IN VITRO LYMPHOREACTIVITY AS ASSESSED BY ^{3}H-URIDINE INCORPORATION

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ABSTRACT

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By

Mary Margaret Lyerly

A new method for the analysis of *in vitro* murine lymphocyte immunoreactivity utilizing ^{3}H-uridine incorporation into RNA was developed. This microculture technique quantifies the responsiveness of a cell population earlier and at a different stage of the cell cycle than does the more widely used measurement of ^{3}H-thymidine incorporation into DNA. The variables of cell density, doses of mitogens, and ^{3}H-uridine dose were first investigated to establish optimal culture conditions. Subsequent studies on the time progression of the response indicated that immunoreactivity could be measured by ^{3}H-uridine incorporation as early as 4 hours. At that time, cultures stimulated with mitogens showed about an 80% increase in ^{3}H-uridine labeling over unstimulated ones. By 8 hours, this mitogen-induced increase of ^{3}H-uridine incorporation had reached over 300% of control values. Through the use of Actinomycin-D and sodium dodecyl sulfate, it was determined that the increased labeling of stimulated cultures was due to incorporation of ^{3}H-uridine into newly synthesized RNA. To explore the usefulness and validity of this technique, three separate approaches were used. First the requirement for serum in the culture medium was tested. The finding that serum was not necessary means that immunoreactivity may be assessed in a chemically defined medium. Secondly, the ability of the technique to selectively measure the responses of T and B cells was investigated. Both purified T and B cell populations were stimulated by mitogens specific for each population. The responses of both populations could be distinguished from each other by the ^{3}H-uridine method. Finally, lymphocyte stimulation was measured by ^{3}H-uridine incorporation at 24 hours and by ^{3}H-thymidine incorporation at 48 hours under otherwise identical culture conditions. A comparison of the results of the two techniques showed a similarity in the shapes of the dose response curves and in the optimal doses, further verifying results obtained with ^{3}H-uridine. Thus, the feasibility of assessing immunoreactivity of lymphocytes by measuring early metabolic events of stimulation has been demonstrated by the development of a microculture assay for ^{3}H-uridine incorporation.

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By

Mary Margaret Lyerly

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INTRODUCTION

The assessment of *in vitro* stimulation of lymphocytes has added valuable knowledge to experimental and clinical immunology in such diverse areas as membrane fluidity, antigen expression, functional differentiation, and antibody synthesis (120, 13, 99, 4). Investigations on lymphocyte activation have provided in-depth understanding of immunoreactivity with respect to metabolic changes occurring as cells enter and pass through the cell cycle and to the populations of lymphocytes involved in normal function and pathological conditions.

Populations of lymphocytes may be induced to divide by antigens or mitogens. Antigen stimulation of primed cells is followed by division of a small clone of cells. These antigen activated cells mount a response, such as the production of antibodies, against the antigen (74). In contrast, mitogen treatment results in the proliferation of a larger percentage of the cell population (58). The immunological response of these stimulated cells is directed against various antigens (4). Regardless of the stimulator, activated lymphocytes undergo similar metabolic changes.

This similarity in biochemical changes of stimulated lymphocytes resides at the level of proliferation which may be measured by the incorporation of ^{3}H-thymidine into DNA. Since cell division occurs simultaneously with the immune response, the measurement of DNA synthesis reflects the potential of a cell population to react immunologically. This immunoreactivity is most easily measured by stimulation of a large percentage of the cell population with a mitogen.

The measure of immunoreactivity has contributed to the knowledge about lymphocyte populations, which include T lymphocytes and B lymphocytes. Murine B cells bear immunoglobulin on their surface, secrete antibodies, and are stimulated by mitogens such as lipopolysacchride (LPS), dextran sulphate, and pokeweed mitogen (134, 27, 75). T or thymus derived cells bear the Thy-1 antigen, produce lymphokines, possess helper, killer and suppressor functions, and are stimulated by various mitogens including Concanavalin A (Con A), phytohemagglutinin (PHA), pokeweed mitogen and periodate (30, 91, 25, 5, 84, 82). The same lymphocyte populations occur in man, but surface markers have been less well characterized. Use of mitogens to define human pathological conditions and T and B cell defects has been widely accepted. For example, mitogen responses have been applied to the study of the mechanism of IgA deficiencies and X-linked agammaglobulinanemea, the prediction of the success of cancer therapy, and the monitoring of preleukemic and remission states (133, 76, 49).

Therefore, the in vitro lymphocyte stimulation as measured by DNA synthesis has contributed important knowledge to the field of immunology. Even greater advantages could be gained if immunoreactivity were assessed by measurement of an event preceding DNA synthesis. The analysis of earlier and different metabolic events could add information about the biochemical mechanisms underlying pathological and deficient immune conditions in addition to yielding results more quickly. Thus far, many mitogen-induced biochemical changes preceding DNA synthesis have been reported, but only changes in protein and phospholipid metabolism have been shown to be specific for responses of T or B populations (40, 102). Lymphocyte RNA metabolism has been extensively studied and it is known that within hours after mitogen stimulation ^{3}H-uridine is incorporated into RNA. In addition, unstimulated T and B cells do not differ in their capacity to incorporate labeled uridine (45). Therefore the use of ^{3}H-uridine incorporation was proposed as another measure of early immunoreactivity and was investigated with respect to microculture conditions and T and B response specificity.

REVIEW OF THE LITERATURE

I. Mitogen-Induced Changes in Lymphocytes

A. Morphological Features

The phenomenon of stimulation was first reported as a series of morphological changes in human peripheral blood leukocytes cultured with PHA, and most of the early work on metabolic changes was done with this mitogen (83). Lymphocytes cultured alone remain viable, but do not proliferate (95). In the presence of a mitogen, some of the cells enlarge, synthesize DNA about 48 hours later and divide (12). The distinctive morphological changes accompanying this stimulation are as follows: The dry mass of cells increases, the cytoplasm becomes basophilic and binds pyronin Y, cytoplasmic vacuoles appear, and the nuclei become more euchromatic (23, 73, 12).

Electron microscope studies have shown that within 15 minutes after the addition of a mitogen, cells agglutinate and are separated by a distance of about 200 angstroms (29). Within 4 hours, nucleolar size and granule concentration increases, but these nucleolar granules disperse by 48 hours (122). At that time, ribosomal aggregates form, mitochondria become more numerous, Golgi complexes enlarge, and the number of cytoplasmic lysozomes increases (20, 122). The formation of nuclear pores in activated cells has also been reported (112). These changes are generally the same for T or B cells responding to their respective mitogens.

B. Biochemical Changes

1. Alterations in Membrane Composition and Transport Properties

The earliest alterations in lymphocyte metabolism occur in membrane composition, followed by changes in transport properties of the stimulated cells. Labeled glucosamine is incorporated into membrane UDP-N-acetyl glucosamine of mitogen-treated cells at twice the rate of control cultures (51). Using ^{14}C-oleate, an increased turnover rate of the fatty acid moiety of membrane lecithin was found in stimulated cell populations (101). A short exposure of cells to various mitogens results in labeled phosphate incorporation into phosphatidyl inositol or phosphatidyl choline and phosphatidyl ethanolamine (35, 71).

These physical changes in the membrane may be important in changes in transport properties. The early influx of several ions following stimulation has been reported. Potassium and possibly sodium influx has been associated with changes in membrane Na-K-ATPase (97). Inhibition of this enzyme prevents potassium influx and subsequent RNA and DNA synthesis (96). Increased calcium influx was first reported following PHA treatment, and later with most types of mitogenic, but not non-mitogenic substances (3, 86). The stimulated membrane appears to have an increased affinity for calcium (129). It has been postulated that Ca^{2+} directly affects the enzymes that regulate cyclic nucleotide levels (128).

Other membrane transport changes include the increased uptake of glucose, uridine, and the nonutilizable amino acid α-aminoisobutyric acid (52, 89, 77). These transport changes are due to an increase in V_{max}, indicating that the affinity of the membrane for the substrate remains constant, but that the number of substrate binding sites has been increased. RNA and protein synthesis are not necessary for these effects.

An increase in membrane permeability as measured by neutral dye uptake has also been reported in stimulated cells (54).

2. Changes in Cyclic Nucleotides, Proteins, and Energy Metabolism

Membrane changes are followed by and in most cases linked to cytoplasmic changes. Intracellular cyclic nucleotide levels shift with mitogen stimulation. The earliest reports indicated a rise in intracellular cAMP, but not in cGMP upon the addition of a mitogen to lymphocyte cultures (88, 114). These studies were followed by the report of DeRubertis, _et al_. showing a rise in cGMP levels of stimulated cells with no change in cAMP (26). Recent work has corroborated the latter finding. Doses of Con A that are optimal for DNA synthesis induce an early increase in cGMP with little effect on cAMP levels, while doses of Con A that produce a decreased DNA response cause increases in cAMP and early decreases in elevated cGMP levels (47). The earliest reports of increased cAMP levels appear to be associated with mitogen doses that did not induce optimal DNA synthesis. There are additional, but less direct reports suggesting the involvement of elevated cGMP and not cAMP in mitogen-induced proliferation. Exogenous cAMP added to mitogen-stimulated cultures inhibits DNA synthesis (28). In contrast, exogenous cGMP can induce lymphocyte DNA synthesis, increases in intracellular cGMP have been associated with histone phosphorylation, and the ratio of intracellular cAMP/cGMP is lower in dividing than in resting lymphocytes (127, 57).

There is an increased energy demand following stimulation which is related to an increase in Krebs cycle activity and glycolysis. A decrease in ATP and ADP and an increase in lactate and pyruvate production has been reported to occur as early as 2 hours after stimulation (107). These changes are accompanied by a corresponding elevation in lactate dehydrogenase (52).

Other enzymes and proteins are synthesized following stimulation. There is an increase in uridine kinase, histone kinase and phosphatase, RNA polymerase, and DNA polymerase (50, 21, 93, 88). New products such as antibodies and macrophage migration inhibition factor are secreted by stimulated cells (4, 116). Labeling with ^{3}H-leucine has shown that there is a detectable increase in protein production at 4 hours by stimulated cultures (40). This increase is almost completely inhibited by Actinomycin-D; evidence that RNA synthesis is necessary for the new protein synthesis (60).

3. Effects on RNA, DNA, and Histones

The major nuclear events involve RNA and DNA in structural, regulatory, and perpetuating functions. It is not known what effect mitogens have on minor subclasses of RNA such as 5.5s, chromosomal, or nucleolar 4-7s RNA. However, mitogen effects on rRNA and hnRNA using ^{3}H-uridine incorporation have been investigated. In addition, mitogen-induced changes in chromatin, histones, and DNA have been reported.

