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TRITIATED-THYMIDINE UPTAKE IN MIXED LEUCOCYTE CULTURES: EFFECT OF SPECIFIC ACTIVITY AND EXPOSURE TIME

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SUMMARY

The degree of *in vitro* transformation of lymphocytes to blast cells can be estimated by measuring the uptake of radioactive precursors into DNA. We have used ^{3}H-thymidine uptake to quantitate blastogenesis in mixed leucocyte cultures. In experiments designed to standardize this procedure, the kinetics of thymidine uptake were studied by adding 4 μCi of ^{3}H-thymidine of varying specific activities to 5-day mixed cultures. With high specific activity (0·194 μg total thymidine/culture), the rate of uptake was constant for only about 6 hr, then declined. There was no further cellular uptake between 8 and 24 hr, even though the total radioactivity of the supernatant medium did not diminish appreciably. This decreasing rate of uptake was at least partly the result of thymidine degradation to thymine and dihydrothymine by cellular enzymes. It is also possible that eventual radiation damage to blast cell nuclei may have retarded DNA synthesis when ^{3}H-thymidine specific activity was high. With decreasing specific activity (up to 19·4 μg thymidine/culture), the rate of uptake became more nearly linear throughout 24 hr exposure to the isotope. The possible effects of thymidine degradation and radiation damage should be considered when measuring radioactive thymidine uptake *in vitro*, and short labelling times should be used whenever feasible.

INTRODUCTION

Many substances can induce lymphocytes to transform to blast cells *in vitro*, and the number of workers investigating this phenomenon is correspondingly large. One problem commonly encountered is that of estimating the degree of response to blastogenic agents. Because transformed blasts are the only cells in short-term leucocyte cultures which synthesize DNA (Bain, Vas & Lowenstein, 1964), the measurement of radioactive thymidine uptake is often used for quantitation. The methods used by different workers have varied with respect to

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the choice of radioactive isotope (^{14}C or ^{3}H), the amounts of radioactive and non-radioactive thymidine added to the cultures, and the length of time the cells are exposed to the isotope. We have tried to standardize the measurement of blast cell transformation by investigating some of these variables.

The experiments described here were done in order to choose a satisfactory technique for the routine measurement of ^{3}H-thymidine uptake. Two obviously desirable criteria are that the counts obtained should be high enough for accurate measurement with reasonably short counting times, and that the various steps in the procedure should be scheduled to fit into regular working hours. Although our experiments have been done with mixed leucocyte cultures, the findings can also be applied to the quantitation of other forms of blastogenic stimulation.

MATERIALS AND METHODS

Preparation of cultures

Normal subjects were used as blood donors in all experiments. Blood was drawn into a syringe moistened with heparin solution (Liquaemin Sodium, Organon, 5000 units/ml), and placed in Falcon No. 2001 disposable plastic tubes, 17 × 100 ml. The tubes were centrifuged for 10 min at 800 *g*. The plasma and the upper portion of the cells were transferred to another tube, and the cells were resuspended in the plasma. This procedure eliminated most of the red cells, while most of the leucocytes remained. The tubes were placed at an angle of approximately 60° to horizontal and incubated at 37°C for 30–60 min, until the remaining red cells had sedimented. The tubes were then placed vertically for a further 10 min, resulting in a distinct demarcation between red cells and supernatant. The supernatant plasma, containing the leucocytes, was transferred to another tube, and total leucocytes and lymphocytes were counted in a haemocytometer. The supernatant was diluted with Medium 199 (Microbiological Associates) containing 100 units of penicillin and 100 μg of streptomycin/ml, so that the final cell suspension contained 1000–2000 total leucocytes/mm^3, and 350–550 lymphocytes/mm^3. The plasma concentration was between 10 and 20%, usually close to 15%. Cell-free autologous or homologous plasma was added if the supernatant had been diluted more than ten times with Medium 199. The leucocytes were cultured in Falcon No. 2001 plastic tubes, 17 × 100 mm size. The unmixed control cultures contained 4 ml of cell suspension from one individual; the mixed cultures contained 2 ml from each of two donors; total volume 4 ml.

All of the above procedures were done using sterile technique.

Measurement of ^{3}H-thymidine uptake

After 5 days' incubation at 37°C, 0·1 ml of ^{3}H-thymidine (20 or 40 μCi/ml) was added to each culture. Specific activities and thus thymidine concentration values varied between experiments and are given in the Results section. The cells were incubated with ^{3}H-thymidine for periods up to 30 hr.

