Studies on the Blastogenic Response of Murine Lymphocytes I. Quantitative Measurement of Stimulation by Phytohemagglutinin (35893)

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Lymphocyte transformation and the induction of subsequent nucleic acid synthesis by specific and nonspecific stimuli has gained prominence in evaluating one of several major immunological defense mechanisms. In some cases, the use of these lymphocyte reactions may serve as one clinical criterion for evaluating the patient's capability of handling malignancy, since this capacity is altered in humans suffering certain malignant diseases (1, 2). This phenomenon then may provide a surveillance mechansim for recognizing and rejecting cells capable of establishing malignant foci. Since the human cannot be utilized effectively as an experimental model, other more appropriate models must be developed.

The laboratory mouse, in view of its genetic characterization, the numerous highly inbred strains available, ease of breeding and housing as well as relatively low maintenance costs provides an excellent model to delve into the mechanisms involved in cellular immune responses.

The major drawbacks to employing the mouse have been a sparsity of appropriate tissue and until recently, the difficulty in establishing suitable culturing conditions for murine lymphoid tissues (3). With these drawbacks in mind, we have developed a culture system that required fewer lymphocytes and less total volume while retaining exceptional reproducibility. Since the conventional "acid-insoluble" methods to extract nucleic acid and to quantitate lymphocyte transformation are tedious and lengthy, we have modified a relatively rapid disk method (4) for the assay of DNA synthesis in lymphocytes.

Methods. Mice. Male and female NIH-BALB/cAnN and C57BL/6N mice between 40 and 60 days of age were used.

Spleen cell preparation. Spleens were removed aseptically and in the presence of 20 ml of medium RPMI 1640 (Grand Island Biological Co.) and gently forced through a 60-mesh stainless steel filter. After a 10-min centrifugation at 275*g*, the cells were washed twice in RPMI 1640 (20 ml). Cell concentration was estimated electronically (Model B, Coulter counter) and cell viability was determined by a dye exclusion technique (5). Microscopic observation revealed the resulting leukocyte suspensions (approx 1×10^8/spleen) to be about 90% mononuclear cells with 95% viability.

Peripheral blood cell preparation. Blood was drawn by cardiac puncture into 1-ml tuberculin syringes moistened with heparin. Erythrocytes were sedimented using Plasmagel by the method of Tridente, *et al.* (6). The leukocyte-rich layer was diluted 2-fold with RPMI 1640 and centrifuged for 10 min at 275*g*. Following removal of the supernatant, the cells were resuspended in 1640, and lymphocyte counts and viability were determined. Generally, there were fewer than 20 erythrocytes/viable lymphocyte in the final suspension with lymphocyte viability consistently greater than 90%. In all experiments, the blood from several mice was pooled to obtain the required number of lymphocytes. The average number of lymphocytes recovered per mouse was approximately 4.0×10^6.

Culture conditions. Lymphocytes were incubated in loosely capped 12×75-mm Falcon plastic tubes in 1 ml of medium RPMI 1640 supplemented with 100 units of penicillin, 100 μg of streptomycin, and 5% heat-inactivated fetal calf serum (Lot No. 00035P, GIBCO). The cell cultures were incubated in an upright position at 37° in a 5%

CO_2–95% air mixture at 80% relative humidity. Serum was selected on the basis of its ability to support transformation in stimulated cultures with minimal spontaneous transformation in unstimulated cultures. Of the numerous sera tested—fetal calf, gamma globulin-free fetal calf; gamma globulin-free newborn calf; calf; human; and homologous mouse plasma—fetal calf serum was found to be the most suitable.

Optimal phytohemagglutinin-P concentration (PHA; Difco Laboratories), for maximum transformation, was determined at several lymphocyte culturing densities.

