of specific T cells that recognize foreign antigens in association with self antigens or mitogenic stimulation leads to maturation of a variety of cells of the effector limb of the immune response. In some cases a maturation of the effector occurs without leading to mitosis (136). Allogeneic cell stimulation produces "natural killer" cells that specifically kill non-self cells (137). In the murine system the inducing T cell has the Ly $1^{+}23^{-}$ cell surface phenotype and the killer cell has the Ly $23^{+}Ly1^{-}$ antigens (138). The natural killer cells slowly disappear in culture but stimulation of the lymphocytes produces an "activated lymphocyte killing" of targets unrelated to the stimulating antigen (162). This new cytotoxic potential may be induced by interferon, occurs proportionally with blast formation and proliferation but does not require cell division (139).

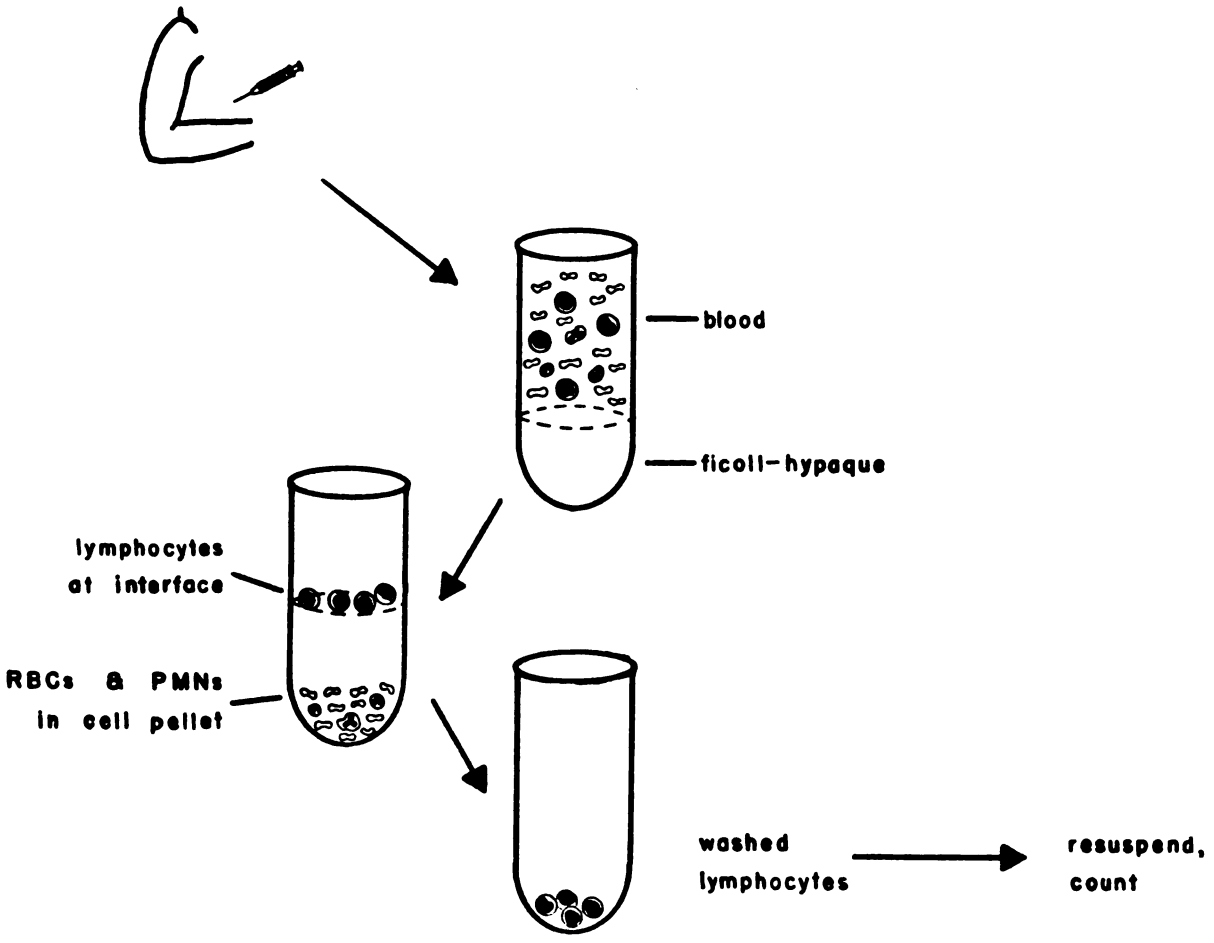
Lymphocyte Isolation
blood
ficoll-hypaque
lymphocytes at interface
RBCs & PMNs in cell pellet
washed lymphocytes
resuspend, count

Figure 1

MATERIALS AND METHODS

Blood donors: 40 healthy volunteers of both sex, 22 to 35 years of age, donated blood (20 to 50 ml) for these experiments.

Cell preparation: Diluted 1:1 (v/v) with RPMI 1640 (Gibco, Grand Island, NY), heparinized (20 ug/ml, Sigma, St. Louis, MO) venous blood samples were carefully layered on the top of Ficoll-Hypaque density medium (p=1.078 ug/ml, Ficoll, MW 40,000,Sigma; Hypaque, sodium diatrizoate, Sigma) in sterile 15 ml polystyrene centrifuge tubes (5 ml of the density medium and 10 m. of diluted blood) and the tubes were centrifuged applying 400 G at the interface for 20 min. (Figure 1). Cells which appeared at the interface were pipetted out, washed twice with the medium by centrifugation (300 G for 10 min.), cell concentration counted with a hemocytometer and adjusted appropriately.