Ribosomal RNA composes over 90% of the RNA in the cell. The 45s molecule is transcribed and methylated in the nucleolus while it is further processed to 32s and 18s macromolecules. The 18s moiety is either rapidly degraded or transported to the cytoplasm to become the 40s ribosomal subunit. While still inside the nucleolus, the 32s molecule is processed to a 28s moiety which becomes the 60s subunit in the cytoplasm. The methylated portion is conserved in the cytoplasm. Labeling with ^{3}H-methyl methionine shows no difference in the methylation kinetics of resting and stimulated lymphocytes. Since the rate of synthesis and degradation of rRNA is slow, longer ^{3}H-uridine labeling is needed to detect changes in this class of macromolecule (18).

In unstimulated cells, most of the RNA is 45s (12). There is some synthesis of new rRNA as measured by the amount of methylation, but few 28s and 18s macromolecules are present, partly because one half of these newly synthesized molecules are degraded in the nucleus (124, 110, 16, 15).

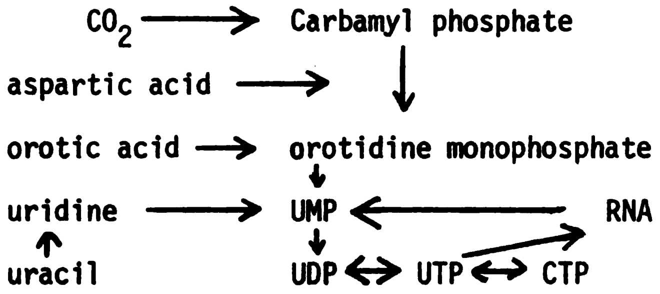
Mitogen treatment induces an increased rate of 45s formation with an increase in RNA polymerase I and II (93, 48). Stimulated lymphocytes show a faster processing of 45s molecules and 32s and 18s and there is no such "wastage" of the 18s moieties (111, 17, 19). The preservation of these newly formed ribosomes is dependent on the synthesis of protein (19).

The first class of RNA to be labeled by a ^{3}H-uridine pulse is polydisperse or heterogeneous nuclear RNA (hnRNA). Its rapid synthesis and degradation accounts for its distribution from 10-100s (18). This labile RNA composes up to 1% of total cell RNA, is not methylated and resembles mRNA in its base composition. For this reason, it has been postulated that after the addition of polyadenylate, the molecule goes to the cytoplasm and becomes mRNA (68, 32). Mitogen stimulated lymphocytes do show an increase in poly A-RNA (108). The pattern of hnRNA labeling is the same for resting and stimulated lymphocytes, except that stimulated cells show a synthesis rate 4 to 5 times higher (14).

After 20 hours exposure to mitogen, the rate of hnRNA synthesis has been exceeded by the rate of synthesis of more stable RNA classes (18).

PHA stimulation causes the cytoplasmic accumulation of a non-methylated 4s moiety that may be a tRNA precursor (61).

^{3}H-uridine incorporation into RNA Uridine nucleotides can be influenced by reactions in the following pathways: (70)



Lymphocytes cannot utilize uracil. There is a low level of incorporation by lymphocytes of labeled CO_2, aspartic acid and orotic acid as compared to labeled uridine incorporation (56, 72). In addition, endogenous carbamyl phosphate may be utilized (56). There is an increase in the amount of RNA due to stimulation even if uridine uptake is inhibited (89). These metabolic studies indicate that uridine is the preferred, but not unique uridine nucleotide precursor.

Uridine crosses the membrane by facilitated transport independent of RNA and protein synthesis. Stimulated cells have an increased V_{max}, but there is no change in affinity for uridine (89). In addition, the rate of uridine entry into a cell is influenced by the extracellular concentration (50, 64). Once inside the cell, incorporation of labeled uridine into the RNA depends on the extent of labeling of the UTP pool and on the rates of RNA degradation and reutilization of internal label.

In summary, the following are some of the events that may be involved in the increased accumulation of labeled uridine in stimulated cells: 1) Increased rate of uridine uptake and incorporation into UTP mediated by increased uridine kinase activity (50, 89). 2) Increased RNA polymerase activity with increased rate of hnRNA and 45s rRNA synthesis (14). 3) Increased rate of degradation of preexisting (unlabeled) ribosomes with an increase in the formation of new (labeled) ribosomes that are not subject to "wastage" (15, 16).

Along with the changes in RNA metabolism, other nuclear events precede DNA synthesis. Stimulated cells appear more euchromatic; this phenomenon has been associated with subsequent DNA synthesis (122). There is an increase in acetylation and phosphorylation of histones which implies increased DNA template availability (92, 21).

The peak of ^{3}H-thymidine uptake by stimulated cells follows the peak of ^{3}H-uridine uptake by about 24 hours (12). Incorporation of thymidine into DNA is related to thymidine kinase, and DNA polymerase whose activities increase following stimulation (70, 88). The uptake of thymidine may be altered by mitogen stimulation and may be the limiting step in DNA synthesis (36). There is also a mitogen induced increase in the repair enzyme polynucleotide ligase whose activity peaks one day after the peak in DNA synthesis (88).

II. Mitogen Activation of B and T Cells

Mitogens act on different lymphocyte populations via different cell receptors. Although the morphological and biochemical changes described above occur during activation, the process results in the production of different end products.

A. Site and Mechanism of Mitogen-induced Activation

The site of mitogen activity seems to be the cell membrane. Using radio-labeled Con A, it has been shown that the label remains at the cell surface (94). Con A bound to Sepharose beads is stimulatory even though it cannot get inside the cells (42). The Con A binding site appears to be a specific cell surface carbohydrate moiety, based on binding inhibition studies using α-D-methyl mannoside (94). This pyrannoside inhibited the response to Con A, but not to PHA or allo-antigens. There are also binding sites specific for PHA. Anti-PHA or N-acetyl-D-galactosamine inhibit the response to PHA, but not to Con A, pokeweed mitogen, antilymphocyte serum, or staphylococcal filtrate (65, 70). Preincubation of cells with PHA inhibits binding of labeled Con A and responsiveness to that mitogen with no effect on subsequent binding or stimulation of the population by LPS (85). However, lacto-peroxidase labeling of surface proteins has shown that some receptors for Con A and PHA are distinct while some are shared (53).

Studies on lymphocyte populations and mitogen receptors have shown that the specificity of the population response does not lie in the mitogen receptor. T and B cells have the same binding capacity of PHA, Con A and LPS (41, 6). Resting and stimulated cells bind the same amount of labeled Con A (121). Therefore, mitogen binding is not the only signal needed by a population for activation.

Nor do the events which follow mitogen binding determine which population will be activated. Both B and T cells show binding, capping, and internalization of Con A, PHA, and endotoxin (42). Inhibitory doses of mitogen prevent capping, but stimulation can occur without capping (81, 42). Thus the relationship between activation and receptor site redistribution is not clear.

There is evidence that activation of a specific cell population is related to the physical form of the mitogen. PHA bound to Sepharose beads stimulates B cells and to some extent T cells (42). Con A bound to Petri dishes activates only B cells (7). This suggests that the conformation of the mitogen determines which population is stimulated and parallels the idea that insolubilized antigen stimulates B cells (34).

B. Mitogens and Lymphocyte End Products

The first indication of differences between activated B cells and activated T cells came from the morphological studies of pokeweed mitogen-treated spleen cells. Most of the stimulated cells possessed euchromatic nuclei and some rough endoplasmic reticulum. The rest of the stimulated cells had a heterochromatic nucleus and well developed rough endoplasmic reticulum. The former cell type corresponded morphologically with PHA stimulated cells while the latter resembled plasma cells (11).

More recent evidence as to T and B cell differences has been obtained by assessing the function of mitogen stimulated cells. B cells produce 19s antibody when stimulated by LPS, or insoluble concentrated Con A (4, 7). Soluble Con A or PHA have no effect (41, 7). In contrast, T cells produce a viral plaque response, interferon, or cytotoxicity against allogeneic cells when stimulated with Con A or PHA (118, 67, 130, 31, 33, 117). LPS seems not to stimulate T cells to perform any function. Therefore, the end product of stimulation is related to the responding cell type.

MATERIALS AND METHODS

Mice: Balb/c females 8 to 20 weeks old from Flow Laboratories, Dublin, VA, were used. Age-matched ninth level backcrossed Balb/c mice homozygous (nu/nu) or heterozygous (nu/+) for the hairless, athymic phenotype were obtained from the colony of Marek Pienkowski, M.S.U., E. Lansing, MI. The presence of a thymus in all heterozygous nude mice and the absence of one in all homozygotes used was verified by autopsy.

Cell Suspensions: Single cell suspensions of spleens were aseptically prepared by passing the organs through a stainless steel mesh. The medium used throughout was RPMI-1640 (Gibco, Grand Island, NY) supplemented with 3.2 mM l-glutamine, penicillin (100,000 units/liter), streptomycin (100,000 ug/liter), and 5% fetal calf serum heat inactivated at 56°C for 30 minutes. In some experiments serum was omitted from the medium. The cells were washed once by centrifugation at 1000 RPM for 10 minutes at 4°C and the supernatant removed. The erythrocytes were lysed during a five minute treatment of each resuspended spleen cell pellet with 0.2 ml ice cold hypotonic solution of 0.155 M NH_4Cl, 0.1 mM EDTA, and 1 mM Hepes (pH 7.35) after the method of Roos et al. (107). This procedure eliminates the variable of mitogen binding to red blood cells, but leaves the lymphocytes viable (70, 2). After three more washings, the cells were counted and resuspended. Viability as assessed by trypan blue exclusion was always above 95%. In experiments using serum-free medium, serum was omitted from the last two washes.

Preparation of Lymphocyte Populations:

T cells: Spleen cells were purified by sequential filtration through glass and nylon wool columns as described by Trizio *et al*. with the following modifications: minimal essential medium (Gibco, Grand Island, NY) with 5% heat-inactivated fetal calf serum (30 minutes at 56°C) was used throughout, the glass wool was Pyrex fine, and the nylon wool was Ft-242, 3 denier, 1.5 inch type 200 (Travenol Lab, Morton Grove, IL). The procedure yeilded 30-40% of the original cell population as effluent (T) cells.

B cells: Spleen cells from athymic nude mice were prepared as described for normal spleen cell suspensions. The lymphocyte populations were characterized as B or T cells by means of their sensitivity to antibody-dependent complement mediated cytotoxicity with anti-Thy 1.2 serum.

Mitogens: Concanavalin A (Con A, Miles-Yeda, Elkhardt, IN) was stored dessicated at -20°C. Lipopolysaccharide (LPS B, *E. coli* 026:B6, Difco, Detroit, MI) and phytohemagglutinin (PHA-P, Difco, Detroit, MI) were stored under refrigeration. All mitogens were weighed and diluted in serum-free medium prior to each experiment. Con A and PHA were passed through a 0.22 u membrane filter for sterilization. LPS was boiled for 1 hour immediately before use. Mitogen doses were given in ug/ml culture.

