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VARIABLES IN THE QUANTITATION OF THE MIXED LEUCOCYTE RESPONSE BY TRITIATED THYMIDINE UPTAKE

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SUMMARY

We have used ^{3}H-thymidine uptake to quantitate the mixed leucocyte response. The technique of ^{3}H-thymidine labelling is an important variable in the total mixed leucocyte culture technique. The effect of variations in the specific activity of ^{3}H-thymidine used, the amount of radioactivity added per culture and the time of exposure to ^{3}H-thymidine was studied using a micro-MLC method. Optimum quantitative and qualitative discrimination was obtained by the addition of high specific activity thymidine for an exposure period of 18–24 hr.

INTRODUCTION

Lymphocytes are stimulated to transform into blast cells in mixed leucocyte culture (MLC). Quantitation of this transformation can be accomplished by measurement of ^{3}H-thymidine incorporation into DNA. There are many variables in the MLC technique including stimulating-responding cell ratios, lymphocyte purification, media, plasma supplements, time of culture and harvest method. The technique of ^{3}H-thymidine labelling is frequently not mentioned in reports of MLC experiments and may not have been carefully considered in planning some experimental protocols. However, the labelling itself is an important variable in the total method and variations in labelling can alter the experimental results and conclusions.

In other studies we have used thymidine of high specific activity for quantitation of the mixed leucocyte response (Etheredge *et al.*, 1972; Shons *et al.*, 1972) and it was our impression that its use afforded better discrimination than other ^{3}H-thymidine labelling techniques. To test this hypothesis we have studied the effect of variations in ^{3}H-thymidine labelling using a micro-MLC method (Hartzman *et al.*, 1971). Because there may be significant difference in the results of cultures set up on different days, even with the use of the same blood donors and technique, our data are based on comparisons of labelling techniques within cultures prepared on a single day.

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MATERIALS AND METHODS

Preparation of cultures

Normal human volunteers served as blood donors. A strongly reacting pair G.T. and D.P., and weakly reacting pair C.D. and B.C. were used. Blood was drawn into a heparinized syringe and allowed to sediment for 2 hr at room temperature. The leucocyte rich plasma was collected and centrifuged for 10 min at 150 *g*. The plasma was decanted and the cell button was suspended in Medium 199, Earls base, with glutamine, 100 mg/l (Gibco, Grand Island, N.Y.), buffered with 0·04 M sodium Hepes (Sigma, St. Louis, Mo.) and supplemented with penicillin, streptomycin, and 25% autologous plasma (199S-Hepes). The leucocytes were purified by the density gradient method of Boyum (1968). After harvesting from the gradient, the cells were washed twice with 199S-Hepes. Cells to be used as responding cells were counted in a haemocytometer and adjusted to a final concentration of 2×10^6/ml in 199S-Hepes. Cells to be used as stimulating cells were suspended in 199S-Hepes and incubated with Mitomycin C, 50 μg/ml of cell suspension for 20 min at 37°C. Following incubation the cells were washed twice with 199S-Hepes and adjusted to final concentrations of 1×10^6, 2×10^6, and 4×10^6/ml 199S-Hepes. Volumes of 0·1 ml from the responding cell pool and from the stimulating cells pools were used to set up 0·2 ml cultures in Falcon microtest plates No. 3040. The plates were covered with plastic covers and incubated at 37°C in a humidified atmosphere.

Labelling of cultures

Radioactive thymidine (^{3}H) of specific activity (SA) 1·9 Ci/mM, 6·0 Ci/mM or 17·3 Ci/mM (Schwarz/Mann, Orangeburg, N.Y.) was added in amounts of total radioactivity of 0·05 μCi, 1 μCi or 2 μCi/culture to cultures of G.T. and D.P. at 120 hr of incubation. The cultures were harvested 4, 8 or 24 hr later. To cultures of C.D. and B.C. after 72 hr of incubation ^{3}H-thymidine of SA 1·9 Ci/mM was added in amounts of radioactivity totalling 1 μCi or 2 μCi culture. Cultures were harvested 8 or 24 hr later.