The cell cultures were pulsed at various time intervals during incubation with 0.5 μCi of tritiated thymidine (2.0 Ci/mmole) or 0.5 μCi of tritiated uridine (2.1 Ci/mmole) in 0.1 ml of RPMI 1640. Nucleoside incorporation was assayed by the method described by Bollum (4) as modified for tissue culture studies by Regan and Chu (7). Incubation was terminated by plunging the cultures into an ice water bath. Subsequently, the cells were disrupted by a 10 kc Raytheon sonic oscillator. Up to six cultures could be sonicated simultaneously. An aliquot (0.1 ml) of the lysate was then delivered onto a Whatman 3MM filter disk. After a 1-min absorption, the disks were submerged in 10% trichloroacetic acid at 2–5°. Then at room temperature, all disks were washed 3× for 10 min in 5% TCA, 3× for 10 min in 95% ethanol, and briefly 2× in ether. The thoroughly dried disks were then placed in glass scintillation vials containing 10 ml of 2, 5-diphenyloxazole and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in toluene and counted in a Packard-Tri-Carb liquid scintillation spectrometer.

Results and discussion. Data illustrated in Fig. 1 indicate that a minimum sonication of 1-min duration was required for effective lysis of cells. With shorter periods of sonication, the relative counts were substantially lower and were directly related to the efficacy of the lysis treatment. Although morphologically dissimilar, stimulated and unstimulated lymphocytes were equally susceptible to lysis by sonication. The reproducibility of replicate cultures was consistently high, and rarely did cultures, lysed by at least a 1-min sonication, differ by more than 15% of the mean estimate. Shorter sonication periods, however, resulted in noticeably poorer replication. The specificity of this technique to measure the incorporation of tritiated thymidine into lymphocyte DNA was verified by the removal of more than 90% of the radioactivity from disks containing extract from stimulated cultures by treatment with DNase.

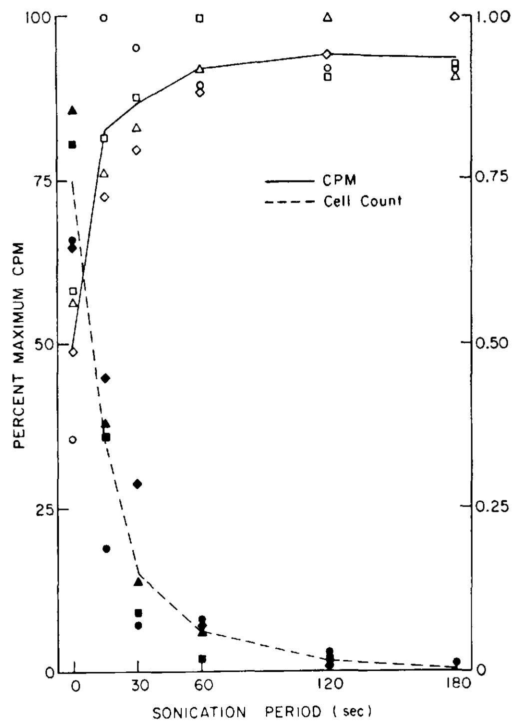

FIG. 1. Effect of culture sonication on the lysis and DNA extraction yields of PHA-stimulated and unstimulated splenic lymphocytes: (identical closed and open symbols) mean values of the same three mice, each measured in triplicate; (triangles and squares) PHA-stimulated cultures; (diamonds and circles) unstimulated cultures. Cultures were terminated after 66-hr incubation. ^{3}H-Thymidine was added at 48 hr.

Use of this modified method to assay nucleoside incorporation eliminates numerous individual culture manipulations, required with the conventional acid-insoluble method (8). The procedure is relatively rapid, and employing the culturing conditions described, it permits up to 150 cultures to be processed and ready for counting within 2 hr after termination of culture incubation.