Preparation of lymphocyte T and B cell subpopulations: T and B cells were separated from the original lymphocyte preparation using sheep red blood cell (SRBC) rosette formation. SRBC pretreated with aminoethylisothiouronium bromide (AET, Sigma) and adjusted to a 10% concentration in phosphate buffered saline (PBS) free of Mg^{++} and Ca^{++}containing 5% dextran (avg. MW 80,700, Sigma)

T and B Cell Preparation

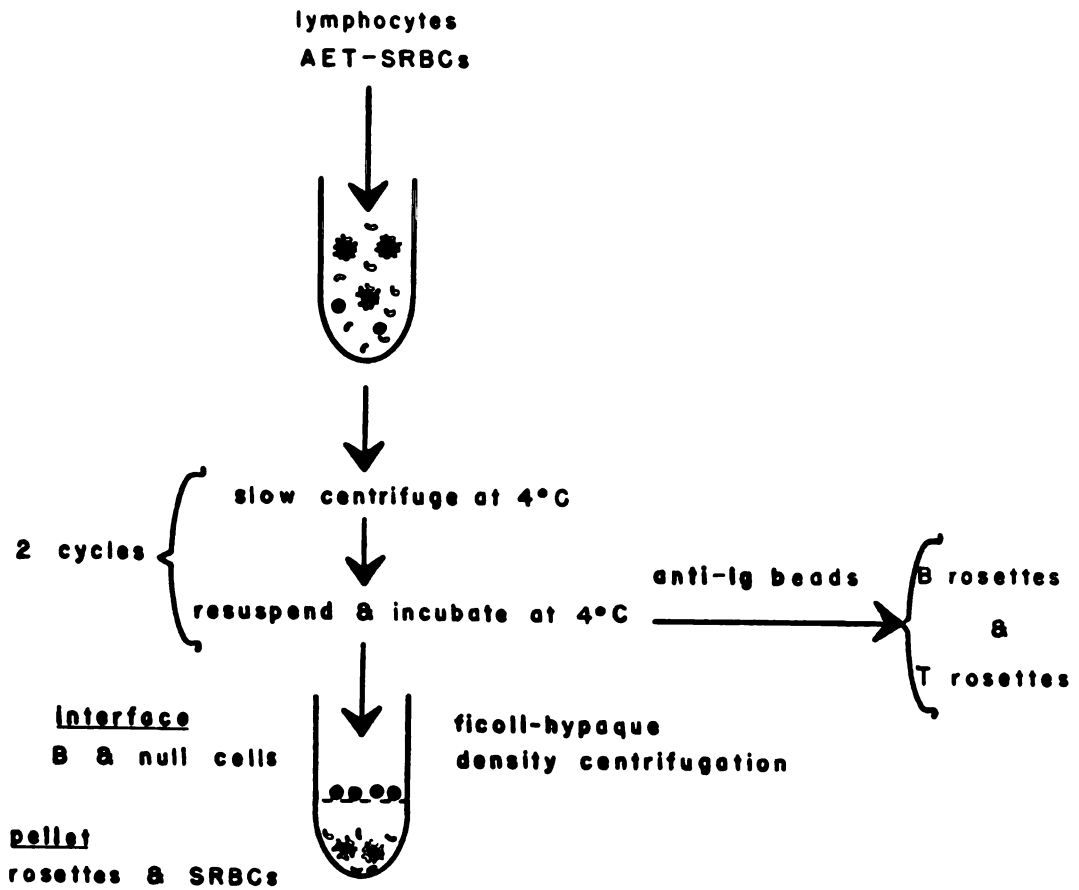

Figure 2

T and B cell characterization

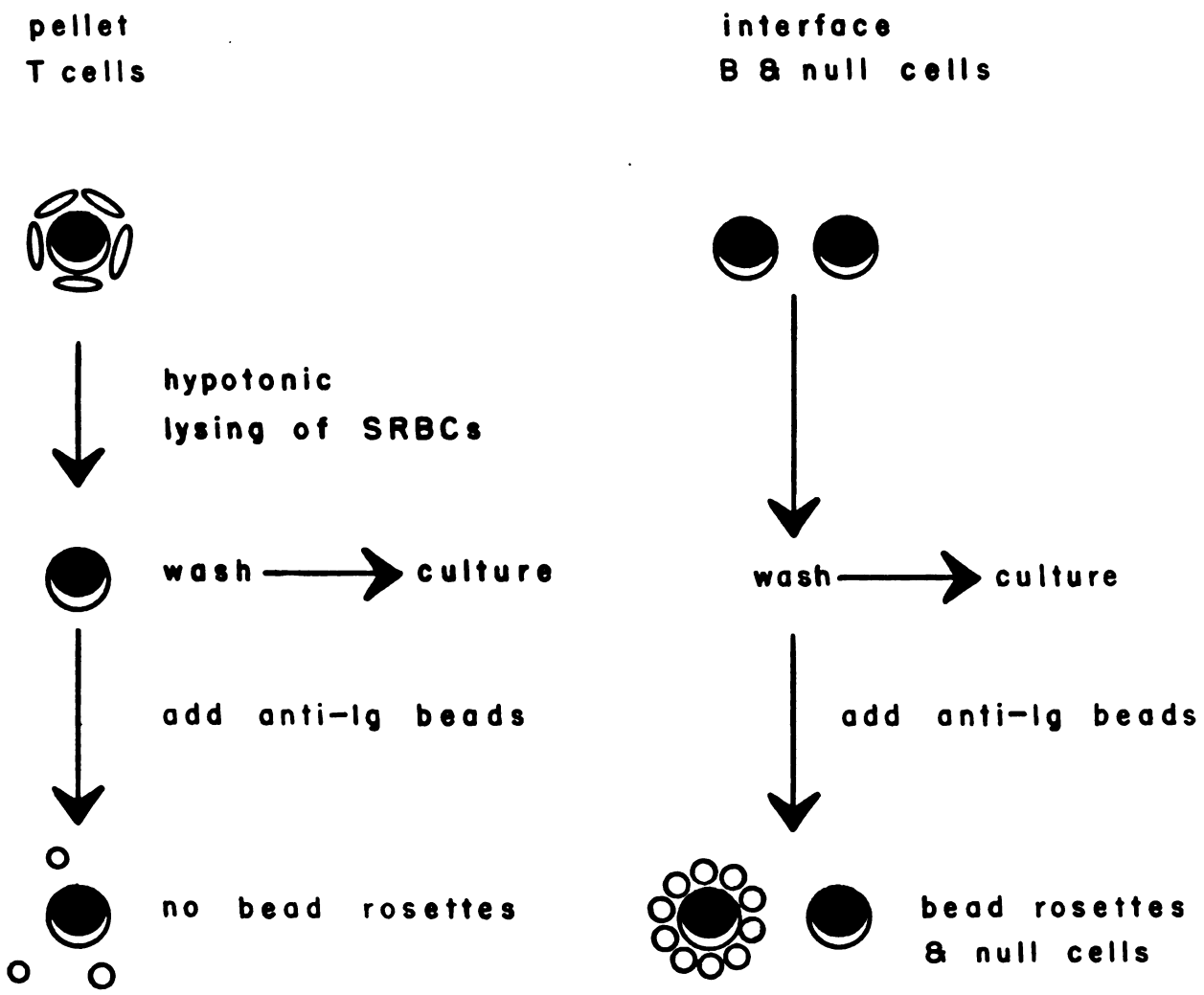

Figure 3

were mixed with an equal volume of lymphocytes at 5 x 10^6 per ml in the same medium and immediately centrifuged (50 G for 10 min. at 4°C), resuspended and incubated one hour on ice (Figure 2). After another 10 min. centrifugation (at 50 G) the SRBC + lymphocyte mixture was gently brought back into suspension and layered on the Ficoll-Hypaque density medum and centrifugated 20 min. at 400 G. The pellet was composed of rosette forming cells (T), while non-rosetted forming cells (B + null) remained at the interface of the density medium. The pellet was treated with hypotonic NH_4Cl EDTA solution and the isolated lymphocyte fractions were washed twice with RPMI 1640. The purity of the subpopulation isolates (Figure 3) was confirmed using polyacrylamide beads coated with rabbit anti-human heavy and light chain immunoglobulins (Immunobeads, Bio-Rad Laboratories, Richmond, California), which form rosettes (3 or more beads or SRBCs bound per cell) with B cells but not with T cells. Monocytes phagocytize the beads and may thus be distinguished from other mononuclear cells. The initial heterogenous lymphocyte preparation was also assessed for B and T cells by double rosetting with polyacrylamide immunobeads and SRBCs.

Cell culture preparation: All manipulations were performed under sterile conditions using a vertical laminar flow hood. Lymphocytes were cultured in microculture plates, 0.35 ml capacity wells (Linbro, New Haven, Conn.) with RPMI 1640 supplemented with 3.2 mM L-glutamine and

containing penicillin (100 IU/ml) and streptomycin (100 ug/ml; both Gibco). 5% heat inactivated fetal calf serum (FCS; Gibco) was added to the medium in some experiments. the cultures were composed of 100 ul of cell suspension per well, 10 ul of ^{3}H-uridine (5,6-^{3}H, 40.8 uCi/mM; New England Nuclear, Boston, Mass.) at 10 uCi/ml and 10 ul of PHA-P solution at appropriate concentration with 3 or more wells used per each experimental variant. The cultures were incubated at 37°C in a CO_2 incubator (5% CO_2-95% air, humid atmosphere). Cell cultures were collected on fiber glass filters (93H, Reeve-Angel, Whatman, NJ) with a semiautomatic multiple sample harvester (Otto Hiller Co., Madison, WI) using 5% TCA or 0.9% NaCl in distilled water for wash and precipitation. Dried filters with cell precipitate were placed in a toluene scintillation cocktail supplemented with Triton X-100 (New England Nuclear). A Searle Model 6890 Delta 300 Liquid Scintillation Counter was used for detection of cell precipitate radioactivity.