Harvest of cultures

The cultures were harvested by precipitation on to glass fibre filters using the apparatus of Hartzman (Hartzman *et al.*, 1971). The filter bearing the precipitate was placed in a scintillation vial and dried for 1 hr at 150°C. Ten millilitres of scintillation fluid (0·0379 g POPOP, 22·74 g PPO in 3·79 l of toluene) were added to each vial and each sample was counted for 1 min in a Unilux II-a Liquid Scintillation Counter (Nuclear Chicago, Des Plaines, Ill.). The incorporation of ^{3}H-thymidine was expressed as counts/min (cpm) and the means of triplicate culture values were calculated. The mitotic index (MI) was calculated for each culture group:

$$\frac{\text{Mean cpm of ABm}}{\text{Mean cpm of AAm}}.$$

For controls, Am cells were present at similar concentrations to Bm cells (1×10^5, 2×10^5 or 4×10^5/culture).

RESULTS

Fig. 1 shows the results of culture G.T. and D.P. By all labelling methods there was

increased uptake after 8 hr of exposure to ^{3}H-thymidine compared to a 4-hr exposure. In virtually all instances ^{3}H-thymidine incorporation after 24 hr of exposure was equal to or greater than after 8 hr of exposure. In general there was a decreased rate of uptake in the 8–24-hr interval compared with the 0–8-hr interval. In every instance except two

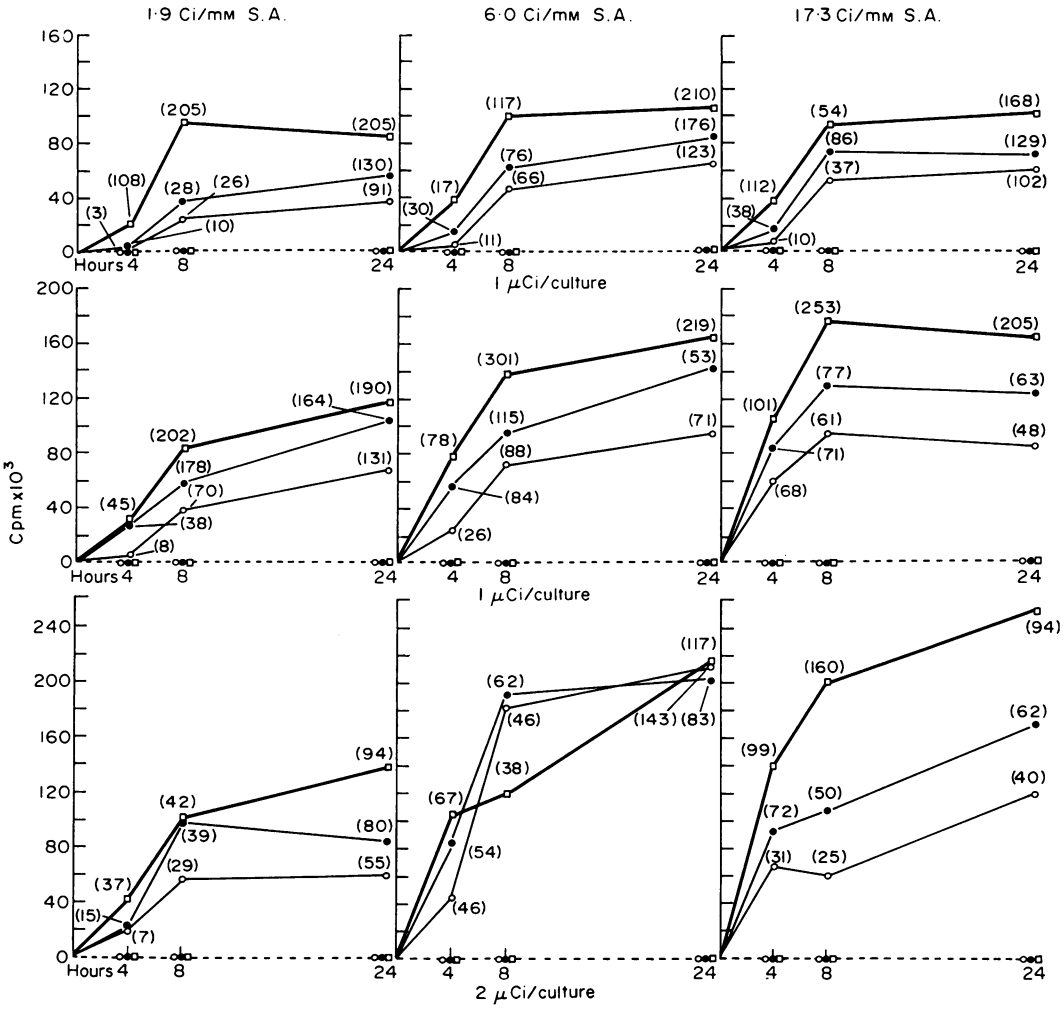

FIG. 1. The effect of varying exposure time, specific activity and amount of radioactivity of ^{3}H-thymidine on incorporation of radioactivity in 5-day cultures of G.T. × D.P. Cpm are mean determinations of triplicate cultures. Mitotic indices are listed in parentheses. Lymphocyte ratios in cultures are: (□) A $2\times10^5\times$Bm 4×10^5; (●) A $2\times10^5\times$Bm 2×10^5; (○) A $2\times10^5\times$Bm 1×10^5. Controls of similar A × Am ratios are shown as dashed lines.