Cultures: Cultures were generally set up in triplicate in Micro Dish Dispo Trays (IS-FB-96-TC, Linbro, New Haven, CN) and kept in a 5% CO_2-95% air humid atmosphere at 37°C in a Hotpack CO_2-incubator. All experiments were repeated 2 to 5 times.

^{3}H-uridine Incorporation: Cultures were composed of 80 ul cells, 10 ul mitogens or medium and 10 ul ^{3}H-uridine (Uridine-5-H^3, S.A. 27.5 Ci/mMole, New England Nuclear, Boston, MA). For determination of the rate of ^{3}H-uridine incorporation, 90 ul cells plus 10 ul mitogen were pulsed with 10 ul prewarmed (37°C) labeled uridine one hour prior to harvesting.

^{3}H-thymidine Incorporation: Cultures of 90 ul cells plus 10 ul mitogens were incubated for 48 hours. ^{3}H-thymidine (Thymidine [methyl-^{3}H], S.A. 20 Ci/mMole, New England Nuclear, Boston, MA) was added in 10 ul (0.1 uCi) and the cultures incubated for an additional 12 hours.

Harvesting of Cultures: A modification of the Q tip method of Williams was used to collect and wash the cells (131). Briefly, each well was swabbed with one 3-inch cotton Q tip (Hardwood Products, Guilford, ME). The wooden end was placed in a clay-filled (Floral Cling, Floral Specialties, Birmingham, AL) well of a Micro Dish Dispo Tray. The tray was filled by placing a Q tip in every other well to prevent contact between adjacent Q tips. The Q tips were air dried for at least 30 minutes and washed by immersion in a 7.5 x 12 cm staining dish containing 275 ml ice cold 5% trichloroacetic acid (TCA). The dishes were kept in an ice bath for the entire procedure. Four fifteen minute TCA washes and three fifteen minute washes in 90-100% ethanol (4°C) were followed by another 30 minute drying period. The cotton end of each Q tip was cut off into a scintillation vial containing 5 ml cocktail (1.5 l toluene, 0.5 l Triton-X 100, 8 g Omnifluor). Samples were allowed to equilibrate for at least 4 hours before counting for 10 minutes each on a Searle Model 6890 Delta 300 Liquid Scintillation Counter. The counting efficiency of the samples was calculated to be about 40%. Results were corrected for quenching using the external standard and are expressed in counts per minute (CPM).

Other Reagents: Actinomycin-D (Calbiochem, La Jolla, CA) was stored protected from light at 4°C. Immediately before addition to culture, Actinomycin-D was diluted in serum-free medium and sterilized by filtration.

Sodium dodecyl sulfate (SDS) (Sequanol grade, Pierce Chemical Co., Rockford, IL) was diluted in distilled water to a final concentration of 5%.

Preparation of Anti-Thy 1.2 Serum: AKR/J mice (Thy 1.1) were injected intraperitoneally with a mixture of 5 x 10^7 AKR/Cum (Thy 1.2) thymocytes and spleen cells once a week for 5 weeks (1). After 2 weeks, they were reinjected and bled one week later. The serum was heat inactivated and frozen (-20°C) in aliquots. A final dilution of antiserum at 1:100 killed 50% thy 1.2 thymocytes and 2% Thy 1.1 thymocytes using the cytotoxicity procedure described below.

Cytotoxicity Test: 90 ul cells (6.25 x 10^6/ml) in culture medium were incubated in 12 x 75 mm tubes at 37°C, 5% CO_2 with 10 ul antisera for 30 minutes. After the addition of 10 ul guinea pig complement (Gibco, lyophilized and reconstituted), cultures were incubated for an additional 30 minutes. Dead cells were stained by a 15 minute incubation at 20°C with trypan blue (final concentration 0.1%) and fixed with formalin (final concentration 3-5%). Cultures were counted within 4 hours. Controls for non-specific killing were prepared by adding normal mouse serum in place of antibody or heat-inactivated guinea complement. These controls were usually above 90% viability. The percent killing with anti-Thy 1.2 and complement was calculated as the percentage of the total number of cells counted that took up dye.

Statistical Analysis: A Monroe Model 1766 Statistical Calculator was used for mean, standard error, student's T test and linear regression analysis determinations.

RESULTS

To develop an applicable microculture system for the routine assessment of immunoreactivity using ^{3}H-uridine incorporation, parameters of the culture conditions were first investigated. The ^{3}H-uridine response in optimal conditions was compared with the ^{3}H-thymidine response with the same cell density and mitogen doses. An effort to confirm the incorporation of labeled uridine into TCA precipitable counts, and to verify specificity of the technique for T and B cell responses was made. The amount of time necessary for a measurable response by stimulated cells was investigated as was the need for serum in the culture medium.

Optimization of Cell Density and Doses of Mitogens

The first set of experiments was carried out to determine the cell density and mitogen concentrations resulting in optimal stimulation as measured by ^{3}H-uridine incorporation. Spleen cells in concentrations ranging from 2.5 to 20 x 10^5 cells/0.1 ml were incubated for 24 hours with 1 uCi ^{3}H-uridine with and without mitogens in the following doses: Con A, 0.25 to 10 ug/ml culture; PHA, 6.25 to 100 ug; and LPS, 31 to 250 ug. The optimal stimulation by all three mitogens was obtained at 5 x 10^5 cells/0.1 ml (Table 1). The response is measured by the stimulation index (SI) which is $\frac{\text{CPM with mitogen}}{\text{CPM without mitogen}}$. The highest index of stimulation, 6.98, resulted from treatment of cells with 2.0 ug/ml culture Con A. The peak of stimulation with PHA, 3.65, was obtained with 25 ug/ml. The LPS dose of 62 ug/ml resulted in a

Table 1. Effect of varying cell densities and concentrations of mitogens in a serum-containing media on ^{3}H-uridine incorporation into Balb/c spleen cell cultures. 0.1 ml cultures in media suplimented with 5% fetal calf serum were incubated in the continuous presence of 1 uCi ^{3}H-uridine for 24 hours.

a. mean CPM ± standard error
b. mean stimulation index ± standard error $(SI = \frac{\text{CPM with mitogen}}{\text{CPM without mitogen}})$

Number of cells/well		2.5×10^5	5×10^5	1×10^6	2×10^6
Mitogen omitted		13,896 ± 573[a]	19,071 ± 405	28,794 ± 1013	39,992 ± 1176
Mitogen added (ug/ml culture)					
Con A	0.25	3.02 ± 0.04[b]	2.71 ± 0.02	2.18 ± 0.11	1.28 ± 0.10
	0.5	4.03 ± 0.09	3.99 ± 0.06	2.99 ± 0.21	1.71 ± 0.17
	1.0	5.85 ± 0.08	5.18 ± 0.25	3.74 ± 0.31	2.46 ± 0.24
	2.0	$\boxed{7.09 \pm 0.08}$	$\boxed{6.98 \pm 0.24}$	$\boxed{4.90 \pm 0.38}$	2.94 ± 0.12
	4.0	5.80 ± 0.16	6.90 ± 0.37	4.60 ± 0.33	$\boxed{3.43 \pm 0.25}$
	5.0		4.89 ± 0.40		
	8.0	1.64 ± 0.03	2.60 ± 0.20	3.26 ± 0.40	2.88 ± 0.26
	10.0	1.38 ± 0.05	1.52 ± 0.80	2.37 ± 0.43	2.66 ± 0.34
LPS	31	1.81 ± 0.04	2.13 ± 0.11	1.56 ± 0.22	1.32 ± 0.04
	62	$\boxed{2.10 \pm 0.04}$	$\boxed{2.31 \pm 0.04}$	$\boxed{1.63 \pm 0.20}$	1.42 ± 0.02
	125	1.79 ± 0.02	1.74 ± 0.24	1.34 ± 0.49	$\boxed{1.49 \pm 0.06}$
	250	0.98 ± 0.02	0.76 ± 0.17	0.79 ± 0.47	1.04 ± 0.10
PHA-P	6.25	2.22 ± 0.02	2.93 ± 0.08	1.61 ± 0.08	1.27 ± 0.02
	12.5	2.85 ± 0.05	3.42 ± 0.13	2.13 ± 0.11	1.34 ± 0.08
	25	3.53 ± 0.05	$\boxed{3.65 \pm 0.35}$	2.12 ± 0.21	1.57 ± 0.16
	50	$\boxed{3.79 \pm 0.04}$	2.75 ± 0.32	$\boxed{2.40 \pm 0.28}$	$\boxed{1.75 \pm 0.20}$
	100		2.18 ± 0.05	1.96 ± 0.02	1.40 ± 0.04

stimulation index of 2.31. The dose range of LPS giving 75% optimal stimulation was 31-125 ug/ml, while 75% optimal stimulation with PHA occurred from 6.25 to 50 ug/ml. An even narrower dose range for Con A was obtained with 75% optimal stimulation occurring between 2 and 4 ug/ml.

Comparison of ^{3}H-uridine with ^{3}H-thymidine Incorporation

In order to compare the incorporation of ^{3}H-uridine to the accepted response as measured by ^{3}H-thymidine uptake, cultures with 5 x 10^5 cells plus doses of Con A varying from 0.16 to 10 ug/ml or LPS from 8 to 250 ug/ml were set up. After 48 hours in culture, a predetermined optimal time, 0.1 uCi ^{3}H-thymidine was added for a 12 hour pulse. A comparison of the results with ^{3}H-uridine at 24 hours and ^{3}H-thymidine at 60 hours under the same culture conditions shows a similarity of responses (Figures 1 and 2). The shapes of the curves and the optimal doses for Con A and LPS are the same for RNA and DNA measurements.

Determination of ^{3}H-uridine Dose

The appropriate dose of labeled uridine to be added to 5 x 10^5 cells/0.1 ml was determined by incubating the cultures for 24 hours with and without 2.5 ug/ml Con A in the presence of 0.1 to 5 uCi ^{3}H-uridine. The amount of label incorporated in stimulated and unstimulated cultures appears to be roughly proportional to the dose of label added (Table 2). The highest stimulation index, 5.305, obtained with 1 uCi ^{3}H-uridine/well is not significantly different as determined in the student's T test from the indices obtained with 0.5 uCi/well, but is different from all other indices by $p<0.01$. A dose of 1 uCi/well was chosen for subsequent experiments since it produces a larger net difference between stimulated and unstimulated cultures than 0.5 uCi/well.

Figure 1. Incorporation of ^{3}H-uridine or ^{3}H-thymidine by spleen cells exposed to various doses of Con A. Cultures of 0.1 ml containing 5 x 10^5 Balb/c spleen cells and mitogen were either incubated with 1 uCi ^{3}H-uridine for 24 hours or pulsed with 0.1 uCi ^{3}H-thymidine at 48 hours for an additional 12 hours. Vertical bars indicate standard error of the mean.

