Incorporation of ^{3}H-Thymidine into PHA-Stimulated Rabbit Peripheral Blood Lymphocytes. Kinetics of the Response[1] (37332)

JOHN M. MANSFIELD AND JOHN H. WALLACE

Department of Microbiology, University of Louisville School of Medicine, Louisville, Kentucky 40201

Two functionally and antigenically different populations of lymphocytes constitute a major portion of the immunological system of higher animals; these two cell populations may act independently of, or cooperatively with, each other in mediating immune responses (1). One population of cells, the bursal-equivalent (B) lymphocytes, is intimately involved in the secretion of antibody molecules following appropriate stimulation (2). Members of the other population, thymus-processed (T) lymphocytes, do not secrete antibody after stimulation but direct cell-mediated types of immunological responses via the production of chemical messengers known as lymphokines (3).

Much attention has been focused in recent years on the T-lymphocyte, since this cell is primarily responsible for allograft rejection, tumor immunity, graft vs host reactions, and other delayed-type hypersensitivity phenomena. Recently, investigators have sought to characterize and quantitate T-lymphocyte functions by evaluating lymphocyte responsiveness *in vitro* to PHA, a phytomitogen with specificity for the T-cell (4, 5). Such studies have indicated that normal ranges of PHA responsiveness can be determined with human lymphocytes on both an individual as well as a group basis (6–8). Further, deviations from normal PHA values have been found in malignancy and other diseases (9–12), and these deviations in PHA responsiveness have been shown to correlate well with a depression of cell-mediated immunity (13, 14).

We have been interested in measuring the PHA response of peripheral blood lymphocytes (PBL) from rabbits exhibiting various pathological conditions. While previous studies have established that rabbit PBL undergo blastogenesis in response to PHA (15–17), they were not concerned with determining either the optimal conditions for mitogen stimulation of these cells or the kinetics of the response with respect to ^{3}H-thymidine (^{3}H-TdR) incorporation into DNA. Consequently, it was of interest in the presently described studies to establish optimal conditions for PHA stimulation and isotope incorporation, kinetics of the response, and individual variations in the response by rabbit PBL.

Methods and Materials. Animals. Adult New Zealand white female rabbits (3.0–4.0 kg body wt) were caged individually and were provided food and water *ad libitum.*

Lymphocytes. Rabbits were routinely bled via cardiac puncture every two weeks. Lymphocytes were separated from whole blood by a modification of the Hypaque-Ficoll technique reported by Perper *et al.* (18). A 34% Hypaque (Winthrop Lab.) solution was prepared by diluting the stock solution with sterile double-distilled water; 34% Hypaque was added to sterile 9% (w/v) Ficoll (Pharmacia) on a 1:2.4 basis to form the gradient material (HF). Freshly drawn, heparinized (100 U/ml) blood was mixed with HBSS at a ratio of 1:2.5. The use of EDTA was specifically omitted in this procedure since preliminary tests showed that it reduced lymphocyte responsiveness to PHA. The HF solution was subsequently layered beneath the diluted blood with a 6-in. spinal-tap needle; in our studies, 10 ml HF was routinely layered under 35 ml diluted blood in sterile 50 ml screw-cap centrifuge tubes. All samples were centrifuged for 25–30 min

[1] Supported by NCI Grant No. CA13658 and Kentucky Tobacco and Health Research Institute Grant No. 73.

with a force of approximately 400*g* delivered to the blood–HF interface. The entire separation procedure was performed at room temperature. Following centrifugation, the lymphocytes, which band with the platelets at the plasma–HF interface, were aspirated to a fresh tube and washed twice in cold RPMI-1640 before being cultured. Lymphocyte populations collected in this manner were characterized by high yield (1–3.5 $\times$ 10^6 cells/ml whole blood), excellent purity ($<$10% blood monocytes, $<$1% PMN's, minimal erythrocyte contamination), and good viability (always $>$95%); the removal of platelets was deemed unnecessary. Whenever lymphocytes from other organs of the rabbit were harvested for the adjunct studies reported here, conventional techniques were employed (17).

Cell cultures. The effects of serum supplementation, cell concentration, culture duration, and PHA concentration on the cellular incorporation of ^{3}H-TdR were determined. Individual and group variations in the responses were noted. Lymphocytes were routinely cultured in 2.0 ml volumes of RPMI-1640 in 16 $\times$ 125 mm upright glass tubes fitted with stainless-steel closures; preliminary tests revealed that RPMI-1640 was superior to other commercially available media (MEM-S, M-199, *etc.*,) in terms of cell viability and response to mitogen. All cultures were incubated at 37° in a 5% CO_2 in air mixture. The PHA source was PHA-P supplied by Difco (lot no. 556513). Several bottles of one lot were reconstituted with double-distilled water, pooled, filter-sterilized, and dispensed in small aliquots to sterile tubes for storage at —20°. As standard procedure, the contents of a tube were thawed and diluted in HBSS to the desired concentrations immediately prior to each test. The desired dosage of PHA was added to cultures in a 0.1 ml volume; controls received 0.1 ml HBSS.