The uptake of radioactive thymidine was stopped by adding 100 μg of non-radioactive thymidine to each culture. The culture tubes were then centrifuged and the medium was discarded. Two millilitres of 5% trichloroacetic acid (TCA) were added to each tube to precipitate the DNA. The precipitate was centrifuged and washed twice with 1 ml of 5% TCA. After the final washing as much supernatant fluid as possible was removed, and 0·5 ml

of Hydroxide of Hyamine solution (Packard Instrument Company) was added to each tube to dissolve the precipitate. The tubes were left at room temperature for 1 or 2 days. The contents were then transferred to liquid scintillation counting vials with two 0·3 ml washings of absolute ethanol. Fifteen millilitres of a toluene solution containing 0·3% PPO and 0·01% dimethyl-POPOP (Packard) were added to each vial.

Thymidine uptake was expressed as counts/min/culture, or as ng thymidine/culture.

The counting efficiency was determined by counting 0·05 μCi of ^{3}H-thymidine (0·05 ml of a 1 μCi/ml solution) in vials containing 1·6 ml of ethanol, 0·5 ml of Hydroxide of Hyamine, and 15 ml of the scintillator solution. The efficiency was 13% under these conditions, and this was assumed to be the value for the TCA precipitates. (Later experiments with TCA precipitates and ^{3}H-toluene internal standards gave values very close to 13%, using the same counter and settings.) Knowing the counts/min, the efficiency, and the ^{3}H-thymidine specific activity, it was possible to calculate the total amount of thymidine taken up by the cells.

Chromatographic analysis of thymidine degradation

Mixed leucocyte cultures were incubated at 37°C for up to 6 days. Two microcuries of ^{3}H-thymidine (specific activity 1·65 μCi/μg) were added to each culture either at the start of incubation or after 5 days. Four millilitres of absolute methanol were added to each tube at intervals of from 0 to 24 hr after addition of ^{3}H-thymidine. The tubes were shaken and centrifuged, and the supernatants were transferred to rubber stoppered glass vials. The vials were stored at −15°C until the end of the experiment. They were then packed in an insulated container with dry ice and sent by air to London where the chromatographic analyses were done (Cooper & Milton, 1964; see Acknowledgments).

RESULTS

^{3}H-thymidine uptake: effect of thymidine concentration and exposure time

In experiments reported previously (Bain & Lowenstein, 1965b), ^{3}H-thymidine (4 μCi; 0·2 or 0·7 μg thymidine/culture) was added to 5-day mixed cultures and controls for 1, 4, 8 and 24 hr. Thymidine uptake in the mixed cultures was approximately linear for the first 4 hr, but usually with a slight lag during the 1st hour. Between 4 and 8 hr, the rate of uptake decreased, and there was little or no additional uptake between 8 and 24 hr. The unmixed control cultures showed a similar pattern, but the counts were much lower and the results were less consistent. Six-day mixed cultures were exposed to ^{3}H-thymidine for 1 hr, and showed no decrease in their ability to incorporate thymidine, compared with 5-day cultures.

In the present investigation, the first 8 hr exposure to ^{3}H-thymidine was studied more closely. Fig. 1 shows one of three experiments, all of which gave similar results. ^{3}H-thymidine solution (4 μCi; 0·11 μg thymidine/culture) was added to 5-day mixed leucocyte cultures and controls for 1, 2, 4, 6 and 8 hr. In the example given, the rate of thymidine uptake was approximately linear for the first 6 hr, and decreased between 6 and 8 hr, but in the other two experiments there was a slight decrease in rate between 4 and 6 hr.

In three other experiments, ^{3}H-thymidine solutions with constant radioactivity (4 μCi/culture), but with different specific activities, were added to 5-day mixed leucocyte cultures. The total amounts of thymidine added were 0·194, 0·582, 1·94, 5·82 and 19·4 μg/culture, and

the cells were exposed to the radioactivity for 1, 4, 8 and 24 hr. Table 1 gives the results from one experiment and shows the range of counts/min obtained. In Fig. 2, total thymidine uptake has been calculated and the averaged values from the three experiments are given.