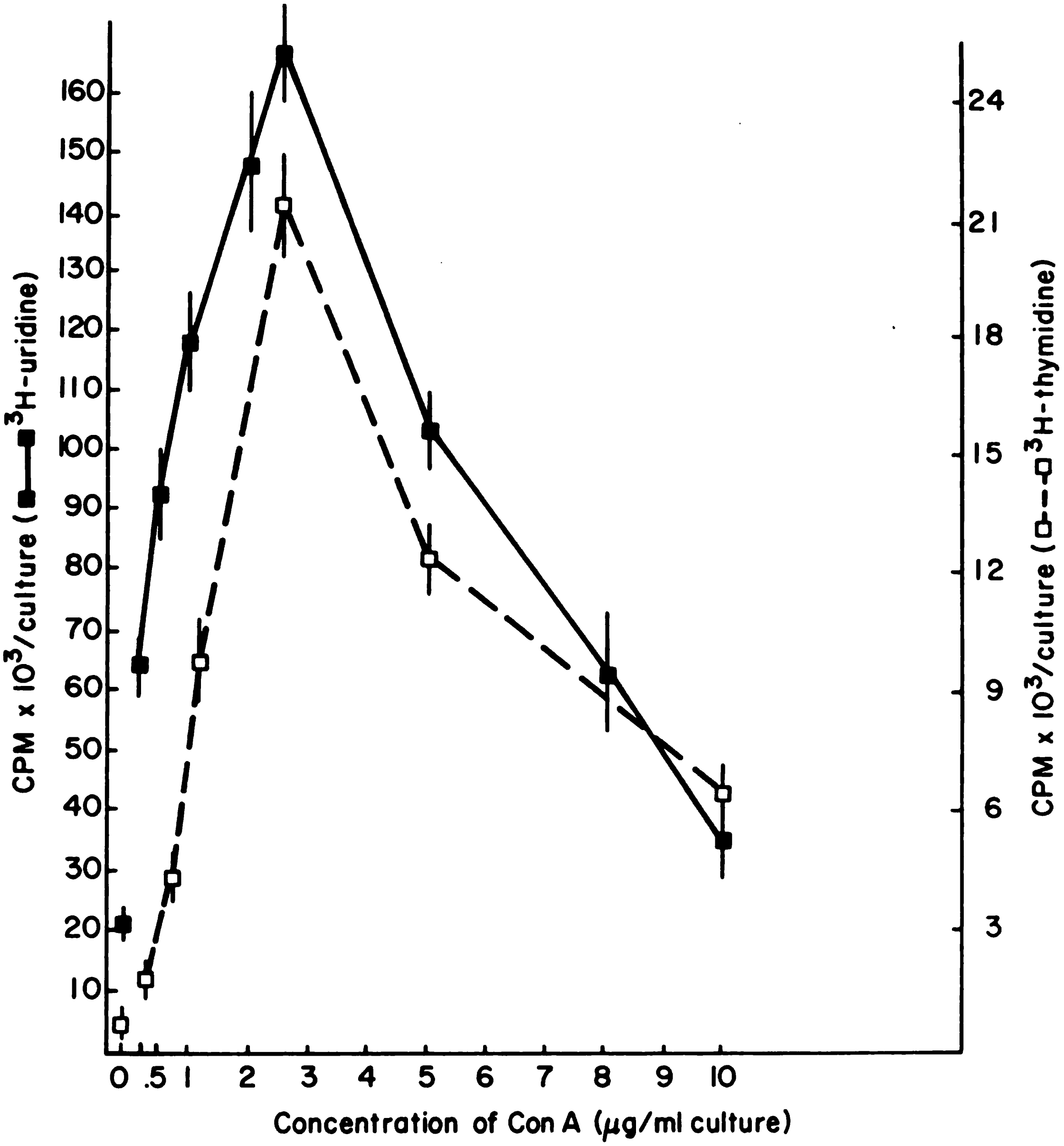

CPM x 10^3/culture (■—■ ^{3}H-uridine)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
CPM x 10^3/culture (□--□ ^{3}H-thymidine)
24
21
18
15
12
9
6
3
0
.5
1
2
3
4
5
6
7
8
9
10
Concentration of Con A (µg/ml culture)

Figure 1

Figure 2. Incorporation of ^{3}H-uridine or ^{3}H-thymidine by spleen cells exposed to various doses of LPS. Cultures of 0.1 ml containing 5 x 10^5 Balb/c spleen cells and mitogen were either incubated with 1 uCi ^{3}H-uridine for 24 hours or pulsed with 0.1 uCi ^{3}H-thymidine at 48 hours for an additional 12 hours. Vertical bars indicate standard error of the mean.

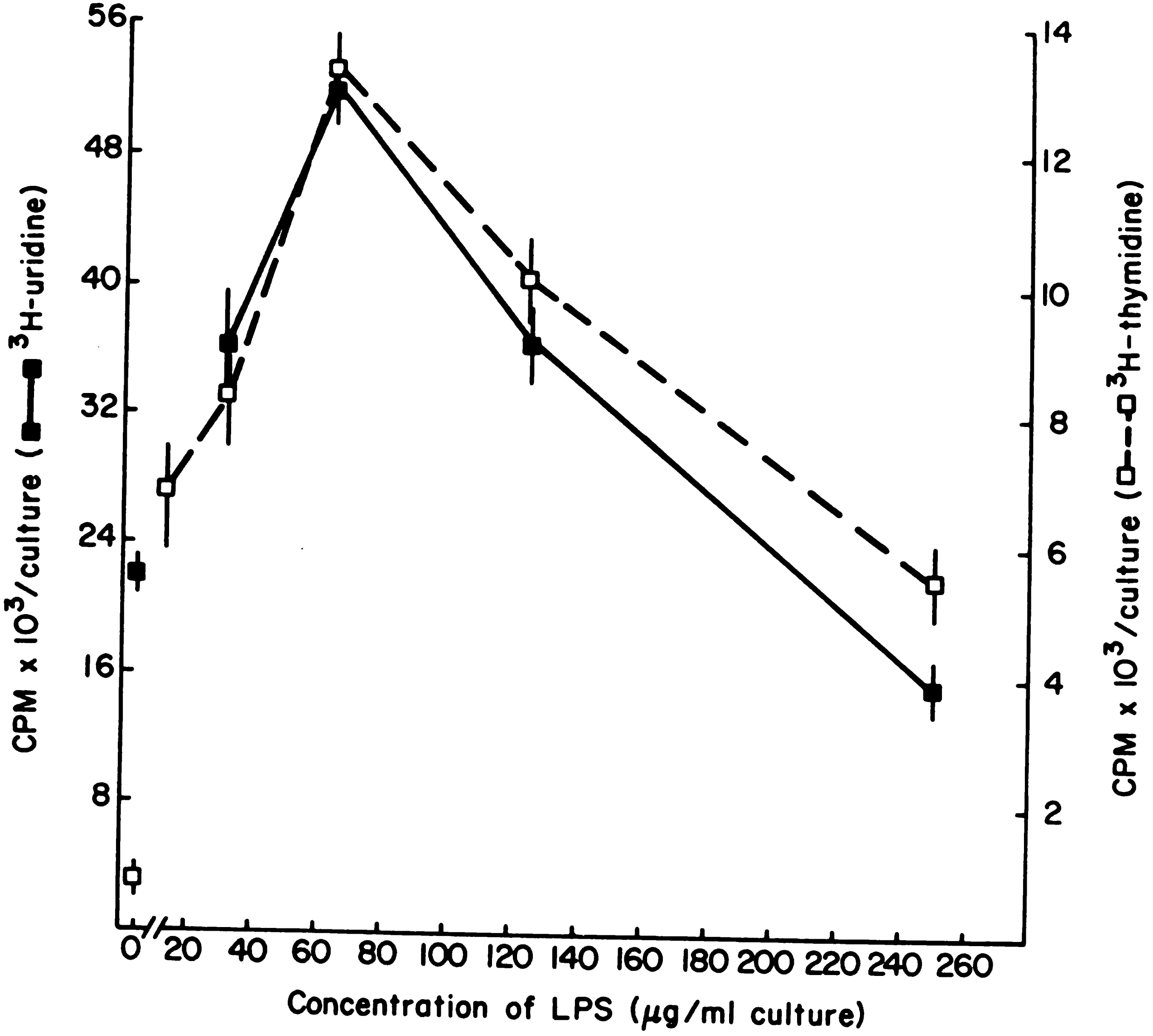

56
48
40
32
24
16
8
CPM x 10^3/culture (■—■ ^{3}H-uridine)
14
12
10
8
6
4
2
CPM x 10^3/culture (□--□ ^{3}H-thymidine)
0
20
40
60
80
100
120
140
160
180
200
220
240
260
Concentration of LPS (µg/ml culture)

Figure 2

Table 2. Relationship between values of SI and amount of exogenous ^{3}H-uridine added. 0.1 ml cultures of 5 x 10^5 Balb/c spleen cells containing various doses of ^{3}H-uridine were incubated for 24 hours with or without Con A.

a. mean CPM ± standard error
b. mean stimulation index ± standard error

uCi ^{3}H-uridine added/culture	CPM ^{3}H-uridine incorporated		SI
	Cells only	Con A (2.5 ug/ml)	
0.1	4,415 ± 76[a]	16,977 ± 386	3.81 ± 0.07[b]
0.5	12,144 ± 541	61,720 ± 975	5.11 ± 0.37
1.0	16,553 ± 432	84,440 ± 1979	$\boxed{5.31 \pm 0.20}$
2.0	34,287 ± 1520	163,674 ± 5848	4.79 ± 0.26
5.0	81,319 ± 1937	337,571 ± 6939	4.15 ± 0.06

Effects of Actinomycin-D and Sodium Dodecyl Sulfate on ^{3}H-uridine Incorporation

Experiments were designed to investigate the relationship between the increase in incorporated counts after stimulation and new RNA synthesis. First, Actinomycin-D at concentrations 0.1 to 10 ug/ml culture was added to 5×10^5 cells with and without 2.5 ug/ml culture Con A and incubated for 24 hours with 1 uCi ^{3}H-uridine. Actinomycin-D at concentrations of 1 or 10 ug/ml inhibits at least 97% of label incorporation induced by stimulation, indicating that the labeled uridine is being incorporated into newly formed RNA (Table 3).

To demonstrate that the increase in labeling is due to incorporation of radioactivity into RNA, but not due to trapping of unincorporated ^{3}H-uridine inside the cells, cultures were treated with sodium dodecyl sulfate (SDS). Two sets of cultures of 5×10^5 cells with 1 uCi ^{3}H-uridine with or without 2.5 ug/ml Con A were established. At the end of 24 hours, one set of cultures was harvested with TCA in the usual way. The other set was treated with SDS to a final concentration of 1%, followed by the usual TCA harvesting. Control and Con A treated cultures harvested with TCA yielded 16,553 ± 432 CPM and 84,440 ± 1,979 CPM respectively. SDS pretreatment resulted in 21,146 ± 321 CPM for control cultures and 89,363 ± 1412 CPM after Con A stimulation. Therefore, SDS pretreatment did not decrease the number of TCA precipitable counts.