Isotope incorporation. All lymphocyte cultures received 1 μCi/ml of ^{3}H-TdR (sp act 1.9 Ci/mmole; Schwarz-Mann) in 0.1 ml HBSS approximately 22 hr before termination. One exception was that isotope was added 8 hr prior to completion of 24-hr cultures. Determination of label uptake was done as described by Colley and DeWitt (19). Briefly, cells were washed twice in ice-cold 0.15 *M* NaCl, precipitated twice with cold 5% (w/v) trichloroacetic acid (TCA), and the final TCA-insoluble material was dissolved in Hydroxide of Hyamine 10-X (Packard). The dissolved material was transferred with a 1.0 ml absolute ethanol wash to 15 ml of scintillation fluid (5.0 g PPO and 0.1 g POPOP per liter toluene). All samples were counted in a Beckman LS-230 liquid scintillation counter at a ^{3}H counting efficiency of 60% with a counting error of 5% or less.

Results. The degree of quenching was constant (35%) for all samples, as determined by the ^{137}Cs external standard ratio technique. Consequently, all data were reported as gross counts per minute (cpm), and conversions to disintegrations per minute were not made.

Serum source and concentration. Figure 1 shows the relationships between serum supplementation of cultures and PHA stimulation.

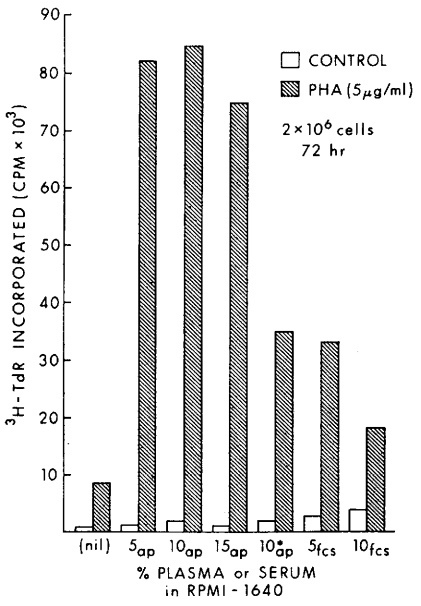

FIG. 1. Responsiveness of rabbit PBL to PHA in medium supplemented with different concentrations of heat-inactivated plasma or serum (ap = autologous plasma; fcs = fetal calf serum; * = not heat-inactivated).

In all instances, the addition of 5–10% autologous heat-inactivated plasma to RPMI-1640 resulted in maximum cell viability (60% or greater at 72 hr) and isotope incorporation. Heat inactivation of plasma was necessary for optimal stimulation and minimum variability in counts. The addition of normal pooled allogeneic serum to cultures gave inconsistent results with respect to cell viability and isotope incorporation. The usage of various lots of fetal calf serum invariably resulted in enhanced stimulation of control cultures, suppressed stimulation of PHA-treated cultures, and low cell viability. All subsequent experiments were performed, therefore, with cells cultured in RPMI-1640 supplemented with 5–10% fresh, heat-inactivated autologous plasma.

Cell concentration. With all doses of PHA tested, maximum isotope incorporation occurred on a cpm/cell basis in PBL cultures containing 1–2 $\times$ 10^6 cells (Fig. 2). Although cell concentrations of up to 8 $\times$ 10^6 cells gave higher gross cpm per culture than lower cell concentrations, the amount of isotope taken up per cell was always considerably lower. Also, nonstimulated (control) culture incorporation of ^{3}H-TdR was always greater with increasing cell concentration,

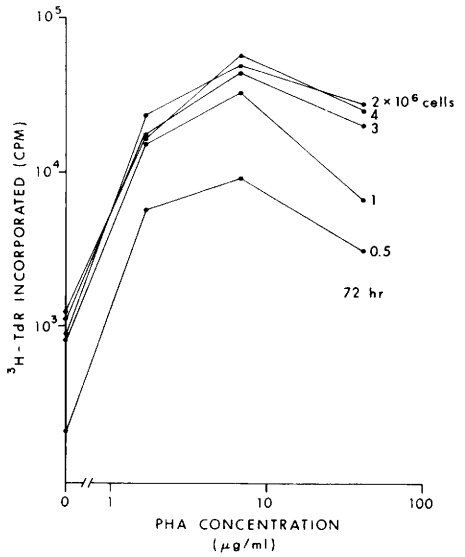

FIG. 2. PHA dose-response curves with rabbit PBL cultures of differing cell concentrations.