PHA-induced lymphocyte transformation was supported by fetal calf serum over a wide range of concentration (1.25 to 20%), with optimal levels generally similar to those reported for supporting allogeneic reactions of mouse lymphocytes (9). It should be noted, however, that the influence of serum concentration on PHA stimulation of lymphocyte thymidine uptake varied markedly between individual mice. Whereas wide variation between sera in their ability to support mouse lymphocyte transformation is well recognized (9–11), no clear indication of the serum-dependent variation among mice of a common strain and age is available. Figure 2 illustrates the results of a typical experiment in which the isotope uptake in splenic cultures of 8 mice was compared at various

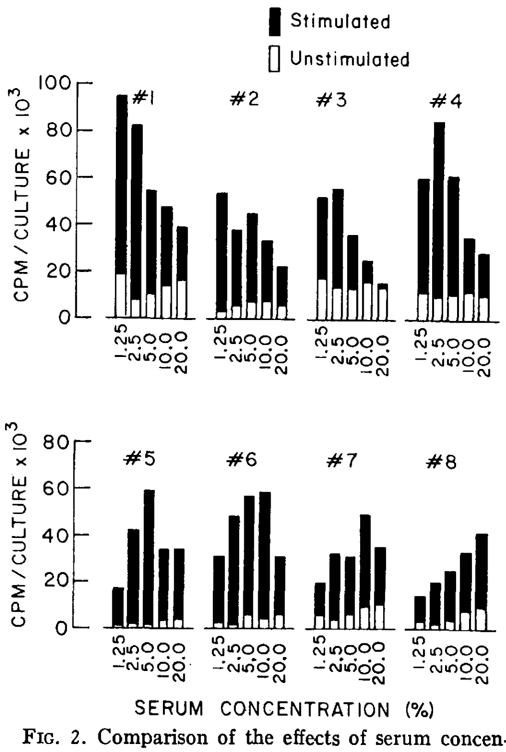

FIG. 2. Comparison of the effects of serum concentration on ^{3}H-Thymidine uptake in PHA-stimulated and unstimulated splenic lymphocytes from eight different mice: Values represent means of triplicate cultures. Cultures were terminated after 66-hr incubation. ^{3}H-Thymidine was added at 48 hr.

serum concentrations. It is evident that the uptake of ^{3}H-thymidine by stimulated lymphocytes from each mouse was highly dependent on serum concentration and furthermore, that optimal serum levels for individual mice differed widely. The underlying mechanisms by which serum promotes lymphocyte transformation and its significance in the interpretation of the *in vitro* response are unclear. From human studies, it has been suggested that autologous serum by its "coating" properties may enhance the mitogen-to-cell and/or cell-to-cell interactions necessary for PHA-induced transformation (12). As heterologous serum contains a variety of beneficial, as well as inhibitory components, it may be reasonable to attribute the observed differences to complex interactions between these serum factors and both the lymphocyte and mitogen. Further confounding the role of serum in lymphocyte cultures, in other experiments performed by us, significant uptake of thymidine (approx 30% of that obtained in cultures supplemented with 5% fetal calf serum) was consistently observed in stimulated lymphocytes cultured in the absence of exogenous serum. Although these data suggest that serum may not be absolutely essential for lymphocyte culturing, it seems possible that certain plasma components indigenous to the donor tissue were not completely removed during the preparatory washing procedures and that these factors, in minute quantities, were capable of contributing to lymphocyte survival *in vitro*. It was noted that the supplementation of these cultures with as little as 1% homologous plasma produced up to a 3-fold increase in thymidine incorporation. In Fig. 3, the magnitude of incorporation by peripheral and splenic lymphocytes at various culturing densities are compared. On the basis of response per 1×10^6 cells, maximum incorporation by stimulated peripheral lymphocytes exceeded that of splenic cells at culturing densities of less than 1×10^6/ml; whereas, above these densities splenic cells were most responsive. Although these data support previous reports that peripheral cells are more responsive to PHA-induced transformation than splenic cells (9), they clearly demonstrate that the