(8- and 24-hr exposure to 2 μCi/culture, 6 Ci/mM SA thymidine), 2×10^5 stimulating cells produced a higher radioactivity uptake than 1×10^5 stimulating cells and 4×10^5 stimulating cells produced a higher radioactivity uptake than 2×10^5 stimulating cells. The addition of 0·5 μCi/culture produced control cpm of 197–2572; control cpm with 1 μCi/culture were

418–3156 and control cpm with 2 μCi/culture were 317–2438. In general higher cpm of ABm combinations corresponded to a higher MI but this was not a uniform finding. Apparently random variations in some control values led to values for the MI which were inconsistent with the radioactivity uptake in an ABm culture. With labelling by the addition of 1 or 2 μCi/culture, increasing SA of the thymidine resulted in higher cpm. This effect was less noticeable with only 0·5 μCi/culture total radioactivity added. Significant stimulation and discrimination between stimulating cell numbers was noted with any label period, SA or total amount of radioactivity combination used.

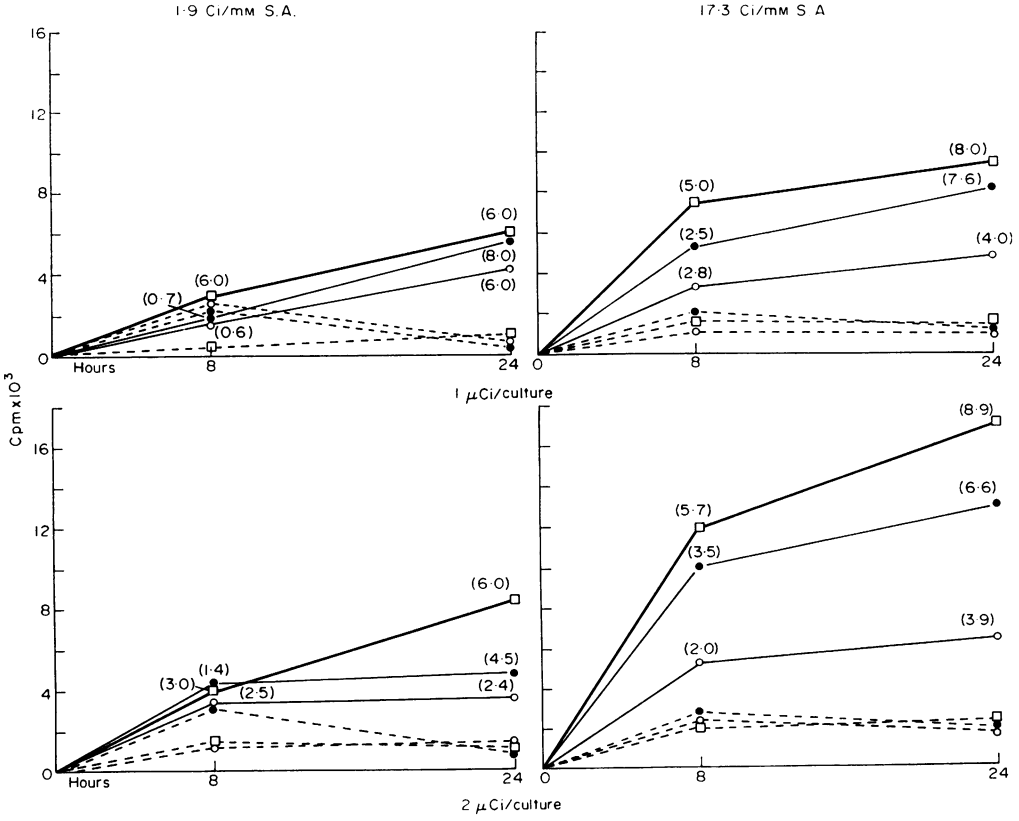

FIG. 2. The effect of varying exposure time, specific activity and amount of radioactivity of ^{3}H-thymidine on incorporation of radioactivity in 3-day cultures of C.D. × B.C. Cpm are mean determinations of triplicate cultures. Mitotic indices are listed in parentheses. Lymphocyte ratios in cultures are: (□) A 2×10^5 × Bm 4×10^5; (○) A 2×10^5 × Bm 2×10^5; (○) A 2×10^5 × Bm 1×10^5. Controls of similar A × Am ratios are shown as dashed lines.