Reagents: Phytohemagglutinin (PHA-P) was purchased from Difco, (Detroit, MI). Dilutions were made in RPMI 1640 medium, sterilized by passage through a 0.22 um membrane filter and used within 10 days. Actinomycin D (Calbiochem, La Jolla, CA) was diluted in serum-free media and filter sterilized just before use.

Statistical analysis: Statistical tests performed were standard error, Student's t-test, coefficient of variation, and analysis of variance.

TABLE 1

EFFECT OF VARYING CELL DENSITIES AND CONCENTRATIONS OF PHA-P IN A MEDIUM CONTAINING SERUM ON (^{3}H) URIDINE INCORPORATION BY HUMAN LYMPHOCYTE CELL CULTURES

Cultures of 0.1 ml in medium supplemented with 5% fetal calf serum were incubated in the continuous presence of 10 µCi/ml (^{3}H) uridine for 20 hrs.

	Number of cells/ml culture	2.5×10^5	5×10^5	1×10^6	2×10^6	4×10^6
PHA dose	1.5 µg/ml	1.2±0.07[a]	1.3±0.09	1.5±0.08	1.4±0.09	1.6±0.12
	3.1	1.7±0.09	2.0±0.21	2.4±0.16	2.5±0.16	2.6±0.21
	6.2	2.0±0.19	2.9±0.22	3.3±0.16	3.5±0.18	3.8±0.28
	12.5	2.8±0.29	4.0±0.30	4.6±0.20	4.4±0.18	4.4±0.27
	25	3.3±0.32	4.6±0.35	5.3±0.22	5.0±0.20	4.9±0.32
	50	4.4±0.37	5.2±0.41	5.6±0.22	5.2±0.19	4.7±0.20
	100	5.0±0.48	4.9±0.31	5.4±0.37	5.0±0.20	4.1±0.15
control culture counts/min ± SE		1,669±75[b]	4,337±241	9,936±411	18,715±842	34,002±2,071

[a]Mean stimulation index ± standard error.

[b]Mean counts/min ± standard error.

EXPERIMENTS AND RESULTS

Establishment of optimum culture conditions

The optimum culture conditions were first established by manipulation of the cell concentration in the fixed 100 microliters/well volume and by varying the PHA dose. The stimulation index (SI) was determined by dividing the counts per minute of radioactivity (cpm) of PHA stimulated cultures by the nonstimulated control which provides a reference of uridine incorporation. 20 hours of culture was chosen as a convenient harvest time from preliminary studies.

Labeled uridine was present continuously during culture. Radiotoxicity is not a severe problem when culture times as short as these are used and label pulses of less duration had insignificant effect on SI. A final concentration of ^{3}H uridine at 10 microcuries/ml was used to give optimum incorporation by stimulated cells while decreasing the variability of background label incorporation by controls. This concentration of ^{3}H-uridine also produces a higher level of radioactive labeling which decreases the amount of time needed for liquid scintillation counting.

The initial cultures contained 5% serum in medium.