These two experiments suggest that the increase in counts following stimulation was due to ^{3}H-uridine that has been incorporated, not trapped, into TCA precipitable material and the action is inhibited by the presence of Actinomycin-D.

Table 3. Inhibition of Con A-induced ^{3}H-uridine incorporation by Actinomycin-D. 0.1 ml cultures containing 5 x 10^5 Balb/c spleen cells, 1 uCi ^{3}H-uridine, and varying doses Actinomycin-D were incubated for 24 hours with and without Con A.

a. mean CPM ± standard error

Actinomycin-D ug/ml culture	CPM ^{3}H-uridine incorporated: Cells only	CPM ^{3}H-uridine incorporated: Con A (2.5 ug/ml)	Net CPM increase	% inhibition
none	13,688 ± 834[a]	49,906 ± 1,516	36,218	--
0.1	10,044 ± 566	14,173 ± 675	4,129	89
1.0	8,302 ± 456	9,238 ± 449	936	97
10	7,733 ± 204	7,825 ± 653	92	99

T and B Lymphocyte Responses Measured by ^{3}H-uridine Incorporation

The stimulation being measured should reflect the immunoreactivity of specific cell populations as opposed to the response of the whole population. Based on the assumption that certain mitogens are specific for T or B cell stimulation, ^{3}H-uridine incorporation was measured in selected cell populations using these agents. The response of normal spleen cells was compared with purified T cells from nylon wool and glass wool columns, and lymphocytes from athymic nude mice which are mainly B cells. Cytotoxicity with anti-Thy 1.2 serum and complement was used as a check on the purity of each cell preparation. Thirty percent of normal spleen cells were killed by antibody and complement treatment, while 94% of the column purified cells were killed, as compared with only 1% of nude spleen cells. Cultures of 5×10^5 cells plus 1, 2, or 5 ug/ml Con A or 50 or 100 ug/ml LPS were incubated with 1 uCi ^{3}H-uridine for 24 hours. The optimal response in all cases was achieved with 2 ug Con A and 100 ug LPS. These results are presented in Table 4. The normal spleen cells respond to both Con A (SI=4.93) and LPS (SI=2.38). Nude cells respond to LPS with an SI of 1.78, but not to Con A (SI=0). The LPS response was abolished in the T purified population (SI=1.11), while the Con A response was retained with an index of 7.14. In addition, the number of counts incorporated per 5×10^5 cells without mitogens is similar for B and T cells as compared with the whole spleen. Therefore, purified cell populations exposed to B or T specific mitogens show a specific response when measured by ^{3}H-uridine incorporation.

Table 4. ^{3}H-uridine incorporation by lymphocyte populations stimulated with selective mitogens. Either 5 x 10^5 normal spleen cells, T cells from column purification, or B cells from athymic mice were cultured for 24 hours with mitogens in the continuous presence of 1 uCi ^{3}H-uridine/well.

a. mean CPM/culture ± standard error
b. mean SI ± standard error

Cell population	CPM ^{3}H-uridine incorporated		
	Cells only	Con A (2.5 ug/ml)	LPS (50 ug/ml)
spleen	18,697 ± 1246[a]	90,389 ± 257 (4.93 ± 0.3)[b]	42,577 ± 1923 (2.33 ± 0.7)
T cells	16,291 ± 850	116,156 ± 5232 (7.14 ± 0.2)	18,104 ± 360 (1.11 ± 0.04)
B cells	18,455 ± 154	15,616 ± 476 (0.84 ± 0.01)	33,023 ± 734 (1.78 ± 0.02)

Rate and Time-dependent Accumulation of ^{3}H-uridine

In the results given thus far the response to mitogens was measured at 24 hours. The question of the minimum culture time required for a response was investigated in two ways. First, cultures of 5 x 10^5 cells were incubated with 1 uCi ^{3}H-uridine in the presence of 2.5 ug/ml Con A, 50 ug LPS, or 25 ug PHA. Cultures were harvested at 4, 8, 12, 16, 20 and 24 hours. Plots of the CPM incorporated at each time with each mitogen showed as much as a 76% increase in response with Con A at 4 hours and 230% increase at 8 hours as compared with unstimulated cultures (Figure 3). The accumulation of ^{3}H-uridine tended to plateau after 8 hours in cultures stimulated with LPS or PHA. Incorporation of radioactivity into Con A treated cultures doubled between 4 and 8 hours. This increased accumulation of label continues to a lesser degree after 8 hours. By 24 hours, the Con A stimulated response is only 50% greater than that at 8 hours.

To further investigate this apparent increase in label incorporation in mitogen-treated cultures, the rate of label incorporation was studied in concurrent experiments. Con A (2.5 ug/ml) was added to 5 x 10^5 cells. Cultures were pulsed with 1 uCi ^{3}H-uridine for one hour prior to harvest at 4, 8, 16 and 24 hours. There is a 34% increase in label incorporation by stimulated cultures as compared with unstimulated ones at 4 hours. By 24 hours, Con A treated cultures show a 46% increase in pulse labeling over controls (Figure 4). Based on these time response studies, stimulation may be measured as early as 8 hours in culture.

Figure 3. Accumulation with time of ^{3}H-uridine by spleen cells treated with various mitogens and cultured in medium containing 5% fetal calf serum. ^{3}H-uridine (1 uCi) was added to 5 x 10^5 Balb/c spleen cells at the initiation to culture. Cells were harvested at various times. ———— , no mitogen; x—·—·x, LPS (50 ug/ml); O·······O , PHA (25 ug/ml); △------△, Con A (2.5 ug/ml). Vertical bars indicate standard error of the mean.

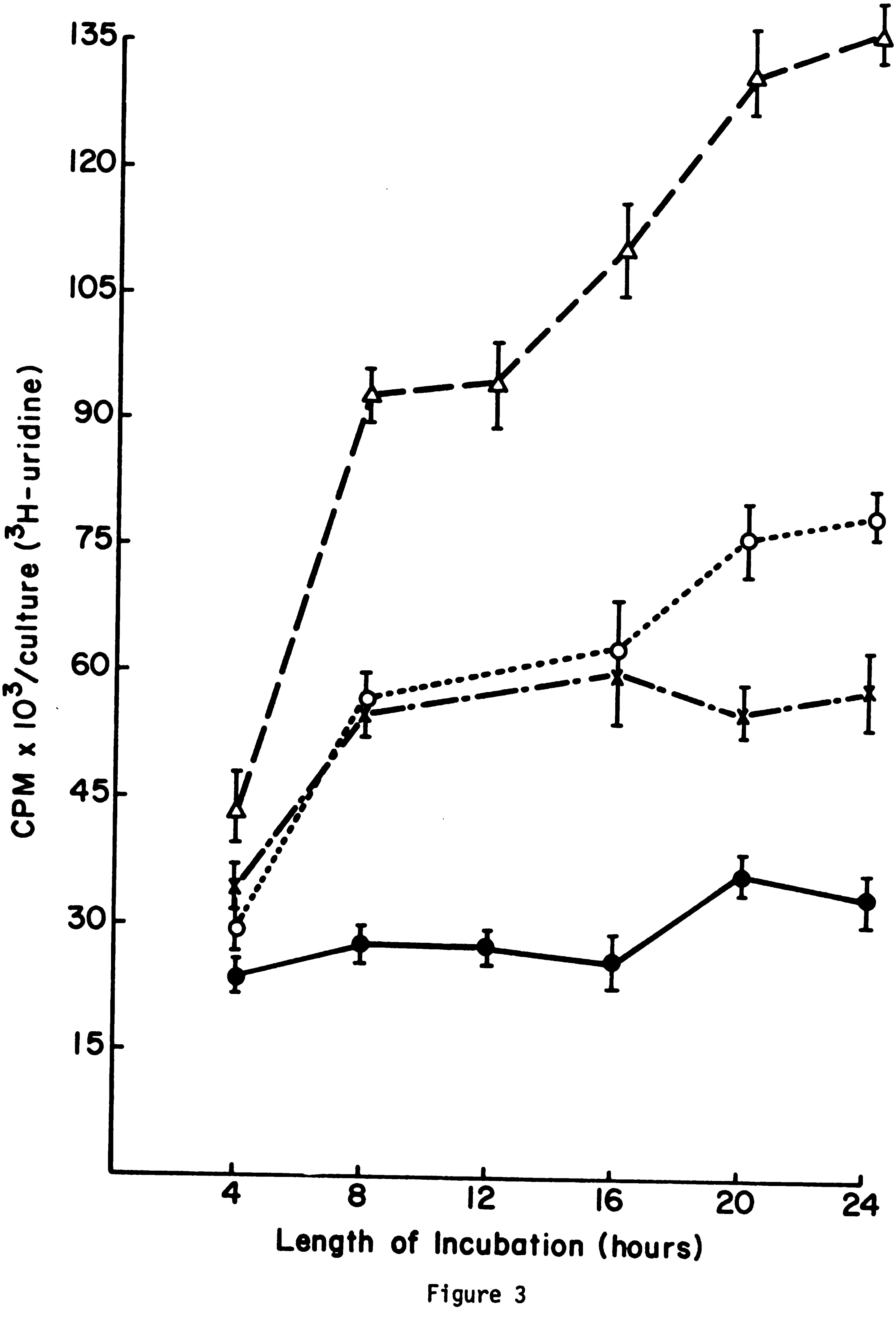

CPM x 10^3/culture (^{3}H-uridine)
135
120
105
90
75
60
45
30
15
4
8
12
16
20
24
Length of Incubation (hours)

Figure 3

Figure 4. Rate of ^{3}H-uridine incorporation by spleen cells exposed to Con A in a 5% fetal calf serum medium. ^{3}H-uridine (1 uCi/well) was added 1 hour prior to harvesting the cultures of 5×10^5 Balb/c spleen cells with or without Con A. ——— , no mitogen; △-------△, Con A (2.5 ug/ml). Vertical bars indicate standard error of the mean.

