A Simplified Technique for *in Vitro* Studies of Lymphocyte Reactivity[1] (36391)

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In vitro studies of lymphocyte reactivity have received a great deal of attention in recent years, and it is generally agreed that measurement of the "transformation" responses of lymphocytes incubated in the presence of mitogens, foreign cells or a variety of antigens offers great promise of mapping immunologic reactivity of the cell donor. Such studies have been used to identify agents responsible for hypersensitivity reactions, to confirm immunologic depression in certain disease states, and in histocompatibility screening.

Conventional techniques for measuring lymphocyte responses (1, 2) involve a cumbersome and time-consuming series of steps before setting up of the cell cultures. These include: (a) sedimentation of erythrocytes from a relatively large volume (20–40 ml) of heparinized blood, (b) preparation of a lymphocyte-rich concentrate of white blood cells from the supernatant plasma, usually incorporating one of several methods to remove excess granulocytes; this step includes at least one and often, two centrifugations, and (c) preparation of cell suspensions containing 1–2 million lymphocytes in culture medium supplemented with autologous plasma or fetal calf serum. In attempting to apply lymphocyte culture techniques to studies of immunologic reactivity of normal individuals and patients with neoplastic disease, we found that this complex procedure for preparing lymphocyte suspensions constituted a major bottleneck in the laboratory and probably, the principal source of errors in the final results. Often, even when 50 ml of blood from patients was used as starting material, there were not enough lymphocytes in the final suspension to permit measurement of responses to a relatively large battery of agents.

Since whole blood incubated with phytohemagglutinin (PHA) has been used successfully to prepare cells for karyotypic analysis (3, 4), it occurred to us that it might also be unnecessary to separate lymphocytes for measurement of responses to antigenic stimuli. Accordingly, we explored the use of whole blood for a variety of lymphocyte incubation studies, and tested other modifications of the conventional technique, in the hope of reducing man-hours and cost, increasing sensitivity, and achieving better reproducibility.

Materials and Methods. Cell suspensions were prepared by adding heparinized whole blood to 10–40 vol of culture medium (RPMI 1640 with penicillin and streptomycin, Associated Biomedic Systems, Buffalo, NY). Experiments were conducted with plain culture medium and medium supplemented with autologous or homologous human plasma or serum, bovine albumin or fetal calf serum. After mixing, 3 ml aliquots of the diluted blood were distributed to 16 × 125 mm culture tubes, mitogens or antigens added, and the stoppered tubes incubated at 37° in an upright position. Mitogen and antigen concentrations were similar to those commonly employed by investigators using conventional techniques.

For two-way mixed-cell culture experiments, 1.5 ml aliquots of diluted blood from each subject were mixed. For one-way experiments, the diluted blood from one of the subjects was irradiated (5,600 R, from a 250 kV source) prior to setting up the cultures.

Lymphocyte stimulation was measured by a ^{3}H-thymidine incorporation method; 1.0

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TABLE I. Paired Mixed-Cell Culture Experiments, in Plain Medium and Medium Containing 20% Fetal Calf Serum.

Cells from subjects[a]	In dilution	Thymidine-^{3}H uptake (10^3 dpm[b])	
		Blood RPMI 1640	In RPMI 1640, with 20% fetal calf serum
A	1:30	1.6	1.9
B	1:30	1.2	1.3
C	1:30	1.0	0.7
A + Bx[c]	1:30	6.7	34.4
A + Cx	1:30	6.8	72.8
B + Ax	1:30	4.3	36.6
B + Cx	1:30	3.5	30.0
C + Ax	1:30	3.8	77.0
C + Bx	1:30	6.3	72.8
A + B	1:30	18.1	70.5
A + C	1:30	28.3	163.4
B + C	1:30	21.1	106.0
B	1:40	1.8	1.5
D	1:40	1.0	1.3
B + D	1:40	4.6	74.0
B	1:20	4.6	4.0
D	1:20	1.8	3.0
B + D	1:20	56.9	175.4

[a] A, B, C, D = different healthy subjects.