Fig. 2(a) illustrates the pattern of uptake over the 24-hr exposure period for each concentration of thymidine. When the lowest concentration was used (0·194 μg/culture), the

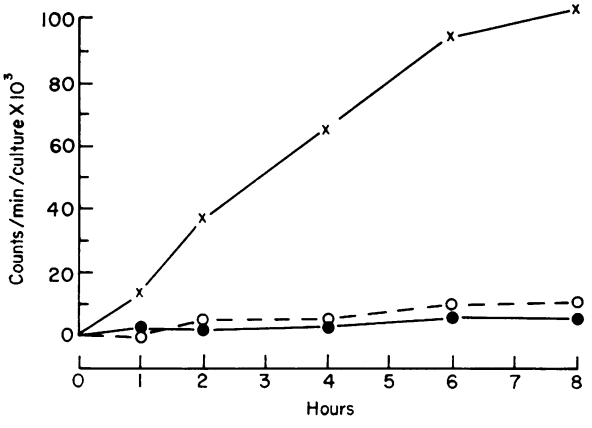

FIG. 1. Effect of length of exposure to ^{3}H-thymidine on thymidine uptake in 5-day leucocyte cultures. ×, Mixed; ○, control No. 1; ●, control No. 2.

results were the same as in Fig. 1, with a diminishing rate of uptake after the first few hours. However, as the thymidine concentration was increased, the rate of uptake became more nearly constant over the entire 24 hr.

TABLE 1. ^{3}H-thymidine uptake in 5-day mixed leucocyte cultures: effect of specific activity and exposure time

^{3}H-thymidine, specific activity: (Ci/mM)	μg thymidine/ culture	Counts/min/culture after exposure to 4 μCi; ^{3}H-thymidine for:			
		1 hr	4 hr	8 hr	24 hr
5·00	0·194	20,085	104,968	167,021	128,938
1·67	0·582	8,851	53,658	83,196	82,133
0·50	1·94	3,685	17,679	29,236	36,293
0·17	5·82	1,575	6,573	11,413	25,239
0·05	19·4	582	2,517	3,705	10,836

These data are re-arranged in Fig. 2(b) to show the effect of thymidine concentration on uptake. With 1, 4 or 8 hr exposure, the amount of thymidine taken up by the cells was not much changed over a wide range of concentrations; the uptake increased only two or three times when the amount of available thymidine was increased by a factor of 100. In contrast, thymidine uptake was proportionally much more affected by concentration when the exposure time was 24 hr.

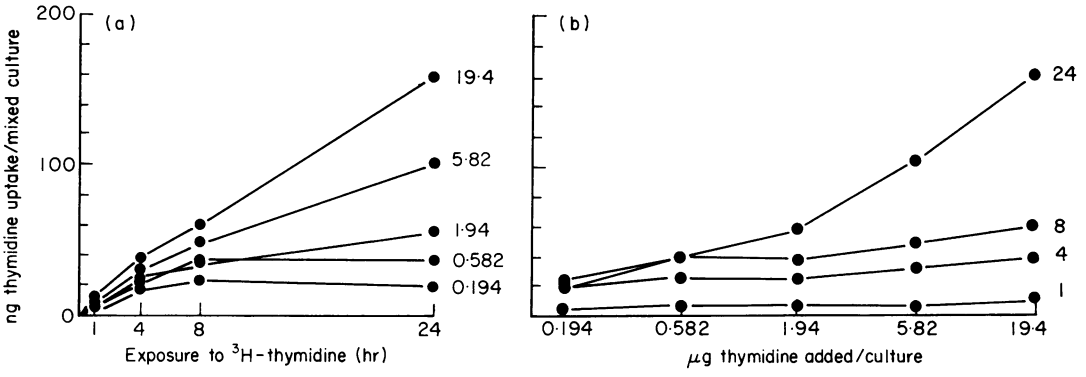

FIG. 2. Effect of thymidine concentration and exposure time on thymidine uptake in 5-day mixed leucocyte cultures. (a) Uptake *versus* exposure time; figures at end of lines are the concentration (μg thymidine added/culture). (b) Uptake *versus* concentration; figures at end of lines are the exposure time (hr).

Thymidine degradation in mixed leucocyte cultures

In three experiments, 4 μCi of ^{3}H-thymidine (0·07 μg thymidine/culture) was added to 5-day mixed leucocyte cultures for 1, 4, 8 and 23–30 hr. Thymidine incorporation by the cells was measured, and radioactive counts were also done on small samples of the supernatant medium. Fig. 3 shows one of these experiments, and the other two experiments gave the same results. The total supply of radioactive material in the medium did not diminish appreciably, but incorporation into DNA became slower after 4 hr and did not continue to rise after 8 hr.