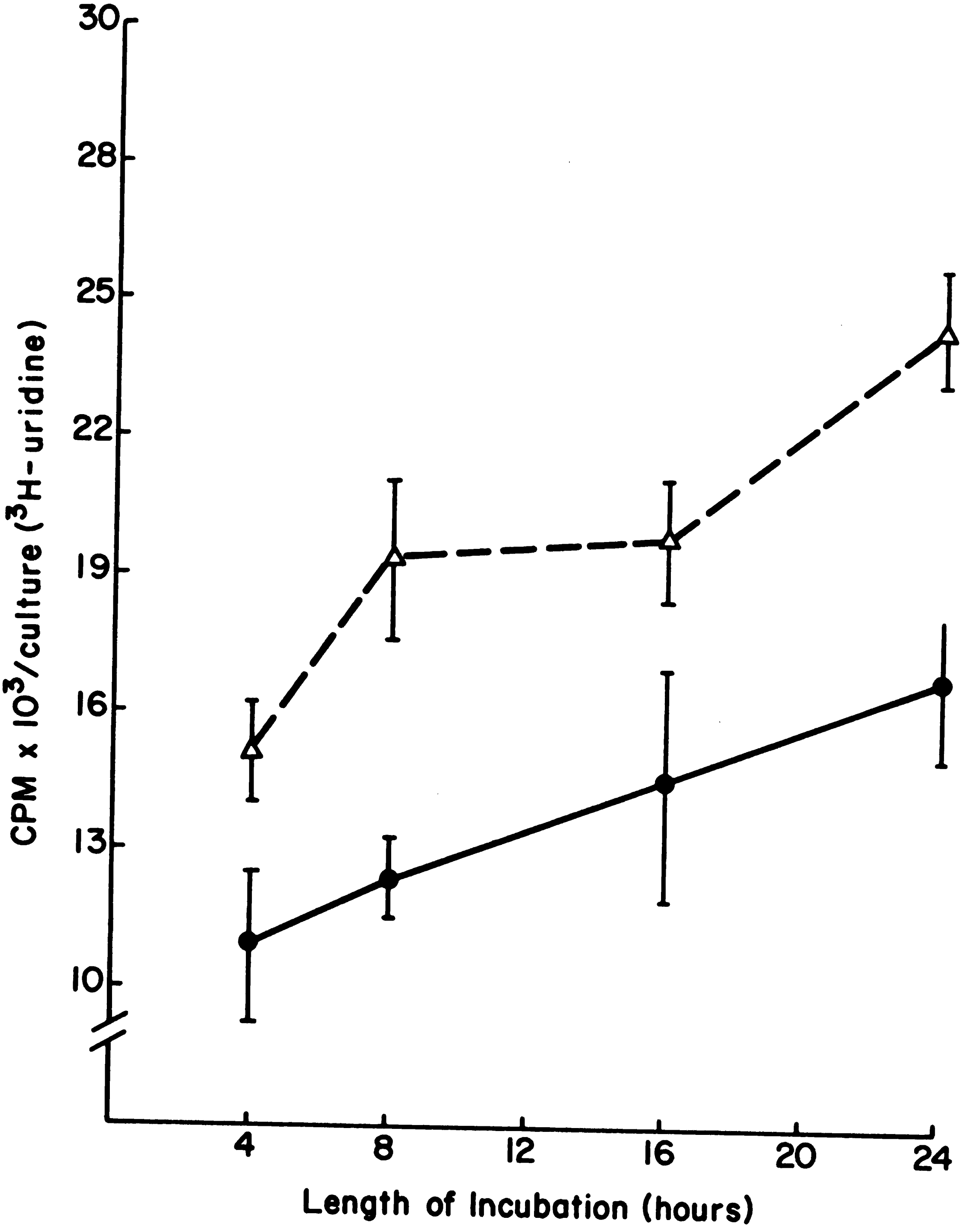

30
28
25
22
19
16
13
10
CPM x 10^3/culture (^{3}H-uridine)
4
8
12
16
20
24
Length of Incubation (hours)

Figure 4

Optimization of Cell Density and Doses of Mitogen in A Serum-free Medium

The potential for application of ^{3}H-uridine incorporation led to the investigation of one final aspect of the culture system. Since the response could be measured at such an early time, the possibility of omitting serum from the culture medium was tested. It is known that serum improves lymphocyte viability in culture, but it also introduces some unknown variables. The same variables of cell density and dose were studied in serum-free cultures. The results without serum are comparable to those with serum (Tables 5 and 1). The optimal cell density of 5 x 10^5 cells/0.1 ml is the same as in cultures with serum. The optimal mitogen concentration is the same for LPS (50 ug/ml) and PHA (12.5 to 50 ug/ml) as with serum, while the optimal Con A dose drops from 2 to 1.25 ug/ml. In addition, the mitogen dose range of the optimal response without serum is similar to those with serum. LPS doses that produce 75% of the optimal response range from about 25 to 100 ug/ml; PHA, from 6.2 to 50; and Con A, 0.62 to 1.25 ug/ml. The highest stimulation indices obtained for each mitogen in the serum-free system were as follows: Con A, 5.41; PHA, 2.43; and LPS, 1.66.

Rate and Time-dependent Accumulation of ^{3}H-uridine in a Serum-free Medium

The time response in the serum-free system was tested in the way described for the serum containing procedure, but the dose of Con A was decreased to 1 ug/ml culture. Pulse labeling for one hour yields a 20% increase in ^{3}H-uridine uptake by Con A stimulated cells at 4 hours and a 53% increase by 24 hours (Figure 5). Mitogen stimulation as measured by constant labeling results in a detectable response with PHA or LPS at

Table 5. Effect of varying cell densities and concentrations of mitogens in a serum-free media on ^{3}H-uridine incorporation into Balb/c spleen cell cultures. 0.1 ml cultures in media without serum were incubated in the continuous presence of 1 uCi ^{3}H-uridine for 24 hours.

a. mean CPM ± standard error
b. mean stimulation index ± standard error

Number of cells/well		2.5×10^{5}	5×10^{5}	1×10^{6}	2×10^{6}
Mitogen omitted		24,861 ± 952[a]	30,514 ± 3291	45,221 ± 663	64,510 ± 2365
Mitogen added (ug/ml culture)					
Con A	0.16	1.64 ± 0.04[b]	1.66 ± 0.14	1.22 ± 0.05	0.95 ± 0.02
	0.32	2.39 ± 0.04	2.51 ± 0.21	1.64 ± 0.01	1.13 ± 0.02
	0.64	$\boxed{4.09 \pm 0.12}$	3.94 ± 0.36	2.63 ± 0.08	1.73 ± 0.02
	1.25	3.85 ± 0.08	$\boxed{5.41 \pm 0.49}$	$\boxed{4.22 \pm 0.09}$	2.61 ± 0.05
	2.5	1.91 ± 0.05	3.17 ± 0.16	$\boxed{4.22 \pm 0.22}$	3.01 ± 0.08
	5.0	1.08 ± 0.00	1.49 ± 0.07	2.22 ± 0.19	$\boxed{3.06 \pm 0.05}$
LPS	12.5	1.23 ± 0.01	1.39 ± 0.08	1.27 ± 0.02	1.17 ± 0.02
	25	$\boxed{1.35 \pm 0.01}$	1.43 ± 0.14	1.20 ± 0.01	1.39 ± 0.02
	50	1.29 ± 0.01	$\boxed{1.66 \pm 0.15}$	1.36 ± 0.03	$\boxed{1.42 \pm 0.02}$
	100	1.15 ± 0.02	1.27 ± 0.04	$\boxed{1.45 \pm 0.03}$	1.35 ± 0.04
PHA-P	6.25	1.98 ± 0.05	2.07 ± 0.12	1.43 ± 0.03	1.17 ± 0.02
	12.5	1.88 ± 0.03	2.23 ± 0.20	1.83 ± 0.02	1.49 ± 0.01
	25	$\boxed{2.25 \pm 0.09}$	$\boxed{2.43 \pm 0.25}$	1.90 ± 0.09	1.52 ± 0.01
	50	2.04 ± 0.04	$\boxed{2.43 \pm 0.08}$	$\boxed{2.06 \pm 0.01}$	$\boxed{1.67 \pm 0.04}$

Figure 5. Rate of ^{3}H-uridine incorporation by spleen cells exposed to Con A in a serum-free medium. ^{3}H-uridine (1 uCi/well) was added 1 hour prior to harvesting the cultures of 5 x 10^{5} Balb/c spleen cells with or without Con A. ————, no mitogen; △------△, Con A (1.25 ug/ml). Vertical bars indicate the standard error of the mean.

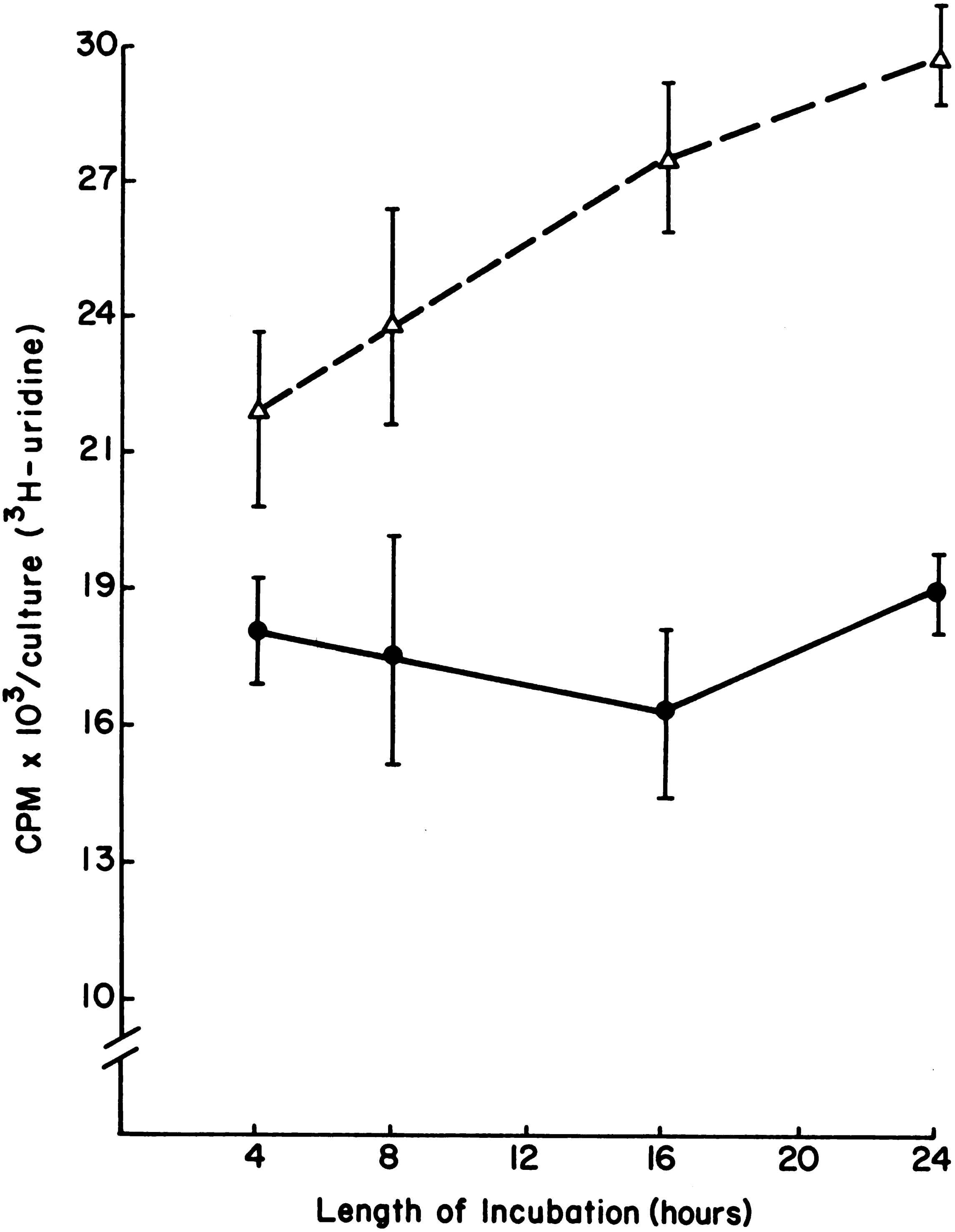

CPM x 10^3/culture (^{3}H-uridine)
30
27
24
21
19
16
13
10
4
8
12
16
20
24
Length of Incubation (hours)

Figure 5

4 hours, and a 70% increase in labeling with Con A at that time (Figure 6). By 8 hours, Con A stimulation appears to be 266% of the unstimulated cultures; PHA, 200%; and LPS, 130%. The increase in labeling plateaus after 8 hours, as in serum-containing cultures.