[b] Cultures harvested at 8 days; 1.0 μCi of ^{3}H-thymidine (sp. act. 18.4 Ci/mmole) added 24 hr. before harvesting.

[c] Ax = irradiated A cells, etc.

μCi of ^{3}H-thymidine, of moderate or high specific activity (2.0, 18.4 Ci/mmole) was added to culture tubes at time intervals ranging from 4 to 6 hr (the conventional labeling period) to 72 hr before termination of culture. For harvesting, 2–3 vol of cold 3% acetic acid were added to lyse erythrocytes, the resulting leukocyte suspension was centrifuged, and the cell button was washed with 12 ml of 3% acetic acid. The final cell button was bleached with hydrogen peroxide, dissolved in NCS solubilizer (Amersham-Searle Corp., Arlington Heights, IL), and prepared for counting in liquid scintillation fluid. Counting efficiency (liquid scintillation counter, Nuclear Chicago Corp.) has consistently been 45% or more, as determined by the channels ratio method.

Results and Discussion. A. Labeling time. Thymidine-^{3}H incorporation increased consistently with labeling time, up to 24 hr. With 48-hr periods of exposure to ^{3}H-thymidine, results were inconsistent. In some experiments, there was further increase in radioactivity, but in others, decreases from the 24-hr values were observed. At 72 hr, ^{3}H counts were usually below the 24-hr values. Therefore, we adopted a 24-hour labeling period for routine use.

B. Supplementation of medium and reactive cell density. For studies of responses to mitogens (PHA, pokeweed mitogen, staphylococcal filtrate, concanavalin A), no supplementation of the culture medium has been required. At all blood dilutions tested, comparable incorporation of thymidine-^{3}H has been observed in cultures containing plain medium or medium enriched with fetal calf serum or other supplements.

When responses to soluble antigens (tuberculin-PPD, Varidase, extracts of monilia and trichophyton) were examined in paired experiments, higher ^{3}H counts were often obtained in medium supplemented with fetal

calf serum, autologous plasma or 1% bovine albumin, than in plain medium. This proved to be related to reactive cell density. At 1:40 blood dilutions, 2- to 6-fold greater ^3H incorporation was usually observed in supplemented medium. In experiments using 1:10 or 1:20 dilutions of blood, however, plain culture medium gave as good results as supplemented medium.

The effect of reactive cell density on lymphocyte response in unsupplemented culture medium was more striking in mixed-cell cultures. This is shown in Table I, which presents data from two experiments comparing plain medium with medium containing fetal calf serum. There were no significant differences in isotope incorporation by unstimulated cells, in the two types of media. However, in all mixed-cell cultures, substantially more thymidine-^3H was incorporated when the culture medium contained fetal calf serum. Reactive cell density appeared to play an important role in plain medium, but not in supplemented medium. In the first experiment, ^3H counts in the two-way cultures, in medium containing fetal calf serum, closely approximated the sums of the corresponding one-way counts. In plain medium, however, two-way cultures (containing twice as many reactive cells) gave 2–3 times as many counts as would have been predicted from the one-way results. This effect was even more evident in the second experiment. In supplemented medium, isotope incorporation in cultures with 1:20 dilutions of whole blood was approximately twice that observed with 1:40 dilutions, as would be expected. In plain medium, however, doubling the number of reactive cells resulted in a 12-fold increase in ^3H counts. A third experiment was performed in which two-way mixed-cell cultures were set up at 1:10, 1:20 and 1:40 blood dilutions. Replicate cultures were harvested on different days, and it was found that peak incorporation of thymidine-^3H occurred somewhat earlier at 1:10 dilutions (6–8 days) than with the more dilute suspensions (8–10 days). At 1:40 dilutions, peak ^3H counts in medium supplemented with fetal calf serum were 6 times greater than in plain medium. At 1:20 dilutions, the advantage for