The PHA concentrations were made by serial dilutions over the range of 1.5 to 100 micrograms/ml of final concentration. Within this range the SI exhibited a dose effect (Table 1). Significant stimulation (at $p<0.01$) was

TABLE 2

EFFECT OF VARYING CELL DENSITIES AND CONCENTRATIONS OF PHA-P IN A MEDIUM WITHOUT SERUM ON (^{3}H) URIDINE INCORPORATION BY HUMAN LYMPHOCYTE CELL CULTURES

Cultures of 0.1 ml in medium were incubated in the continuous presence of 10 µCi/ml (^{3}H) uridine for 20 hrs.

	Number of cells/ml culture	5 x 10^5	1 x 10^6	2 x 10^6	4 x 10^6
PHA dose	1.5 µg/ml	1.76±0.09[a]	1.74±0.12	1.31±0.02	1.20±0.05
	3.1	1.76±0.19	1.63±0.02	1.37±0.12	1.38±0.09
	6.2	1.87±0.21	1.8 ±0.17	1.67±0.15	1.72±0.15
	12.5	2.91±0.46	2.99±0.32	2.56±0.17	2.42±0.11
	25	3.27±0.49	3.44±0.26	2.93±0.11	2.68±0.04
	50	2.79±0.33	3.34±0.16	3.09±0.05	2.30±0.17
	100	2.51±0.29	3.13±0.13	3.23±0.27	2.27±0.18
control culture counts/min ± SE		5,281.3±556[b]	10,856±1,088	25,617±3,055	41,675±8,841

[a]Mean stimulation index ± standard error.

[b]Mean counts/min ± standard error.

found at all concentrations of PHA for 1 x 10^6 cells/ml.

The control cpm exhibited a linear increase proportional to the increase in cell concentration over the range of 2.5 to 40 x 10^5 cells/ml. The highest SI value 5.6 was obtained at 1 x 10^6 cells/ml and 50 micrograms/ml of PHA, although there was no statistical difference ($p>0.4$) between SI for the upper and lower limits of the cell dilutions for this PHA concentration. With increasing cell numbers per culture well there is decreasing variation within the pooled data. The coefficient of variation (CV) varies from 24% for 2.5 x 10^5 cells/ml to 12% for 4 x 10^6 cells/ml at 50 micrograms/ml of PHA.

Table 2 shows the data for the same set of experiments when the lymphocytes were cultured in a medium without FCS. The overall SI are still obtained at significant levels while being lower values than that obtained with serum present. Similar trends are observed within the data when serum is present or omitted from the medium. The highest variation of SI between wells is found at the lowest cell concentration. 1 x 10^6 cells/ml again presents the highest stimulation at a dose of PHA that is however 50% the optimal amount when serum is present.

Time-dependent incorporation of ^{3}H-uridine

A series of experiments were performed to study the relationship of uridine incorporation by PHA stimulated lymphocytes with time of culture. 5 x 10^5 cells/ml were incubated with 100 micrograms/ml of PHA and 10 microcuries/ml of ^{3}H-uridine. Incorporation of radioactivity

Figure 4. Incorporation of ^{3}H-uridine by PHA stimulated lymphocytes with time of culture. Lymphocytes were cultured at a concentration of 0.5×10^{6}/ml with 100 ug/ml of PHA in RPMI 1640 containing serum. 10 uCi/ml of ^{3}H-uridine was added at the beginning of culture. The stimulation index is the ratio of cpm for PHA stimulated cultures to cpm for nonstimulated (control) cultures. Vertical bars indicate the standard error of the mean. ——— cpm for + PHA, .—.—. cpm - PHA, – – – stimulation index.

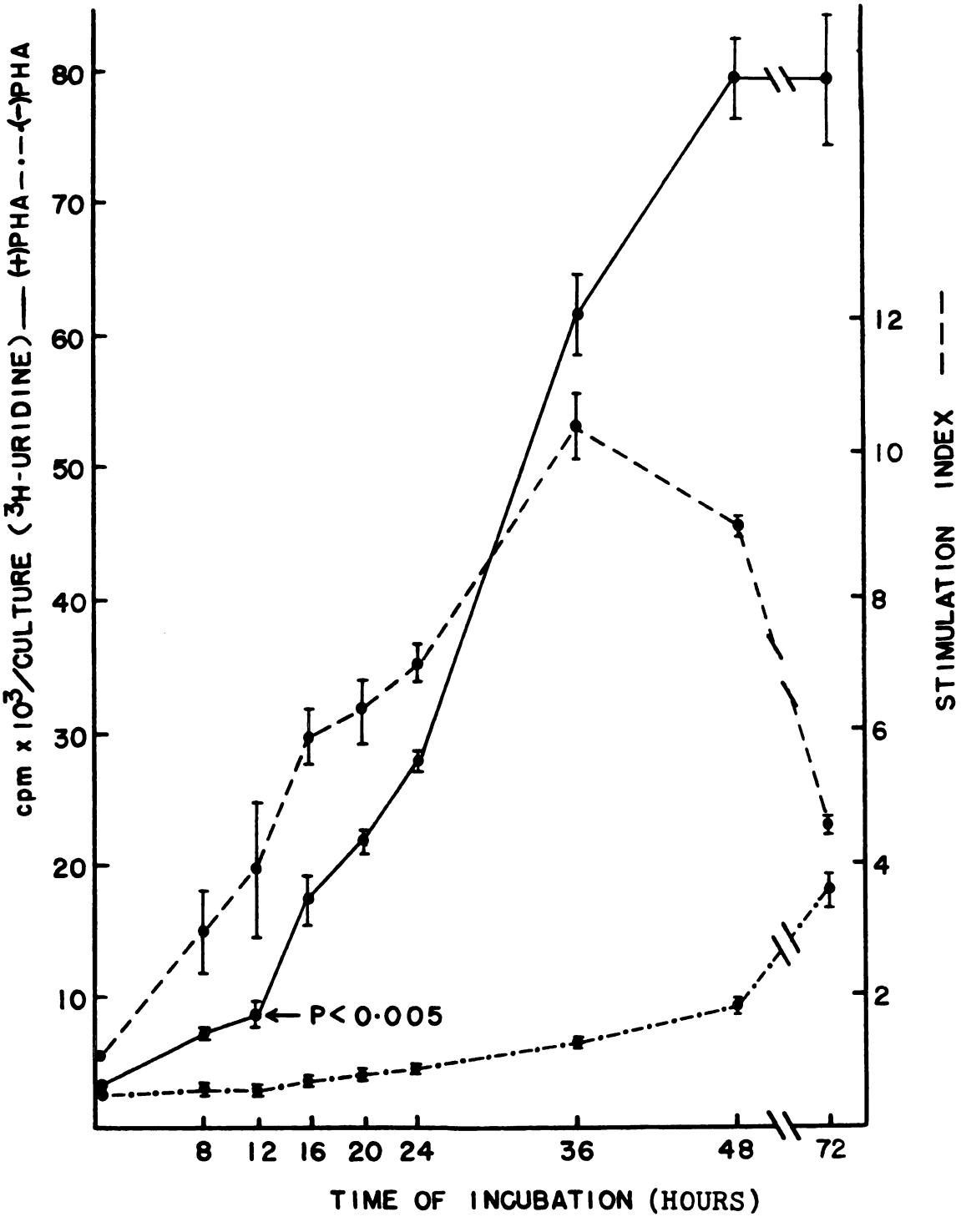
cpm x 10³/CULTURE (³H-URIDINE) —(+)PHA -·-(-)PHA
STIMULATION INDEX ---
80
70
60
50
40
30
20
10
12
10
8
6
4
2
P<0·005
8
12
16
20
24
36
48
72
TIME OF INCUBATION (HOURS)