Results presented here suggest that the measurement of ^{3}H-uridine incorporation in mitogen stimulated cultures measures specific responses of T and B cell populations and is feasible at 8 hours with or without serum. The stimulation being measured appears to be related to ^{3}H-uridine incorporation into newly formed RNA and similar with respect to dose-response curves to that measured by DNA synthesis.

Figure 6. Accumulation with time of ^{3}H-uridine by spleen cells treated with various mitogens and cultured in a serum-free medium. ^{3}H-uridine (1 uCi) was added to 5 x 10^5 Balb/c spleen cells at the initiation of culture. Cells were harvested at various times. ———— , no mitogen; x—·—·—x , LPS (50 ug/ml); O·······O , PHA (25 ug/ml); △ ----- △ , Con A (1.25 ug/ml). Vertical bars indicate standard error of the mean.

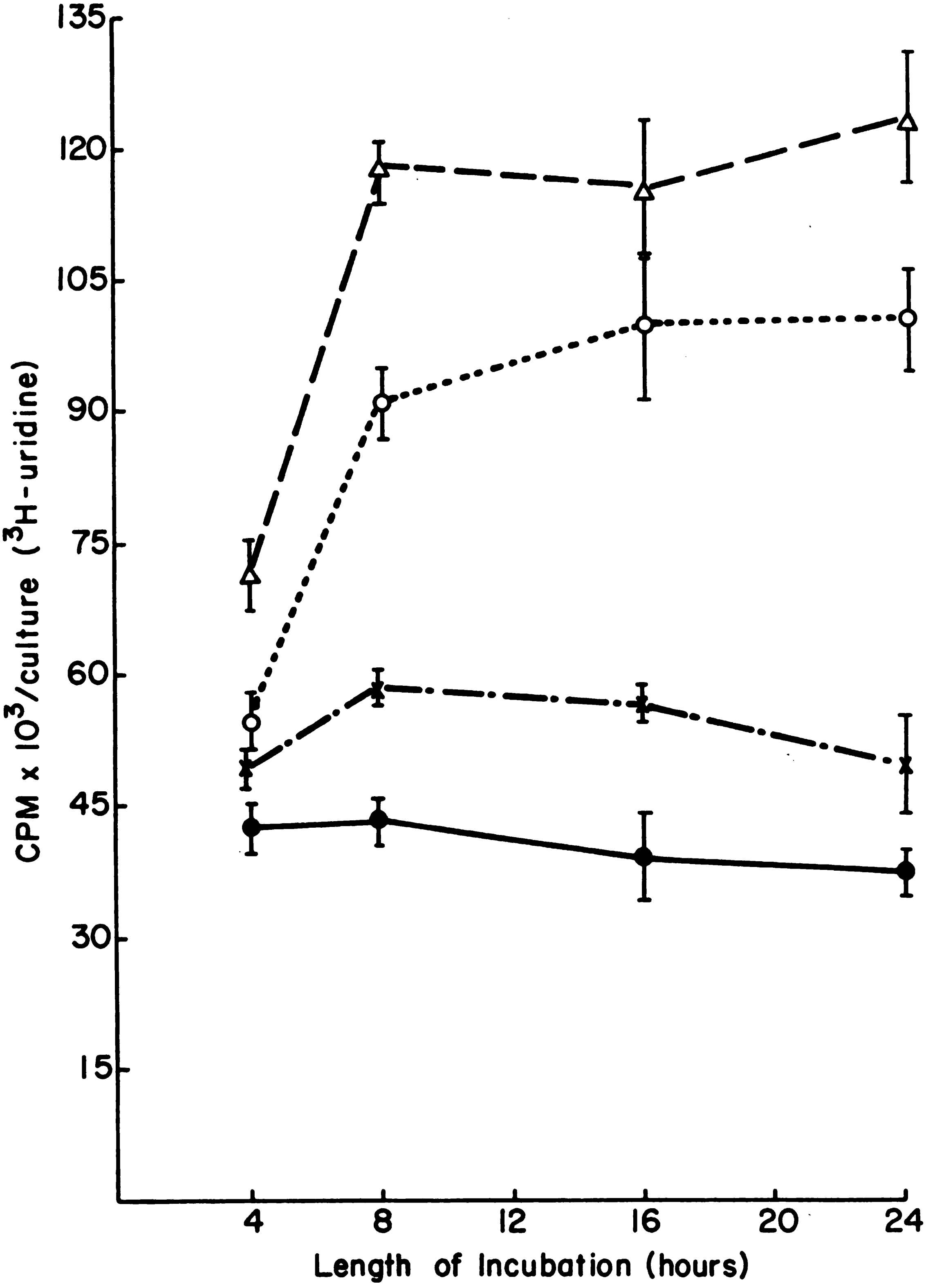

135
120
105
90
75
60
45
30
15
CPM x 10^3/culture (^{3}H-uridine)
4
8
12
16
20
24
Length of Incubation (hours)

Figure 6

DISCUSSION

A microculture technique measuring ^{3}H-uridine incorporation into lymphocyte RNA was developed to assess in vitro immunoreactivity. An investigation of the culture conditions followed by studies on the rate and time course of the response indicate that early increases in RNA synthesis may be measured by 8 hours. In addition, responses of T and B lymphocytes are distinct as measured by ^{3}H-uridine incorporation. A comparison of the results obtained by this method with those of ^{3}H-thymidine utilization show that ^{3}H-uridine incorporation is as precise a tool for measuring immunoreactivity as the generally accepted assessment of DNA synthesis. Moreover, this comparison reveals some advantages and possible applications of ^{3}H-uridine incorporation.

An analysis of the data presented in Table 1 indicates a relationship between cell density and mitogen stimulation. It may be noted first that the number of counts precipitated in unstimulated cultures is roughly doubled as the cell concentration increases from 2.5 to 5 x 10^5 cells/well. The lack of a proportional increase in labeling at higher cell concentrations could be due to crowding and depletion of media nutrients (44). Secondly, as the cell density increases, the dose of mitogen required for maximal stimulation shifts slightly. As the cell density increases from 2.5 to 20 x 10^5 cells/well, the optimal dose of Con A goes from 2 to 4 ug/ml culture, while that for LPS varies from 62 to 125 ug and PHA from 25 to 50 ug. The amount of stimulation as measured by the stimulation index changes with the cell density. Less than optimal culture conditions above 5 x 10^5 cells/well could account for increasingly lower indexes of stimulation. Below 5 x 10^5 cells/well, the slightly lower indexes of stimulation may be due to lack of cell contact which is necessary for mitogen stimulation as measured by DNA synthesis (90).

The results obtained with ^{3}H-uridine are similar to those reported for ^{3}H-thymidine uptake with respect to mitogen potency. The greatest amount of stimulation occurs with Con A, followed by PHA and LPS (Table 1). It has been estimated that 29-45% of mouse spleen cells respond to Con A, and 11-26% to PHA (58).

The broad dose range for optimal LPS stimulation and narrower ranges for PHA and Con A are common features observed when their effects on DNA synthesis are measured (115). Those patterns are seen for Con A and LPS with ^{3}H-uridine, but not for PHA (Table 1). The variation in dose range for each mitogen may be related to the way the mitogen interacts with the cell surface and the purity of the mitogen preparation. LPS acts via its Lipid A portion which is thought to integrate randomly into the cell membrane and cause some conformational changes which lead to cell division (70). The number of LPS molecules required per cell for activation varies over a wide range. In contrast, PHA and Con A bind specific cell receptors. The number of molecules per cell that bind to provide maximum stimulation has been estimated to be 1-3 x 10^6 Con A molecules per cell (6). Inhibition of DNA synthesis at high doses of Con A was not due to toxicity since inhibition could be reversed, but may be due to elevation of intracellular cAMP (94, 47). High doses of PHA have been shown by some to be toxic (104).

The purity of the Con A and PHA preparations affect the shape of the dose response curve and the amount of mitogen necessary for optimal stimulation (38, 39, 105). The Con A used in the above experiments was purified by 3 crystallizations, while the PHA was cruder. The optimal stimulation of highly purified PHA preparations is obtained with 1 to 5 ug/ml, while the preparation used here gave a maximum response at about 25 ug/ml (70 and Table 1). The action of impurities in the PHA preparation could account for its broad dose range.

The dose response curves for Con A and LPS as measured by ^{3}H-uridine were compared with those obtained with ^{3}H-thymidine (Figures 1 and 2). All culture conditions were held constant except for the assay technique. There was essentially no difference between results with labeled uridine and labeled thymidine with respect to the shape of the dose response curve or the optimal dose for either Con A or LPS. This implies that ^{3}H-uridine measures the response of the same cell populations, T and B respectively, as ^{3}H-thymidine. The level of stimulation as measured by DNA synthesis is related to the number of cells responding since the length of the S phase is constant (58). The similarity of results of ^{3}H-uridine with ^{3}H-thymidine is further indication that ^{3}H-uridine incorporation reflects not only the specific population responding, but also the percentage of the total cell population stimulated.

A variation in indices of stimulation between experiments as measured by ^{3}H-uridine incorporation was noted. Results in Table 1 show a maximum index of about 7 with 2 ug/ml Con A, while the index for 2.5 ug/ml Con A was about 5 in Table 4 and 4 in Table 3. This variation was not due to differences between normal and heterozygous (nu/+) Balb/c since age and sex-matched animals exhibit similar responses in both radioactivity incorporated and indexes of stimulation to Con A and LPS. Although the exact nature of this variation is not clear, it was probably related to individual and age differences in animals between experiments (115).

Dose response and cell density results for ^{3}H-uridine uptake in a serum-free system were similar to those with serum (Tables 5 and 1). In the serum-free medium, the higher counts in unstimulated cultures and a possible lack of serum nutrients could account for consistently lower optimal indices of stimulation. The dose of mitogen necessary for optimal stimulation was the same for LPS and PHA with and without serum. The lower dose of Con A required in the serum-free system was probably due to a lack of serum glycoproteins and glycolipids that this mitogen would normally bind (70). PHA also binds to serum components, but the amount of PHA required and the broad dose response curve prevent detection of this dose shift in the serum-free system.

