A Micromethod for Lymphoblastic Transformation of Mouse Lymphocytes from Peripheral Blood (37242)

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Although several micromethods for culturing human peripheral lymphocytes exist (1–3), attempts to develop similar procedures for small laboratory animals have not been equally successful (4–6). First, mouse lymphocytes appear less responsive to PHA stimulation than human lymphocytes under their respective culture conditions (7, 8). Second, because cell suspensions from whole organs have generally been used from mice, direct comparisons to human peripheral lymphocytes are difficult; also, the mice had to be killed, thus eliminating them from further use or breeding experiments. Third, the culture systems for mouse lymphocytes employed varying amounts of heterologous serum (5, 6) or lacked appropriate standardization with respect to optimal culturing conditions and culture time. The lack of a fast and reproducible micromethod for lymphoblastogenic transformation in mice is regrettable because it precludes studies on the genetic control of transformation and initiation of cell proliferation without sacrificing the animals.

We now describe a reliable method for culturing mouse peripheral lymphocytes that (a) requires only 50 μl of blood, thus allowing the preparation of several cultures from individual mice without interference with their metabolism or reproduction, (b) avoids the need for tedious cell separations, thus retaining erythrocytes in culture which may be beneficial for successful blastogenic transformation (9), and (c) excludes the use of heterologous sera. The method should be useful in investigations of the function of peripheral lymphocytes and allow for study of their genetic control including linkage studies for the genes governing their response to PHA or other antigens.

Materials and Methods. The following strains of mice were used, AKR/J, C57BL/10ScSn, C57BL/10J, and C57L/J; unless stated otherwise, they were 10 ± 2-wk-old females.

Phytohemagglutinin-M (PHA-M) was obtained from Difco Laboratories, and culture media consisting of Trowell's T-8 and Ham's F10, TC 199, McCoy's 5a modified, Waymouth's MD705/1, and RPMI 1603, 1629, 1630, 1634, and 1640 (10, 11), were purchased from Grand Island Biological Laboratory (Gibco). The pH of all media was adjusted to 7.2–7.4 with 20% sodium bicarbonate solution. Tritiated thymidine (^{3}H-5-TdR) with a specific activity of 2 Ci/mmole was obtained from New England Nuclear, Boston, MA.

The mice were placed in a warm restraining block and their tails were wiped first with 70% and then with 95% alcohol. After the tail had been incised with a sterile razor blade, the first drop of blood was discarded to avoid possible contamination; the blood was then collected in a sterile plastic culture tube (12 × 75 mm, Falcon No. 2058) containing 50 USP units of preservative-free sodium heparin/10 drops of blood. Usually, between 0.2 and 0.5 ml of blood was collected from each mouse to give 4–10 cultures, but in some experiments blood was pooled from several mice. The culture tubes were transferred to a sterile laminar flow hood, tapped lightly to mix the blood and heparin, and an appropriate amount of the mixture was pipetted into new culture tubes. Various culture media containing 100 μg of streptomycin and 100 units of penicillin/ml were added, followed by an appropriate amount of diluted PHA-M (see Results). We used the standard dilution recommended by the manufacturer which consisted of adding 5 ml of distilled H_2O to the PHA vial and resulted in about 10 mg total protein/ml of distilled water. In each experimental series the same batch of PHA-M was used. Control cultures were set up containing no PHA-M. The tubes were capped loosely, shaken gently, and then placed upright in an incubator at 37 ± 0.5° with 5% CO_2 in air for various time intervals. Then ^{3}H-TdR was added, and after appropriate periods of labeling, the cultures were removed from the incubator; erythrocytes were lysed by adding 2 ml of distilled water to each culture and passing several times through a pasteur pipette. The contents were collected on glass fiber filters (No. 934AH, Reeve Angel) using a Millipore sampling manifold (No. 3025), washed twice with 0.85% NaCl, and four times each with 0.5 *N* perchloric acid (PCA), absolute ethanol, and anhydrous ethyl ether. Each filter was allowed to dry and placed in a scintillation vial with 1 ml of Soluene (Packard Instrument Co., Inc.). The vials were allowed to stand for at least 12 hr at room temperature before adding 15 ml of Omnifluor–toluene Cocktail (New England Nuclear). After 0.5 hr of standing in the dark at 4°, the tubes were counted in a liquid scintillation counter Unilux II (Nuclear Chicago). The external standard method was used to determine the quenching effect (12).