Figure 4

TABLE 3

^{3}H-URIDINE INCORPORATION BY LYMPHOCYTES OF A SINGLE NORMAL INDIVIDUAL SAMPLED OVER AN EXTENDED TIME PERIOD

Cultures of 0.1 ml supplemented with 5% FCS at 1 x 10^{6} lymphocytes/ml were incubated 20 hours in the continuous presence of 10 uCi/ml of ^{3}H-uridine.

Day of experiment	Optimal dose of PHA-P	CPM of control cultures	Stimulation index
0	25 ug/ml	21,718	5.6
16	50	8,855	4.6
121	50	10,416	4.4
127	50	7,199	5.2
135	100	12,672	8.0
139	100	7,061	8.2
		12,424±2199 [a]	5.8±0.61 [a]

[a]Mean values ± SE

was determined at time intervals of 1 to 72 hours from start of cultures. Figure 4 illustrates the results in radioactive counts and SI. The cpm of the PHA stimulated cultures increases nearly linearly to 48 hours where it appears to plateau. The unstimulated cells cpm increases slowly over the course of the 72 hours. The maximum SI appears at 36 hours and beyond this time the increase of control label incorporation produces a net decrease in the SI. At 12 hours of culture the first statistically significant (at $p<0.005$) SI is obtained although there is an obvious increase in label incorporation before this time.

Lymphocyte stimulation in a single individual over an extended time of observation

Lymphocytes from a single normal individual were repetitively isolated over a period of a few months (Table 3) to determine possible fluctuations in level of ^{3}H-uridine incorporation occurring with time. By analysis of variance there is significant difference (at $p=0.01$) between the results obtained on different days both in terms of cpm and respective SI. In total variation the ratio of stimulated to control cultures cpm remained a more stable index of significant immunoreactivity than a simple comparison of cpm (CV=26% for the SI and 43% for raw cpm). Subtracting the control cpm from the stimulated cpm as a means of correcting for the background uridine incorporation produces a skewed value showing less precision between experiments (CV=55%).

TABLE 4

CELL SURFACE MARKERS ANALYSIS OF LYMPHOCYTES SUBPOPULATIONS

Proportion of cells demonstrating		Cell population		
		Initial Lymphocyte preparation	"B"-celled preparation	T-cell preparation
SRBC rosetting		79.5(79-80)[b]	0	92.5(91-94)
Immunobead rosetting		9.0(8-10)	74.5(68-81)	10.0(5-15)
Non-rosetting		11.5(10-13)	ND	ND
Non-rosetting with immunobeads	non phagocytizing	N D[a]	23.5(18-29)	81.0(70-92)
	phagocytizing	N D[a]	2.0(1-3)	9.0(3-15)

[a]Phagocytosis of beads not determined because simultaneous use of SRBC and immunobeads requires incubation at 4°C.

[b]Average value from 3 experiments and the range of values shown in parentheses.

TABLE 5

^{3}H-URIDINE INCORPORATION BY LYMPHOCYTE POPULATIONS EXPOSED TO PHA-P IN VITRO

Either 5 x 10^{6}/ml heterogeneous lymphocytes, T cells, or B cells were cultured for 20 hrs. with PHA in the continous presence of 10 uCi/ml ^{3}H-uridine.

Cell populations[a]	Experiments #1	#2	#3	#4
	Stimulation Index[b]			
Heterogeneous lymphocytes	3.36±0.08[c]	3.57±0.11[c]	5.02±0.34[c]	2.56±0.20[c]
T cell populations	5.42±0.05[d]	5.23±0.08[d]	7.34±0.11[d]	5.50±0.16[d]
B cell populations	1.65±0.03[e]	0.93±0.02[e]	0.87±0.10[e]	1.06±0.05[e]
Reconstituted peripheral blood lymphocyte populations[g]	3.92±0.10	4.32±0.02	ND[f]	ND[f]

[a]Cell populations were characterized by means of surface markers (see methods).

[b]Mean stimulation index ± standard error.

[c,d,e]Statistically significant (at $p<0.05$) differences between c and d, c and e, d and e within each experiment.

[f]not determined.

[g]composed of 90% T-enhanced and 10% B-enhanced lymphocyte populations.

Cellular specificity of ^{3}H-uridine incorporation

The initial lymphocyte preparation was separated into SRBC-rosetting and non-rosetting subpopulations by density centrifugation. Maximal rosetting occurred when two gentle centrifugation steps were used. The isolated subpopulations were characterized as T cells by the presence of SRBC receptors and absence of surface Ig, and B cells with surface Ig and lacking SRBC receptors. Monocytes were identified by phagocytosis of immunobeads. As shown in Table 4 approximately 80% of the lymphocytes obtained were T cells, 9% B cells, and 11% null cells which exhibit neither characteristic marker. Within the B cell preparation no SRBC rosettes were observed while over 90% occurred in the cell pellet. There were approximately 2% monocytes in the B cells and 9% present in the T cell preparation.