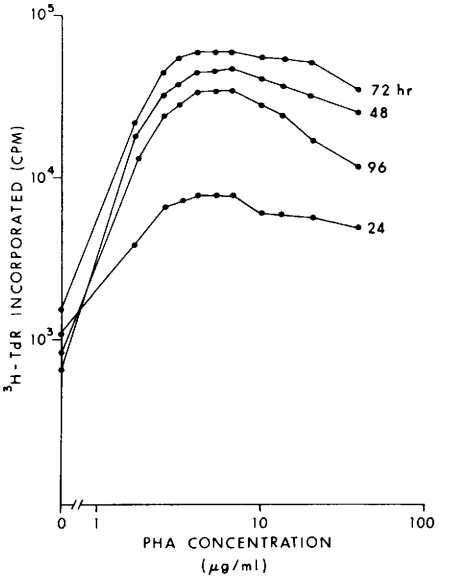

FIG. 3. PHA dose-response curves with rabbit PBL cultures of differing duration (2 $\times$ 10^6 cells/culture).

resulting in significantly lower indices of stimulation (SI)[2]. For the present study, cell concentrations of 2 $\times$ 10^6 per culture were employed not only for reasons of maximal stimulation, but also since small changes in viability or cell numbers would not appreciably affect culture isotope incorporation.

Culture duration and PHA dosage. Figure 3 depicts results of a representative experiment with PBL from a single rabbit on one occasion. In this instance, as in all tests, maximum isotope incorporation occurred in 72-hr cultures with 48, 96, and 24 hr cultures incorporating less, respectively. The dosage of PHA giving maximum stimulation in each case was approximately 5 μg/ml. Although 72-hr cultures had greater maximum cpm than 48-hr cultures, the 48-hr cultures displayed greater SI than 72-hr cultures due to lower control levels of isotope incorporation. This relationship is more clearly seen in Table I.

Variability in the response to PHA. The least variation in group PBL responses to PHA occurred in 48-hr cultures (Fig. 4). For example, counts of 48,000 ± 7,000

[2] SI = $\text{cpm}_{\text{PHA}}/\text{cpm}_{\text{Control}}$.

TABLE I. Mean Counts per Minute (cpm) and Indices of Stimulation (SI) for PHA-Treated Rabbit PBL Cultures of 24–96 hr Duration.

Culture duration (hr)	cpm × 10^3 (PHA/control)[a]	SI
24	7.5/1.1	7
48	48 /0.9	53
72	60 /1.5	40
96	34 /1.0	34

[a] 2 × 10^6 cells/culture; 5 μg/ml PHA.

(mean cpm ± SD) at 48 hr are compared to counts of 60,000 ± 17,000 at 72 hr for PBL from ten rabbits tested at the optimum dosage of PHA; the corresponding coefficients of variation (CV) in cpm are 15% at 48 hr and 28% at 72 hr. Also, repeated tests with PHA on single animal's PBL over a 3-month period were less variable with 48-hr culture periods (46,000 ± 5,000 cpm for a typical animal) than with 72-hr cultures. Individual animal CV's in cpm with peak isotope incorporation were always less than group CV's in 48- or 72-hr cultures.

Adjunct studies with rabbit lymphocytes. The peak responses of rabbit PBL to PHA were compared to the peak responses of lymphocytes from bone marrow (BMC), spleen (SPC), and lymph node (LNC). Data presented in Table II show that LNC and SPC were stimulated by PHA to levels greater than PBL, but that BMC responded to PHA at a level considerably lower than lymphocytes from the other sources. Also, rabbit PBL were tested for reactivity *in vitro* with a variety of different stimulants. PBL could be stimulated by Concanavalin A (SI = 155), pokeweed mitogen (SI = 38), activated supernatant fluids from antigen-stimulated lymphocyte cultures (SI = 8), as well as by antigen for which the cells were sensitive (PPD; SI = 10) when PBL were prepared and cultured in the manner outlined in this study as being optimal for PHA stimulation.

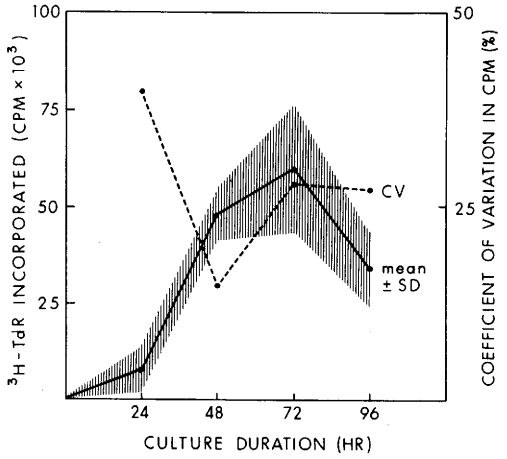

FIG. 4. Mean responses (± SD) and coefficients of variation with optimal PHA stimulation of rabbit PBL cultures of differing duration.