Results were expressed either in absolute counts of ^{3}H-incorporation (dpm) or as stimulation index (SI) or both; the latter is defined as the ratio of radioactivity in PHA-stimulated cultures over controls. A total of 1550 cultures was analyzed.

Results. Effect of different media and influence of medium volume on PHA response. Optimal responses to PHA of whole blood cells occurred with Trowell's medium T-8 and RPMI 1640; the SI ranged from 20 to 50. The two media performed equally well. Of the other media tested, Ham's F10 and RP MI 1603 yielded good SI but the absolute counts were much lower than those obtained with T-8 or RPMI 1640. All other media were unsatisfactory with respect to both SI and radioactive counts. These tests were performed using 50 μl of blood, 0.5 ml of medium, 25 μl of PHA-M solution, and 2 μCi of ^{3}H-TdR as label. Incubation time was 36 hr followed by a 16-hr labeling period.

In order to determine the optimal volume of medium for PHA responsiveness we used 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 ml of T-8 medium; 25 μl of PHA-M solution was added and 1 μCi of ^{3}H-TdR was used as a DNA tracer. The best stimulation was obtained with 0.5 ml medium/50 μl of blood, whereas all other dilutions were inhibitory to the PHA response (Fig. 1). In unstimulated cultures the nonspecifically bound activity rose with volumes greater than 0.5 ml.

PHA dosage. The optimal PHA dose was determined in cultures containing 25, 50, 100, 250, 500, 1000, and 2500 μg PHA, respectively/culture and 50 μl of whole blood in 0.5 ml of T-8. The highest lymphocyte re-

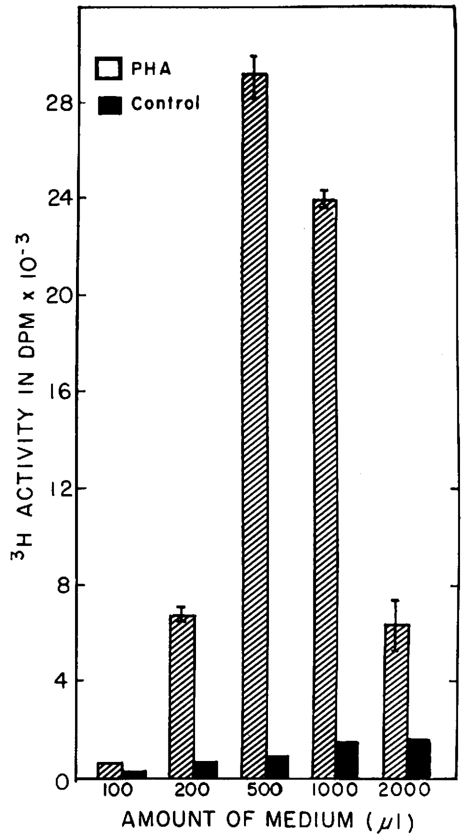

FIG. 1. Effect of medium volume on the growth of mouse lymphocytes. The conditions were optimal except for varying volumes of T-8 medium; highest blastogenic transformation of mouse peripheral lymphocytes occurred with 0.5 ml.

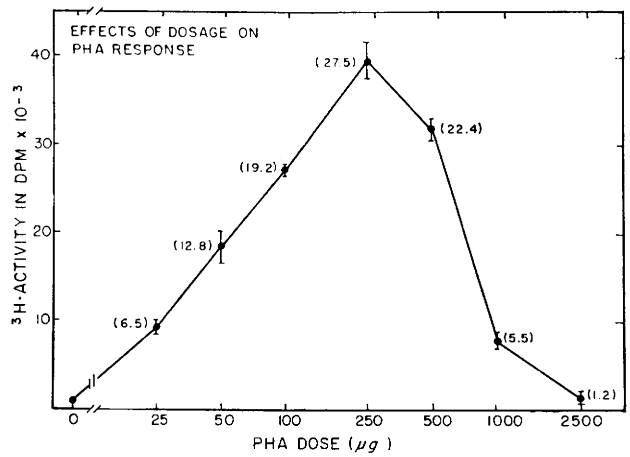

FIG. 2. Dose–response curve revealing that optimal PHA-M transforming dose is 250 μg added in a volume of 25 μl to 0.5 ml T-8 medium containing 50 μl of blood. Labeling was done with 2 μCi ^{3}H-TdR/culture.

sponse occurred with 250 μg (25 μl) of PHA-M/culture, *i.e.*, 500 μg/ml of medium. A greater concentration inhibited ^{3}H-incorporation (Fig. 2). The osmolality of the final culture mixture containing 50 μl of blood, 0.5 ml of T-8, and 250 μg of PHA-M was 301 mOsm/kg and in the controls containing no PHA-M solution, 305 mOsm/kg.