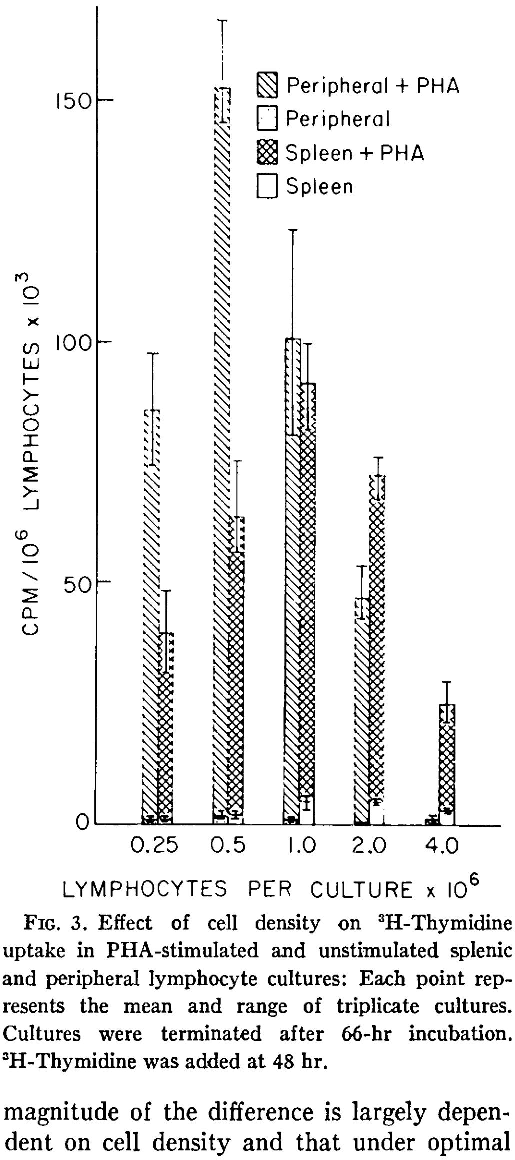

FIG. 3. Effect of cell density on ^{3}H-Thymidine uptake in PHA-stimulated and unstimulated splenic and peripheral lymphocyte cultures: Each point represents the mean and range of triplicate cultures. Cultures were terminated after 66-hr incubation. ^{3}H-Thymidine was added at 48 hr.

magnitude of the difference is largely dependent on cell density and that under optimal conditions the difference is substantially less than originally reported. In these and earlier experiments, maximum incorporation per 1 $\times$ 10^6 peripheral lymphocytes was consistently obtained at culturing densities between 0.5–1.0 $\times$ 10^6 cells/culture while 1.0–2.0 $\times$ 10^6 cell were required in splenic cultures. At concentrations exceeding these optimums the thymidine uptake was noticeably suppressed. To examine the possibility that PHA concentration limits the response of stimulated cultures at higher cell densities, optimal PHA levels were determined over a range of cell densities (0.5 to 4.0 $\times$ 10^6 cells). Maximum incorporation was achieved at a concentration of 1 μl/culture. At higher levels (10 μl/culture) PHA-P was cytotoxic and transformation was greatly reduced, although to a lesser degree at higher cell concentrations. In contrast, lower concentrations (0.1 μl/culture) produced no significant transformation.

The time course of nucleoside incorporation by peripheral and splenic lymphocytes was generally similar (Fig. 4). As previously noted (10) we similarly observed a transitory surge of thymidine uptake, most likely due to the large lymphocyte, during the first 18 hr of incubation in unstimulated splenic cultures. As early as 6 to 12 hr after cell incubation, increased ^{3}H-uridine uptake was demonstrable in all stimulated cultures. Maximum uridine incorporation in PHA-