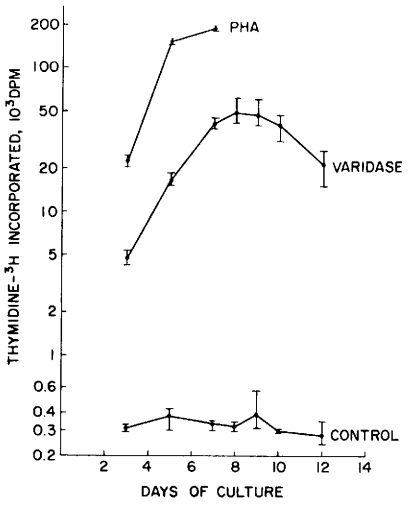

FIG. 1. Time course of ^3H-thymidine incorporation into leukocytes, with the whole-blood technique. The blood donor was a healthy young woman with a strongly positive delayed skin test reaction to Varidase. Blood was diluted with 39 vol of culture medium containing no supplements. ^3H-thymidine (1.0 μCi, 2.0 Ci/mmole) was added 24 hr before harvesting. Vertical lines represent range of values at each point (of quadruplicates, for the control and Varidase cultures).

supplemented medium was only twofold, and at 1:10 dilutions, slightly *higher* counts were obtained in plain medium. Thus, it appears that at appropriate cell densities, no supplementation of culture medium is required for any type of lymphocyte stimulation study.

At the higher cell densities, of course, significant quantities of autologous plasma are present in the diluted blood and thus, the medium is, in fact, "supplemented." However, it seems unlikely that this could account for our findings. If some minimal concentration of plasma protein were required for optimal lymphocyte responses, one would expect that this concentration would be effective regardless of the stimulant used. However, we found systematic differences, according to type of stimulant. Furthermore, the plasma concentrations in the one-way and two-way mixed-cell cultures shown in Table I (first experiment) were identical; thus, the disproportionally greater ^3H incorporation in the latter can only be ascribed to the higher

TABLE II. Responses of a Healthy Subject to a Battery of Lymphocyte Stimulants.[a]

Stimulant	Thymidine-^{3}H uptake 10^3 dpm; mean ± SE[b]
Harvested at 5 days	
None	1.9 ± 0.3
Phytohemagglutinin	430.5 ± 10.4
Pokeweed mitogen	100.1 ± 9.2
Staphylococcal filtrate	158.1 ± 5.1
Concanavalin A	213.5 ± 6.9
Harvested at 8 days	
None	1.5 ± 0.1
Tuberculin-PPD	64.9 ± 4.4
Trichophyton extract	39.8 ± 1.7
Monilia extract	25.6 ± 6.2
Varidase	7.3 ± 1.7
Two-way, mixed-cell[c]	25.8 ± 1.0
One-way, mixed-cell[d]	8.2 ± 3.8
One-way, with homologous cultured cells[e]	18.3 ± 3.3

[a] Delayed skin test responses of subject: intermediate PPD, 3.7 cm; trichophyton extract, 3.4 cm; monilia extract, 3.1 cm; Varidase, 1.9 cm.

[b] Blood dilution, 1:20; unsupplemented medium; 1.0 μCi of ^{3}H-thymidine (sp. act. 23.7 Ci/mmole); quadruplicate cultures.

[c] With equal volume of 1:20 diluted blood of another healthy subject.

[d] Second subject's blood irradiated to 5600 R.

[e] Cultured lymphoid cells (RPMI 1788), irradiated to 5600 R.

reactive cell density.