Effect of incubation time. The greatest incorporation of ^{3}H-TdR occurred in cultures incubated for 36 hr before the addition of 1 μCi of label/culture. A second smaller peak occurred after 60 hr of incubation (Fig. 3). We used a labeling period of 16 hr. Volumes of blood, medium, and PHA were as described.

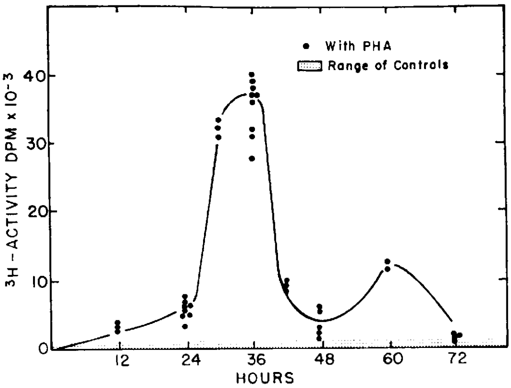

FIG. 3. Incorporation of ^{3}H-TdR is shown at various time intervals of incubation of the blood cultures. Incubation time exclusive of 16-hr labeling period with 1 μCi of ^{3}H-TdR is plotted.

Length of labeling period and effects of ^{3}H-TdR dose. Labeling periods of 1, 4, 8, 16, 20, and 24 hr were used. The incorporation of ^{3}H-TdR was found to level off or slightly decrease after 16 hr. We attribute the lower incorporation at 24 hr to cell damage or death from prolonged exposure to beta-irradiation (Fig. 4).

Levels of ^{3}H-TdR greater than 2 μCi/culture were toxic as indicated by a decline of incorporation. Although incorporation of radioactivity still increased above 2 μCi in PHA-stimulated cultures, the increase of nonspecifically bound activity was much more pronounced when 5 μCi or more were added to the control cultured without PHA.

Effects of mouse genotype on PHA response. Optimal culture conditions were established with blood from 10 ± 2-wk-old C57BL/10J mice. The following conditions were adopted: 50 μl of whole blood, 0.5 ml of T-8 medium or RPMI 1640, 250 μg of PHA-M or 25 μl of standard dilution, incubation for 36 hr prior to addition of 1 μCi of ^{3}H-TdR/culture, and a labeling period of 16 hr.

A comparison of the PHA responses of AKR/J, C57L/J, and C57BL/10J mice is shown in Table I; the PHA stimulatory response was greater in C57L/J than in AKR/J mice; that of C57BL/10J was in between

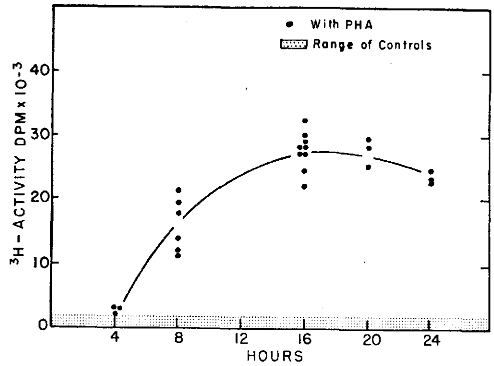

FIG. 4. Effect of the pulse duration on the incorporation of ^{3}H-TdR into peripheral mouse lymphocytes. After 16 hr, a maximum is reached.

these strains. No differences were found between C57BL/10J and C57BL/10ScSn. With the onset of thymic leukemia in the susceptible AKR/J strain, the peripheral lymphocytes can no longer be stimulated by PHA.

Discussion. The conditions of our method are similar to those described for human lymphocyte cultures (1); they differ, however, in two important respects: (a) a tenfold greater dilution of cells and (b) no supplementation of the cultures with fetal calf serum. Our cultures grow in very low concentrations of autologous plasma, thus avoiding the possible presence of natural antibodies directed against various murine cell types (13) and of possible nonspecific stimulating effects of heterologous serum.