Discussion. The rabbit is estimated to have 20–40% T-lymphocytes in its PBL population, compared to much higher values for the peripheral blood of man and other animals (20, 21). One might expect rabbit PBL, therefore, to respond to PHA at lower levels than PBL from other species and at lower levels than lymphocytes from lymphoid organs such as spleen and lymph node. Earlier studies by others have indeed demonstrated that rabbit PBL respond to PHA at very low SI (17, 22), compared to levels of stimulation attained with PBL from humans (7). Furthermore, Singhal *et al.* (17) reported that rabbit PBL could not be stimulated by PHA to the same degree as lymphocytes from bone marrow, appendix, spleen, or lymph node. Fanger *et al.* (4) relate the necessity of using reduced glutathione with rabbit cells as an "enhancer" of the mitogenic response *in vitro*.

The present study, however, shows that rabbit PBL may be stimulated to predictably high SI's by PHA, without the addition of mitogenic enhancers. Further, PBL respond

TABLE II. Comparison of the Responses of Rabbit Lymphocytes from Various Organs to PHA *in Vitro*.

Lymphocyte source[a]	SI[b]
Bone marrow	1.5
Peripheral blood	53
Spleen	250
Lymph nodes	450

[a] 2 × 10^6 cells/culture; 5 μg/ml PHA.

[b] Optimal indices of stimulation.

to PHA at a significantly greater level than lymphocytes from bone marrow. Since bone marrow is supposedly devoid of mature T-cells (23), the graded order of responsiveness to PHA by lymphocytes from the various sources tested (LNC > SPC > PBL > BMC) may reflect their relative T-cell composition in the rabbit. Also, rabbit PBL prepared and cultured in the manner described here respond well to stimulation by Concanavalin A, PWM, activated supernatant fluids, and antigen for which the cells are sensitive.

Part of the inconsistency of the early work with rabbit PBL may have been due to suboptimal culture conditions. For example, culture periods ranging from 24 to 108 hr, concentrations of 1–8 $\times$ 10^6 cells per culture, and media supplemented with high concentrations of allogeneic or xenogeneic serum are reported. Our investigation indicates that the conditions for optimal PHA stimulation of rabbit PBL as measured by the subsequent incorporation of ^{3}H-TdR include: (a) 48 hr culture period; (b) 2 $\times$ 10^6 PBL per culture in RPMI-1640 containing 5–10% heat-inactivated, autologous plasma; (c) 5 μg/ml PHA-P; and (d) 1 μCi/ml ^{3}H-TdR added to cultures 22 hr before termination. Under these conditions, individual and group variations in the response to PHA are slight.

Probably another significant factor in obtaining optimal stimulation was the method employed for separating lymphocytes from rabbit blood. Preliminary work showed that sedimentation of cells in dextran (24) or gelatin (4), or differential centrifugation of whole blood (25), resulted in unsatisfactory yield and purity of the cell populations obtained. Erythrocyte, blood monocyte, and polymorphonuclear neutrophil (PMN) contamination was high in these preparations; adverse effects on the stimulation of PBL have been reported as being due to large numbers of erythrocytes or PMN's present in culture (26). The Hypaque-Ficoll technique employed in the present study gave a consistently high degree of lymphocyte recovery and purity and is recommended as the method of choice.

In conclusion, rabbit PBL appear to be very useful for immunologic studies. Data presented here indicate that normal values may be established for ^{3}H-TdR incorporation into PHA-stimulated PBL when optimal culture conditions are employed. Tests for deviations from normal PHA values, on an individual or group basis, could be used as indicators of altered T-lymphocyte function. Indeed, preliminary evidence from our laboratory suggests that PBL from rabbits with suspected immunosuppressive disease have markedly impaired PHA responses *in vitro,* as well as suppressed cell-mediated immune responses *in vivo.*

Summary. Rabbit PBL were shown to incorporate predictably high levels of ^{3}H-TdR following stimulation with PHA. The conditions necessary for optimal stimulation of rabbit PBL were: (a) separation of lymphocytes from whole blood by the Hypaque-Ficoll technique; (b) 2 x 10^6 PBL per culture in RPMI-1640 containing 5–10% heat-inactivated, autologous plasma; (c) 48 hr culture period; (d) 5 μg/ml PHA-P; and (e) 1 μCi/ml ^{3}H-TdR added to cultures 22 hr before termination. Under these conditions, individual and group variations in the response to PHA were slight, and PBL responsiveness to other mitogens as well as to antigen were good.

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Received Feb. 9, 1973. P.S.E.B.M., 1973, Vol. 143.