Table 5 presents the results obtained when 5×10^{6} cells/ml of the separated B and T cells were cultured with 50 micrograms/ml of PHA for 20 hours. Within four experiments there was significant incorporation of ^{3}H-uridine by T cells and the original unseparated lymphocyte preparations. The SI for the B cell cultures remained near control values. In two experiments when the original heterogenous preparations were reconstituted by combining the isolated T and B cells at a ratio of 90 T cells to 10 B cells the resulting SI were near the original values(Table 5).

Association of ^{3}H-uridine incorporation with RNA synthesis

Lymphocytes were cultured with the RNA synthesis inhibitor actinomycin D at a range of 0.1 to 10.0 micrograms/ml for 20 hours, with media containing 5% FCS, to examine the relationship between increased radiolabel uptake in PHA stimulated cultures and RNA synthesis. There was a dose-dependent decrease in recovery of the label from all cultures, with complete inhibition appearing at 10 micrograms/ml of actinomycin D. Cell viability near control in actinomycin D treated cultures was confirmed by trypan blue exclusion by viable cells at 20 hours of culture. Inhibition of the increase in PHA stimulated radiolabel uptake and the fact that the recovered label was incorporated into TCA and saline precipitable material suggested that ^{3}H-uridine incorporation was a result of RNA synthesis stimulation.

DISCUSSION

Lymphocytes originate from pluripotent stem cells present in embryonic liver, blood, or yolk sac, or adult bone marrow that through selective induction give rise to progenitor cells committed to a specific lineage of B or T cells (156). These precursor cells become immunocompetent T cells after traffic through the thymus (196). The mature small lymphocyte recirculates rapidly in a cycle through the blood and lymphoid tissues (cell"traffic"), blood residency time being measured in hours (197). B cells are transformed by immune stimulation into plasma cells which actively secrete immunoglobulin (Ig).

Peripheral blood leucocytes may be delineated into three broad categories of cells: T cells, B cells, and null cells, based on the distinction of unique cell surface markers. T cells possess receptors for sheep red blood cells (SRBC) with no surface Ig, while B cells are marked by the presence of surface Ig and absence of SRBC receptors. Null cells are the remainder of cells possessing neither receptor, probably composed of a mixed population of cells of lymphoid and non-lymphoid origin.

B lymphocytes and null cells possess receptors for complement (157-159) and B, T, and null cells all have receptors for the Fc portion of immunoglobulins. In peripheral blood 60-70% of T cells have IgM Fc receptors and less than 20% have receptors for the Fc portion of IgG (194). These T

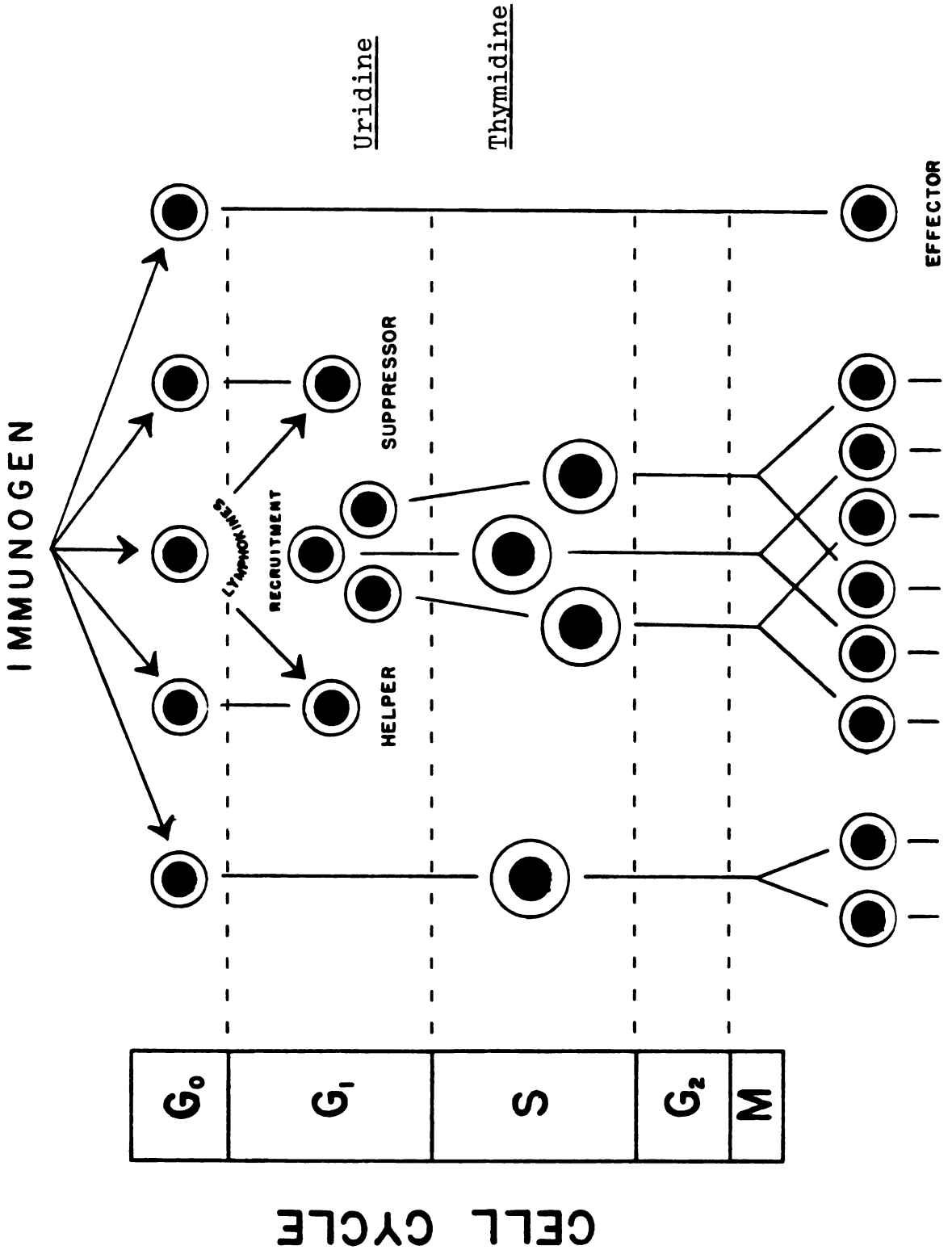

Figure 5 Lymphocyte Stimulation

cell subpopulations differ in PHA stimulation response and in vitro modulation of B cell Ig production (195). The Ia antigens are distinct alloantigens on B cell membranes normally occurring on only 1-2% of T cells (160). After stimulation with mitogens, antigens, or alloantigens a high percentage of T cell blasts become Ia positive, but this new phenotypic expression occurs after the peak of thymidine incorporation.