We found that the optimal conditions depend upon the type of medium, its volume, the PHA dose, and the incubation time. Although Trowell's T-8 medium (14) and RP MI 1640 (10) differ considerably in their composition, they performed equally well. All other media permitted only a small (RPMI 1603 and 1629, Ham's F10, Waymouth's MD705/1) or no response (TC 199, McCoy's 5a, and RPMI 1630 and 1634) to PHA-M stimulation. Another factor is cell density (Fig. 1); for example, the 1:1 ratio of blood to medium employed by others (1) for human cultures is unsatisfactory for mice. The PHA dose dependence of blastogenic transformation is well known (7, 8), and our findings (Fig. 2) are similar to those of man (1). Despite the fact that our results are reproducible with different PHA batches, it is advisable to evaluate each batch in a known strain of mice, *e.g.*, C57BL/10J. Also, the PHA dilution must be carefully adjusted as peripheral lymphocytes are more sensitive to PHA toxicity in a serum-free medium (15). Although the system described here is not free of autologous plasma, its concentration in the final culture mixture is very low (*i.e.*, $< 5\%$).

Similar effects of incubation time were observed to those found by Park and Good (1), although these authors only show the ascending slope to the plateau. Despite the lengthy labeling period of 16 hr, our curve is sharp and distinct, perhaps because of the accumulation of toxic metabolites or deprivation of essential nutrients blocking DNA synthesis or both. Several other reasons could be cited, but no decision has as yet been made on the various possibilities.

Like others (1), we found that the activ-

TABLE I. Strain Differences in Incorporation and SI.[a]

Group	^{3}H-Activity in acid-insoluble fraction (dpm)	
	With PHA	Controls
AKR/J normal	27,155 ± 810 (27)[b]	433 ± 42 (12)
AKR/J lymphatic leukemia	492 ± 93 (6)	458 (2)
C57L/J normal	57,440 ± 5929 (13)	800 ± 67 (11)
C57BL10/J normal	32,943 ± 1739 (12)	773 ± 67 (8)

[a] Strain differences between the high leukemia strain AKR/J and the low tumor strains C57L/J and C57BL/10J. The mice were 2–4 mo old except for the AKR/J leukemic mice which were between 9–10 mo of age. Culture conditions were as follows: 50 μl of blood, 0.5 ml of T-8 medium and 250 μg of PHA-M; 36 hr of incubation prior to 16 hr of labeling.

[b] In parentheses, number of tests made.

ity in the acid-insoluble fraction increased up to 16 hr after adding the DNA tracer, then leveled off and declined probably because of cell death from the extended ^{3}H-TdR exposure; toxicity effects were clearly observed with increasing ^{3}H-TdR dose. Therefore, our culture procedures should readily lend themselves for studies of the relative radiotoxicity of different isotopes (16).

It is generally agreed that circulating lymphocytes represent a heterogenous cell population of several functional types (1, 7, 17). The cells undergoing lymphoblastic transformation following PHA stimulation are generally considered to be thymus-derived or T cells (1, 18). In this case, whole blood culture may be used for a quantitative measure of these cells. T cells are primarily engaged in the cellular immune response, which is known to be governed by specific host genes (8, 19). Indeed considerable strain differences exist in both the stimulation index and the total uptake of radioactivity (Table I). The determination of a more complete strain distribution as well as the genetic analysis of the factors controlling PHA response will be published elsewhere. Attempts are also being made to determine their relationship to spontaneous tumorigenesis and life-span, inducibility of murine type C RNA virus and its group-specific antigen, and the effect of hormones and various metabolites on blastogenic transformation. Presently the method is also being tested for its suitability in mixed lymphocyte culture to elucidate the mechanisms of action of histocompatibility genes.

Leukemic mouse cells are unresponsive to PHA possibly because of a severe reduction of circulating normal lymphocytes; also, they may be blocked (20) from reacting with PHA or lack membrane PHA binding sites. Another possibility is the presence of inhibitory serum factors (21) in leukemic mice. The numerous leukemic cells in the culture medium (about 40,000/μl) do not synthesize DNA as the radioactive counts are equally low in controls as in the PHA containing cultures. In man no major differences exist in PHA response of cells from acute leukemia patients and normal individuals (22). In contrast, the PHA response is either abolished (23) or delayed (24) in chronic lymphoid leukemia as it is in AKR mouse leukemia.

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