Another parameter of the ^{3}H-uridine incorporation technique investigated was the effect of exogenous ^{3}H-uridine on the amount of label incorporated. A roughly proportional relationship between the amount of label added and the amount incorporated was found (Table 2 and 45). This probably reflects equilibrium of labeled and unlabeled uridine in the UTP pool. Low amounts of label, 0.1 and 0.5 uCi, are not diluted by serum uridine suggesting that the serum level of this nucleoside is low. The statistically significant drop in stimulation indices above 1 uCi/well could be due to cell death by radiotoxicity.

Additional observations on the ^{3}H-uridine incorporation technique may be made based on the time response results (Figures 3-6). The purpose of pulse labeling and label accumulation experiments was to determine the earliest time the response could be measured and to see if the increased stimulation index was due to an increased labeling of stimulated cells or to a decreased labeling of unstimulated cells. Therefore, the pertinence of these studies to RNA metabolism cannot be assessed.

One hour pulse-labeling data of control and Con A stimulated cultures with and without serum was analysed by the student's T test and linear regression (Figures 4 and 5). The slope of the time-dependent response of non-stimulated cells in a serum-free medium is not significantly different from zero. This suggests a lack of increase in the rate of RNA synthesis of unstimulated cells and is in agreement with the findings of others (123). The slopes of the responses of Con A treated cultures both with and without serum are significantly different from zero ($p<0.01$), implying an increased rate of RNA synthesis during stimulation as has been previously reported (18). However, the slope of the response of unstimulated cells in the presence of serum is also significantly different from zero ($p<0.01$). Since some preparations of fetal calf serum may be slightly mitogenic due to bacterial products, it is possible that this increased rate of RNA synthesis is due to stimulation by the serum. In addition to the mitogen-induced increase in the rate of RNA synthesis, the magnitude of the mitogen response with or without serum is significantly different from the respective control response at all time points ($p<0.01$). This difference probably reflects the increased size of the responding population.

In contrast to the pulse-labeling experiments, the addition of ^{3}H-uridine at the beginning of culture measures label accumulation over time (Figures 3 and 6). As predicted by the pulse-labeling results, unstimulated cultures without serum show little change in incorporated counts over time, and serum containing control cultures do exhibit a slight increase in label accumulation. Con A stimulated cultures with or without serum show the expected accumulation of ^{3}H-uridine as predicted by pulse-labeling up to 8 hours. After that time, the decrease in proportional accumulation may be related to a decrease in exogenous ^{3}H-uridine. Previous findings indicate that the level of label is proportional to the amount of label added and that the same number of cells are able to incorporate at least three times as much ^{3}H-uridine (Table 2). Therefore, the decrease in expected accumulation of label after 8 hours is not due to saturation with ^{3}H-uridine but probably is related to a depletion of extracellular label. This may be complicated in the serum-free system by a lack of other nucleotides which are necessary for RNA synthesis (72). In general, it may be concluded from both of these time response studies that the measured indices of stimulation are due to increased rates of labeling and accumulation of counts by mitogen-treated cells, rather than to decreased labeling of unstimulated cultures. The response both with and without serum appears by 4 hours and is easily read at 8 hours.

Actinomycin-D inhibition of RNA synthesis was used to investigate the fate of the incorporated ^{3}H-uridine. The drug appears to act by complexing with the DNA template and interfering with RNA polymerase and RNA transcription (100). In stimulated lymphocytes, Actinomycin-D treatment reduces basophilia and the number of detectable nucleoli (63). The synthesis of 18s and 28s, but not 4s or polydisperse DNA is inhibited (119, 63). Data presented in Table 3 represents about a 40% reduction in incorporated ^{3}H-uridine in unstimulated cells and a 97% inhibition of the ^{3}H-uridine increases induced by mitogens with 1 or 10 ug/ml. This agrees with Kay <u>et al</u>. who found a 25% decrease in labeled RNA in unstimulated cells and 100% inhibition of the PHA induced increase (63). The reduction of inhibition to 89% with 0.1 ug/ml Actinomycin-D may reflect labeling of 18s moieties which are not as sensitive to the drug as are 28s (62). Actinomycin-D inhibition of ^{3}H-uridine incorporation by stimulated cells implies that the label is going into newly synthesized RNA.

Since stimulation of lymphocytes causes an increase in uridine uptake and in UTP pool size, the possibility that these two labeled molecules, rather than labeled RNA, were being measured was investigated (89, 18). Although TCA does precipitate uridine or UTP, these moieties could have been trapped inside TCA-fixed cells (106). If this were the case, pretreatment of cultures with SDS, which lyses cells and denatures protein while leaving RNA intact, should have resulted in fewer TCA precipitable counts (59). Results indicate that SDS pretreatment does not alter the amount of TCA precipitable label. The stimulation measured appears not to be the result of changes in internal uridine pool size, but rather label incorporation into RNA.

The stimulation by T or B cell specific mitogens of purified cell populations was investigated to determine whether ^{3}H-uridine incorporation measured responses of specific segments of the lymphocyte population. Without the ability to distinguish cell population responses, the method would have little application since mitogen stimulation has been widely used to analyze the ontogeny and function of T and B cell populations (75, 6). Moreover, mitogen stimulation of specific cell populations has recently demonstrated the presence of the immune response antigen (Ia) on the surface of stimulated T cells and the existence of at least two subpopulations of both T and B cells (27, 81). Without a defined specificity of response, none of these experiments could have been done.

In this study, purified B cell populations were obtained from nude mice and T cells from column separation. Although these cell sources have been widely tested by others, a check of their composition by complement-dependent cytotoxicity with anti-Thy 1.2 serum (37, 43, 125). This technique confirmed the lack of Thy-1 bearing cells in nude mice and the removal of B cells from column purified spleens. Assuming that LPS is specific for B cell stimulation, and that Con A activates only T cells, data presented in Table 4 agrees with the cytotoxicity results on the composition of each cell population. More importantly, these data indicate that ^{3}H-uridine incorporation measures the responses of selected cell populations to specific stimuli.

Up to this point it has been shown that the culture conditions for the ^{3}H-uridine technique have been optimized and that results are comparable to those for ^{3}H-thymidine. The ability to obtain results by 8 hours and the specificity for T and B cell responses suggest some advantages and applications of the technique.

The short culture time obviates the need for serum in an otherwise chemically defined medium. Preparations of fetal calf serum have been shown to possess varying amounts of bacterial products which may be mitogenic (113). Two serum α globulins, low density lipo-proteins and α-fetoprotein have been shown to inhibit lymphocyte proliferation (78, 22, 80). In addition to various enzymes, serum contains insulin and thymic hormone which induce Thy 1 negative cells to become Thy 1 positive, and a thymic hormone inhibitor (9). The lack of a serum requirement eliminated these and other unknown factors that may effect the culture.

The comparatively short culture time required has the advantage over the measurement of DNA in that secondary effects of stimulation are less likely to be detected. The effects include the production by stimulated lymphocytes of a blastogenic factor which increases proliferation and α-fetoprotein which inhibits it (120, 66). In longer cultures, mitogens have been shown to induce suppressor cells which inhibit proliferation, newly Thy 1 positive cells which may react with T cell mitogens, and T cell Ia antigens whose function has not been clarified (103, 100, 30, 13, 24).

The population specificity demonstrated previously may be utilized in one of the several possible applications of the ^{3}H-uridine technique. That is, the selective removal of responding cells by radiotoxicity with high levels of labeled uridine. The exact mechanism of radiotoxicity is not known, although the damage by a free radical appears to be at the nuclear level. The maximum range of a ^{3}H-beta particle is 0.6 u, so both nucleus and cytoplasm of a ^{3}H-uridine labeled lymphocyte are irradiated. It has been shown that cytoplasmic irradiation by incorporation of ^{67}Ga-citrate is not as toxic as nuclear incorporation of ^{3}H-thymidine (55).

Two methods for radiolysis of lymphocytes have been used. They are the incorporation of ^{125}I UDR into DNA, which requires cells in the S phase and the exposure of cells to ^{125}I antigens to which the animal must first be primed (10). ^{3}H-uridine mediated radiotoxicity would have the following two advantages over present methods: time in culture could be decreased since DNA synthesis is not required and antigen priming of animals would not be necessary. Therefore, the addition of a high amount of ^{3}H-uridine to stimulated populations would quickly eliminate the responding population leaving the remaining cells availabe for functional testing.

The ability to assess immunoreactivity with ^{3}H-uridine, presumably during G_1, has one final advantage over measuring ^{3}H-thymidine during S. Although there is a similarity in immunoreactivity results obtained by measuring RNA and DNA synthesis, events occurring in G_1 do not necessarily have to lead to events in S. For example, antibody, macrophage migration inhibition factor or interferon production can occur even if DNA synthesis is inhibited (132, 126, 8). In addition, it has been shown that Con A stimulation of lymphocytes from immature animals induces fatty acid incorporation, an early event, with no effect on DNA synthesis. Cells from older animals respond to Con A in both stages of the cell cycle (102). In another study, cells were cultured with Con A, and anti-Con A was added at various times up to 24 hours. Analysis of protein, RNA and DNA synthesis showed normal protein and RNA synthesis, but no DNA synthesis, if the antibody were added in the first 20 hours of culture. The removal of Con A and anti-Con A and the addition of PHA restored the DNA response (69). This suggests that induction of cells into G_1 does not guarantee continuation into S and measurement of DNA synthesis alone may not be adequate. Therefore, there is a need for assays of immunoreactivity at different stages of the cell cycle such as incorporation of ^{3}H-uridine during G_1.

SUMMARY

An *in vitro* microculture assay for murine spleen lymphocyte immunoreactivity that utilizes ^{3}H-uridine incorporation was developed. The parameters of the culture technique with respect to cell density, doses of LPS, Con A and PHA, ^{3}H-uridine dose, and length of culture time were investigated. It was found that a response was detectable at 4 hours for all three mitogens with labeling ranging up to 176% of the control value. By 8 hours there was a 100 to 230% increase in mitogen-induced incorporation of radioactivity. Similar increases were observed in a serum-free system. The responses in both media were due to increased incorporation of label by stimulated cultures and not to decreased labeling of non-mitogen treated cultures over time. The validity of the ^{3}H-uridine incorporation method was tested in three ways. It was found that labeled uridine is incorporated, not trapped, into TCA precipitable material that is probably RNA since labeling was inhibited by Actinomycin-D. Results with 24 hour ^{3}H-uridine labeling were shown to be similar to those obtained with ^{3}H-thymidine labeling at 48 hours with respect to optimal mitogen dose and the shapes of the dose response curves. Moreover, the ^{3}H-uridine technique was demonstrated to be specific for the responses of stimulated T or B cell populations.

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