Figure 5 illustrates the possible consequences of lymphocyte interaction with an immunogen, here defined as any antigen or substance producing immunological stimulation. A generalized response for a lymphocyte could be entering G_1 and blast formation, movement through the DNA synthesis stage and tetraploidy, followed by division into daughter cells and an expansion of the mitotic response as long as the inducing stimuli are still present. There is at least one stage of the cell cycle, the G_1/S boundary, that requires additional stimulus to traverse as the ratio of mitotic cells to blast cells is always low. An immunogen-responsive lymphocyte is not obligated to progress through S phase, as is the case in some instances for effector antibody producing cells(139) and activated cytotoxic cells which are induced without noticeable mitotic activity (136). Interleukin 1 (LAF-IL 1) provides the stimulus when present with immunogen for activated T cells to enter S phase (174).

In an antigen-specific response the actual number of cells responding to the primary stimulus is low but through recruitment of other lymphocytes by lymphokines produced during the

initial stages of stimulation (G_1), or cell contact and other mechanisms,the immunological response is amplified. B cells may also be recruited into cellular immune responses (192). In a system where only recruitment progressing to mitosis of non-stimulated lymphocytes by stimulated cells occurs or clonal expansion of responding cells, measuring the DNA synthesis phase provides a sensitive indicator of stimulation of the small primary responding population. Augmenting or diminishing the extent of proliferation or effector cell induction however are the DNA synthesis-dependent suppressor lymphocytes and the helper cells. Suppressor cells are generated in vivo by immunization with a high dose of antigen, probably overriding the antigen-presenting function of macrophages (164), or by desensitizing doses of antigen (123). Helper T cell induction is regulated by other T cell subpopulations (165,166). Within the cell culture system each distinct human T helper, suppressor and monocyte subpopulation (175) or animal regulatory and effector cells (166,176) can now be identified specifically by use of monoclonal antibodies or specific antisera.

In a continuous lymphoid cell line it was demonstrated that lymphokine secretion (MIF) occurred maximally in the S phase of the cell cycle (196). It is possible that other immune regulatory factors are released primarily at the late stages of the cell cycle also. Blast cell formation and actual increases in lymphocyte numbers may not always correlate with thymidine incorporation (193). For accurate determination of stimulation potential quantitation of events

preceding S phase is required. In the study presented significant RNA synthesis occurs at 12 hours of culture, prior to the DNA synthesis phase and as such is removed from regulatory mechanisms functioning with extended time in culture.

In developing this assay it was first necessary to define the optimal conditions for maintaining peripheral blood lymphocytes. Generally 1 x 10^6 lymphocytes were isolated per 1 cc of blood. Using the standardized density centrifugation procedure for isolation of lymphocytes from other contaminating cell types a certain percentage of cells may be damaged by temperature changes, physical disruption and by other indeterminate factors. Because of the fastidious nature of lymphocytes in vitro the number of viable cells in culture declines with time. The presence of serum in the medium has a beneficial but not totally understood effect on maintenance of mammalian cells in culture. In this system 5% FCS was used and increasing the total amount present to 15% had no significant effect on the stimulation index. Substitution of FCS with 5% autologous or pooled human AB serum similarly had little effect on the SI. When lymphocytes were cultured in medium with serum present (Figure 1) there is an apparent stimulatory effect increasing with time in culture for the control lymphocytes. This increase of uridine incorporation with time does not occur when lymphocytes are cultured in serum-free medium (187). Mitogenic factors present in the serum (191) and possible primary sensitization of cells (178,179) in response to serum antigens may contribute to this increased label uptake.

Without serum a higher label uptake by unstimulated cultures (Table 2) was evident, which reduced the net SI for PHA-stimulated lymphocytes. The optimum SI for the serum-free cultures was obtained at a lower PHA concentration (25 microgram/ml vs. 50 microgram/ml) for 1 x 10^6cells/ml while the highest label incorporation was 30% lower than when serum was present. The sensitivity of the assay may be decreased by culturing without serum but since significant indices are still obtained being able to use a chemically-defined system may outweigh the disadvantages, especially in variability between lots of serum.

The lymphocytes cultured without PHA presented a basal rate of uridine incorporation that is directly proportional to the cell concentration. This may reflect the reduced but significant turnover of RNA in the resting cell (85). This control uptake is consistent between experiments with a CV of 12% for 1 x 10^6cells/ml, therefore slight increases above control in uridine uptake for stimulated cultures become significant. This level of background label incorporation also provides an internal control for detection of any technical problems. Generally background ^{3}H-thymidine incorporation in nonstimulated cells is very low.

For most of these experiments 10 cc of blood were ordinarily drawn yielding approximately 1-2 x 10^7 lymphocytes. For testing all of the PHA concentrations at each of the cell dilutions listed in Table 2.5 x 10^6 cells were required. In a clinical setting adequate quantities of blood may not be drawn from infants, elderly, or leukopenic patients.

However, the concentration of cells cultured may vary from 2.5-4.0 x 10^6 cells/ml with little significant change in the amount of ^{3}H-uridine incorporated with PHA present at the optimal dose, although less precision is obtained at the lower concentrations.

The time-course of ^{3}H-uridine incorporation by PHA stimulated cultures in media with 5% FCS was followed over a 72 hour time period (Figure 4). Enhanced incorporation is observed in PHA treated cells at eight hours of culture and significant incorporation (at $p<0.005$) occurs by 12 hours. In this system the maximum incorporation of either ^{3}H-uridine or ^{3}H-thymidine by stimulated lymphocytes is evident at the same PHA concentration but significant incorporation of thymidine followed uridine incorporation by at least 24 hours. With thymidine uptake the rate of increase of incorporation is proportional to the number of cells that remain unstimulated (172) and the number of cells not in S phase declines exponentially. Slight changes in the exponential rate at which cells traverse the S phase will produce large variations in thymidine incorporation (173). Over the range of exponential growth of stimulated lymphocytes as judged by thymidine incorporation there occurs a substantial responder cell recruitment (47). Uridine incorporation assessed during short-term culture should reflect the rate of increase of isotope incorporation that is directly proportional to the number of cells entering G_1.