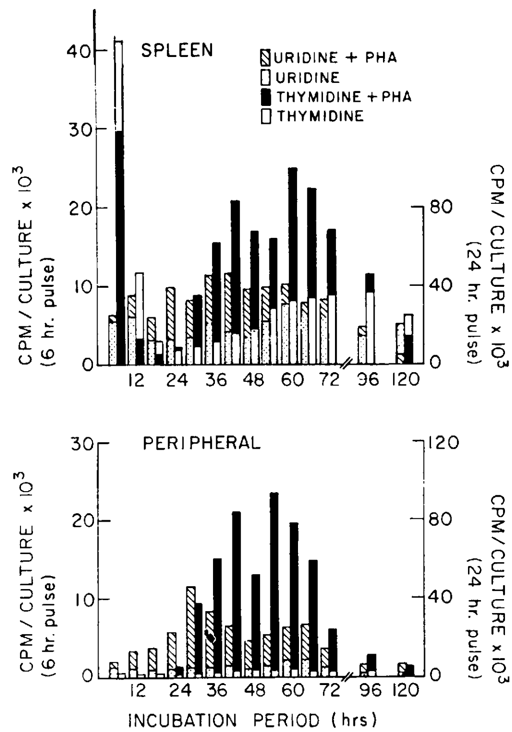

FIG. 4. Comparison of the rates of ^{3}H-Thymidine and ^{3}H-Uridine incorporation by PHA-stimulated and unstimulated splenic and peripheral lymphocytes during a 5-day incubation period. Tritiated nucleoside was added as indicated for the terminal 6- or 24-hr incubation. Values represent the mean of triplicate spleen cultures and duplicate peripheral cultures.

stimulated cultures occurred between 30 and 42 hr and was observed 6 to 12 hr earlier in peripheral cultures than in splenic cultures. In contrast, maximum DNA synthesis in stimulated cultures took place between 42 and 60 hr of incubation, approximately 18 to 24 hr after the initial period of maximum RNA synthesis. After 72 hr of incubation, little nucleoside was incororporated into nucleic acid by either stimulated or unstimulated cells.

In the present studies, both splenic and peripheral lymphocytes cultured under optimal conditions proved useful in studying PHA-induced lymphocyte transformation in the mouse. On the basis of the discrimination ratio of stimulated to unstimulated cells, the peripheral lymphocyte is, however, the preferred cell for study of this phenomenon. The peripheral lymphocyte is not only more responsive than the splenic cell to PHA stimulation, but the level of nucleoside incorporation in unstimulated cultures is extremely low. Although techniques have been described to increase the yield of leukocytes (9), the major disadvantage of peripheral blood as a source of lymphocytes is the limited number of cells obtainable from a single mouse. This difficulty may be largely avoided using spleens, though this cell population contains various leukocytic substances and erythroid cells known to inhibit cell culture and thereby may confound interpretation. Furthermore, other lymphoid, epithelial, and connective tissue cells present in splenic cultures may contribute to the total amount of radioactivity incorporated.

Summary. A sensitive and reliable system to culture mouse lymphocytes stimulated to transform by phytohemagglutinin is described. This system permits the convenient use of a relatively rapid modified "acid-insoluble" method to assay nucleoside incorporation. Under the conditions described, stimulated splenic and peripheral lymphocytes demonstrated elevated RNA synthesis 18–24 hr prior to maximum DNA synthesis. Nucleoside incorporation of stimulated peripheral lymphocytes exceeded that of splenic cells and occurred earlier. The degree of transformation was dependent on cell density, phytohemagglutinin-P concentration, and serum level.

1. Savel, H., Cancer **24**, 56 (1969).

2. Sharman, C., Crossen, P. E., and Fitzgerald, P. H., Scand. J. Haematol. **3**, 375 (1966).

3. Knight, S., Ling, N. R., Sell, S., and Oxnard, C. E., Immunology **9**, 565 (1965).

4. Bollum, F. J., J. Biol. Chem. **234**, 2733 (1959).

5. Phillips, H. J., and Terryberry, J. E., Exp. Cell Res. **13**, 341 (1957).

6. Tridente, G., Cappuzzo, G. M., and Chieco-Bianchi, L., *in* "Histocompatibility Testing" (E. S. Curtoni, P. L. Matting, and R. M. Tosi, eds.), p. 67. Wilkins and Wilkins, Baltimore (1967).

7. Regan, J. D., and Chu, E. H. Y., J. Cell Biol. **28**, 139 (1966).

8. Ling, N. R., "Lymphocyte Stimulation," p. 47. North-Holland, Amsterdam (1968).

9. Hayry, P., and Defendi, V., Clin. Exp. Immunol. **6**, 345 (1970).

10. Adler, W. H., Takiguchi, T., Marsh, B., and Smith, R. T., J. Exp. Med. **131**, 1049 (1970).

11. Mangi, R. J., and Mardiney, M. R., J. Immunol. **105**, 90 (1970).

12. Alford, R. H., Proc. Soc. Exp. Biol. Med. **133**, 1443 (1970).

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