C. Use of the simplified technique. Reproducibility of isotope incorporation has proved very satisfactory with the whole-blood technique and 24-hr ^{3}H-thymidine labeling. In an experiment with blood from a subject with strong delayed hypersensitivity to Varidase, 20 replicate cultures with this antigen, using unsupplemented medium, a blood dilution of 1:30 and thymidine of high specific activity, gave ^{3}H incorporation ranging from 137 to 190 $\times$ 10^3 dpm, with a mean of 164 $\pm$ 21 (SD) $\times$ 10^3 dpm. This standard deviation (13% of the mean) compares favorably with those we have obtained using lymphocyte concentrates. Control cultures in this experiment yielded 1.47 $\pm$ 0.45 $\times$ 10^3 dpm, and the antigen:control response ratio was 112.

Figure 1 presents a study of the time course of thymidine-^{3}H incorporation into cells of another healthy subject who gave a strongly positive delayed skin test reaction to Varidase. The smoothness of the Varidase curve demonstrates that this technique provides good reproducibility. Peak ratios of isotope incorporation in stimulated vs control cultures were approximately 150 for Varidase and 560 for PHA. These values are at least as good as those obtained in various laboratories using lymphocyte concentrates. Peak incorporation of isotope occurred somewhat later than would have been expected with conventional techniques; maximal response to PHA was observed at 5–7 days and to Varidase, at 7–10 days.

Table II presents the response of a healthy subject to our full battery of stimulants. A total of 52 cultures were performed with 8 ml of blood. The responses to mitogens and antigens were at least equal to those we had obtained previously, using separated lymphocytes from this individual. Lower responses were obtained in mixed-cell cultures and especially, the one-way reaction with cultured lymphoid cells; in our experience, such cultured cells approach PHA in stimulating activity (5). These decreased responses are attributable to the suboptimal cell density for unsupplemented medium (see above).

We were rather surprised to find that with the whole-blood technique, final radioactivity counts were of the same order of magnitude we and others had previously obtained with separated leukocyte preparations, despite the fact that the culture tubes contained as little as one-tenth the number of lymphocytes used in conventional methods. The same observation was made by Junge *et al.* (6), who reported experience with a similar technique while this study was in progress. This appears to be due to two factors: (a) use of longer ^{3}H-thymidine labeling times, often not feasible with separated leukocyte preparations, because of degradation of the nucleoside (7, 8), and (b) apparently, better functional status of the reacting cells.

We have defined many, but not all, of the conditions necessary to yield optimal responses with this technique. However, it is already evident that in our hands, it provides

results clearly superior to those we obtained previously with methods using separated leukocytes, in addition to affording substantial savings in time, effort and amounts of blood required. Reproducibility has been better, response ratios have been higher and responses to a battery of mitogens and antigens, in over 100 studies of patients with lymphoma and leukemia, have shown better correlations with clinical status and delayed skin test reactions than we observed previously with conventional techniques. We believe that this superiority is due to: (a) more uniform distribution of cells among individual culture tubes, when centrifugation–resuspension of leukocytes is eliminated, (b) avoidance of unpredictable and varying cell damage, which must occur during the manipulations involved in preparing lymphocyte concentrates, (c) measurement of a significantly larger fraction of the peak lymphocyte response, with a 24-hr thymidine labeling period, and possibly (d) maintenance of more stable pH and oxygen tension, when culture tubes contain fewer leukocytes and relatively large numbers of erythrocytes. Use of unsupplemented medium affords greater convenience and significant savings in cost, as well as avoiding the artifactual errors which may be encountered when fetal calf serum (9) or other supplements are used. Because of its greater sensitivity, this technique has proved particularly useful in studies for which only limited volumes of blood are available, as of small laboratory animals (10).

Summary. In vitro studies of lymphocyte reactivity to mitogens, soluble antigens and homologous cells were performed by diluting whole blood with culture medium and using 24-hr labeling with ^{3}H-thymidine. Sensitivity, reproducibility and correlations with delayed skin test responses of the blood donors were superior to those obtained with conventional techniques using lymphocyte concentrates. At appropriate reactive cell densities, supplementation of the culture medium with agents such as fetal calf serum has not been necessary. This simplified technique offers considerable savings in effort, cost and volumes of blood required.

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