Lymphocytes from a single normal individual were isolated at random intervals over a five month period of time.

All conditions for blood collection, lymphocyte isolation and culturing were kept constant. Uridine incorporation was significantly different between times of blood collection over the course of the observation period. Lymphocyte proliferative capacities have been observed to fluctuate on a daily (180) and hourly (181) basis when assessed by thymidine incorporation or blast formation. The proliferative response appears to conform to intrinsic individual day to day and circadian cycles. The day to day variability of ^{3}H-uridine uptake is consistent with the variability of thymidine uptake from these results. The stimulation index is a moderately stable measure for comparison of data within experiments. There is however considerable variation in SI between experiments, in agreement with data obtained by others for thymidine incorporation.

Age differences also affect the stability of the stimulation response. The subjects used in this study were not age-matched. In older patients there is observed a decline of *in vitro* stimulation response as a consequence of fewer responding cells and impaired division of the responding cells (163). Mitogen responses have been found in fetal thymocytes as early as 10-12 weeks of gestation, and an apparent antigen-specific response by cord blood and bone marrow lymphocytes at 19 weeks (161). The significance of this innate variability in PHA stimulated uridine incorporation *in vivo* is not known.

In order to determine that this system presented a valid test of cellular immunoreactivity it was of critical importance

to confirm that the enhanced uridine incorporation in cultures stimulated by the T cell mitogen PHA was specific to the T cell subpopulation of lymphocytes. T cells possess specific receptors not present on B cells that bind sheep red blood cells (SRBC) forming rosettes with multiple SRBC (182). Pretreatment of the SRBC with the sulfhydryl reagent AET produces consistant percentages of T cell rosettes using SRBC obtained from single or multiple lots (183). Lymphocyte binding affinity is increased by incubation with thymosin (184) and decreased by theophylline (185). Rosettes were stable when kept at 4°C and formation time was considerably decreased by using two steps of mild centrifufation. Slides of Giemsa stained smears were used to obtain the differential count of SRBC rosettes. For differentiating Ig-positive rosettes wet mounts of 3% formalin fixed cells were read using conventional light microscopy. Fab fragments of anti-Ig (heavy and light chains) antibodies covalently bound to polyacrylamide beads of one micron average diameter visualized surface membrane Ig molecules on lymphocytes. Use of Fab fragments as the reagent precludes the labeling of Fc receptors on cells, however the presence of residual Ig molecules bound to Fc receptors before incubation with beads may be a minor source of error for quantitating surface Ig.

The distribution of T, B, and phagocytic cells (Table 4) is consistant with literature values (160,186). When the purified B and T lymphocytes were mixed together, 9 T cells per 1 B cell, and cultured in the presence of PHA the SI

obtained were statistically different from the unpurified preparation only for experiment 2 ($p<0.025$). This implies that both lymphocyte subsets remained functionally intact throughout the separation procedure. Significant stimulation was obtained only with the purified T cell preparation but not for B cells, i.e. ^{3}H-uridine incorporation by PHA stimulated lymphocytes was specific to the T cell subpopulation. The degree of stimulation of the T cells was actually significantly greater (at $p<0.05$) than the unpurified preparation, possibly because of a slight suppressive effect exhibited by normal B cells (126).

With a residual macrophage population still present the purified T cells could enhance the sensitivity of the assay to detect weaker lymphocyte stimulation. The B cells cultured without PHA had a slightly higher background incorporation of label than the T cell control but not sufficiently high enough to affect the SI. There are no B cell-specific mitogens for human lymphocytes currently available so the alternative approach of studying uridine incorporation by specifically stimulated B cells was not possible. Using a murine system with spleen cells Pienkowski et al (187) demonstrated Con A stimulated ^{3}H-uridine incorporation by specifically stimulated T cells while increased label incorporation exclusive to the B cells occurred in response to the B cell-specific mitogen Escherichia coli lipopolysaccharide.

Cell collection and isolation of tritiated polyribonucleotides were greatly expedited by use of a semiautomatic

multiple sample harvester (Otto Hiller Co.). A row of twelve wells of the microculture plate are harvested simultaneously by repeated agitation and flushing of cells with 0.9% NaCl or 5% TCA (189) and drawn off by vacuum. Presumably the cells are disrupted by water pressure as they pass through glass fiber filters. RNA in the medium strength ionic solution is precipitated and remains bound to the glass fibers (188). Repeated washings with saline will flush free any unincorporated uridine. Semiautomatic multiple harvesters are currently used by numerous laboratories in processing lymphocytes labeled with radioactive thymidine for quantitation of DNA synthesis.

In addition to being TCA and saline precipitable it was necessary to prove that increased uridine incorporation could correlate with increased levels of newly synthesized RNA. At low concentrations of actinomycin D RNA chain elongation is inhibited while at higher concentrations initiation is also inhibited (190). rRNA synthesis is the most actinomycin D sensitive RNA species at low inhibitor concentrations. 1.0 microgram/ml of actinomycin D inhibited control lymphocyte cultures uptake of ^{3}H-uridine by approximately 60%, while the PHA induced increase was inhibited by 90%. At 10 microgram/ml the inhibition was nearly complete. This diminishing of label incorporation with no apparent effect on cell viability indicates that PHA stimulates RNA synthesis at an early stage of the cell cycle.

SUMMARY

The detection of early RNA synthesis by phytohemagglutinin stimulated human peripheral blood lymphocytes has been investigated as a rapid means of assessing immunoreactivity *in vitro*. RNA synthesis was quantitated by incorporation of ^{3}H-uridine, with significant incorporation achieved by 12 hours of culture and a 10 fold increase above controls observed by 36 hours. This study has established the parameters of a semi-microassay and has shown that enhanced ^{3}H-uridine incorporation by phytohemagglutinin stimulated lymphocytes is exclusive to the T cell subpopulation as determined by the presence of characteristic cell surface markers. Serum supplements to the culture media were not required to obtain significant and reproduceable results. Quantitation of early RNA synthesis is a practical method for assessing cellular immune competence.

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