Different results were seen in cultures of weakly reacting pair C.D. and B.C. after a shorter incubation period (Fig. 2). All ABm combinations revealed lower cpm and a lower MI. Significant stimulation and adequate discrimination between stimulating cell numbers was not seen after 8 hr exposure to 1 or 2 μCi/culture of SA 1·9 Ci/mM thymidine. Using 17·3 Ci/mM SA thymidine definite stimulation and discrimination was noted with the addi-

tion of 1 or 2 μCi/culture after exposure periods of either 8 or 24 hr. Control cpm for cultures of C.D. and B.C. ranged from 518–2815 cpm, a range similar to that for cultures of G.T. and D.P.

DISCUSSION

^{3}H-thymidine is commonly supplied in specific activities of 1·9, 6·0, and 17–20 Ci/mM and at concentrations of 0·5 or 1 mCi/ml. By using a Hamilton syringe multiple microcultures may be easily labelled with 1–4 μL/culture providing 0·5–4 μCi total radioactivity/culture. Cultures may be conveniently labelled for a short period (up to 4 hr) with harvest the same day or the ^{3}H-thymidine can be added one day, with harvest the next day, giving an exposure period of 18–24 hr. The microcultures may also be labelled at the beginning of the day, frozen at −20°C 8 hr later and harvested the next day. In previous experiments we have shown no effect of short term freezing on subsequent cpm of each culture. From our data we conclude that prolonged exposure to thymidine of high specific activity is desirable.

Somewhat different conclusions were reached in a previous study of ^{3}H-thymidine labelling variables using a micro-MLC technique. Bain (1970) found the addition of 1 μCi of SA 0·05–1·67 Ci/mM thymidine/350,000–550,000 responding lymphocytes resulted in increasing uptake over a 24-hr period. However when SA 5 Ci/mM thymidine was used the uptake after 24 hr was lower than that at 8 hr. In our experiments we have used similar quantities of radioactivity (1 μCi/100,000–400,000 responding lymphocytes) yet we did not find the decreased uptake of 24 hr using high SA thymidine. With all combinations used we did see a decreasing rate of uptake in the 8–24-hr interval. This might be explained on the basis of decreased availability of thymidine, due to DNA incorporation or breakdown to thymine or dihydrothymine. There might also be inhibition of DNA synthesis due to high levels of radioactivity in the nuclei.

Using the micro-MLC technique and 1·9 Ci/mM SA thymidine, Hartzman (1971) found that adding increasing amounts of radioactivity resulted in increasing cpm after a 16–20-hr exposure period. But at about 1·5 μCi/100,000 responding lymphocytes a plateau was reached beyond which the addition of greater amounts of radioactivity did not increase the cpm. The total amounts of radioactivity of 1·9 Ci/mM SA thymidine we used were below this plateau level yet we noted little increase in cpm when 1 μCi was added to 100,000 responding lymphocytes compared to the addition of only 0·5 μCi to 100,000 responding lymphocytes.

The use of high SA thymidine and large amounts of radioactivity/culture increases both AAm and ABm cpm. The resulting MI is similar to that calculated on cultures to which a smaller quantity of radioactivity of lower SA was added. However, the higher cpm allows a shorter measurement period with acceptable counting variability. It is necessary to record 5,000–10,000 counts to minimize random counting variations. Thus the use of higher SA thymidine and a larger quantity of radioactivity allows a more efficient utilization of scintillation counting equipment. The rapidly increasing uptake seen in the first 8 hr after the addition of ^{3}H-thymidine logically rules out using exposure periods of only a few hours. Small difference in harvest times between cultures in a single group might cause significant differences in cpm between otherwise equivalent cultures. Using longer exposure periods of 8–24 hr differences in harvest times would have a minimal effect on culture to culture comparisons within a harvest group.

In summary, the technique of radioactive thymidine labelling is an important variable in an MLC method. Maximum qualitative and quantitative discrimination in MLC is possible only by optimizing all variables. This study indicates that optimum radioactive thymidine labelling in a micro-MLC method is accomplished by the addition of 2 μCi of 17·3 Ci/mM SA thymidine/200,000 responding lymphocytes for an exposure period of 18–24 hr.

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