One possible explanation for the decreasing rate of ^{3}H-thymidine uptake during 24 hr

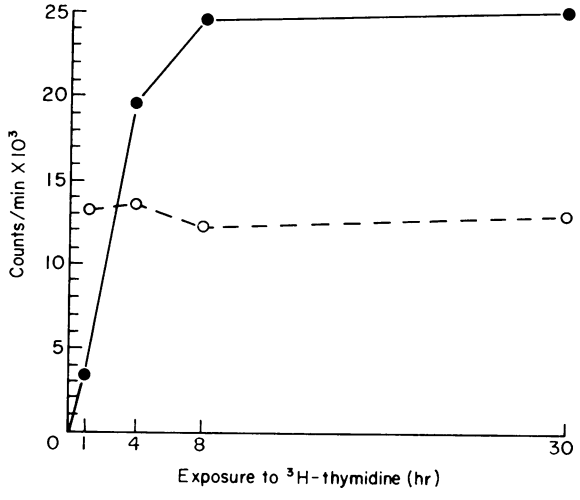

FIG. 3. Effect of length of exposure to ^{3}H-thymidine on thymidine uptake by cells and on radioactivity in supernatant culture medium. Five-day mixed leucocyte cultures. ●, ^{3}H-thymidine uptake by cells in 5-day mixed leucocyte cultures; ○, radioactivity of supernatant medium (0·05-ml samples).

incubation was that the thymidine was converted to other substances not incorporated into DNA. This possibility was tested by removing medium from mixed cultures after 24 hr exposure to the isotope, and adding this medium to other (6-day) mixed cultures for 1 hr. Thymidine uptake in these 6-day cultures was compared with that of parallel cultures incubated for 1 hr with ^{3}H-thymidine that had been incubated for 24 hr in Medium 199 with 10% plasma, but had not previously been exposed to cells. These experiments have been reported in detail elsewhere (Bain & Lowenstein, 1965b). Pre-incubation of ^{3}H-thymidine with mixed leucocyte cultures diminished its subsequent incorporation into TCA-precipitable material. This indicated that cells in mixed leucocyte cultures could modify thymidine so that it was no longer used in DNA synthesis.

TABLE 2. Thymidine degradation in mixed leucocyte cultures

Length of incubation before addition of ^{3}H-thymidine (days)	Length of exposure to ^{3}H-thymidine (hr)	Cellular uptake of radioactivity (counts/min/mixed culture)	Percentage composition in supernatant medium of:*		
			^{3}H-TdR	^{3}H-T	^{3}H-diT
0	0	25	97	3	0
0	20	791	16·5	40	43·5
5	0	157	97	3	0
5	4	9,065	83	17	0
5	7	13,828	71	28·5	0·5
5	24	28,741	44	48·5	7·5

* ^{3}H-TdR, ^{3}H-thymidine; ^{3}H-T, ^{3}H-thymine; ^{3}H-diT, ^{3}H-dihydrothymine.

The products of thymidine degradation were then investigated. Two microcuries of ^{3}H-thymidine (1·2 μg thymidine/culture) were added to 0- and 5-day mixed leucocyte cultures for various periods of time up to 24 hr. ^{3}H-thymidine uptake by the cells was measured. Samples of supernatant medium were analysed chromatographically by Dr E. H. Cooper (Cooper & Milton, 1964), and the distribution of radioactivity between thymidine and some of its degradation products was determined (non-radioactive thymidine was not added in this case).

The results are given in Table 2. The ^{3}H-thymidine solution added to the cultures was relatively pure (97% thymidine), but during the first 20 hr of culture there was marked conversion to thymine and dihydrothymine. When radioactive thymidine was added to the cultures after 5 days' incubation, degradation to thymine occurred as before, but the formation of dihydrothymine was much slower. The cellular uptake of radioactivity in 5-day mixed cultures showed the same pattern as in the earlier experiments where similar thymidine concentrations had been used.

DISCUSSION

An important point to be considered in quantitating blastogenesis by thymidine uptake is that thymidine added to cells may not persist *per se*, but may become converted to substances that are not incorporated into DNA. Neutrophils can degrade thymidine to thymine and dihydrothymine, and transformed lymphocytes can affect its conversion to thymine (Milton,

Cooper & Halle-Pannenko, 1965). This was seen to be the case in the present experiments (Table 2). During the 1st day of culture when neutrophils were present and still viable, large and approximately equal amounts of both thymine and dihydrothymine were formed. Much less dihydrothymine was produced between the 5th and 6th days, but there still was very active thymine formation. At this time, most of the neutrophils had degenerated and the cultures contained mainly lymphocytes and blast cells.

Our experiments showed a gradually diminishing rate of thymidine uptake into DNA. This might be explained by exhaustion of the thymidine supply, partly by incorporation into nuclear DNA and partly by conversion to other substances. Fig. 3 shows that a maximum of only 2·5% of the total radioactivity was removed from the medium even when the total supply of thymidine was very low (0·07 μg/culture). Therefore, it is unlikely that uptake into DNA played any significant part in limiting the amount of thymidine available for DNA synthesis.

Degradation probably did cause a significant loss of available thymidine, but a careful examination of our results suggests that additional factors may also have caused the rate of incorporation into DNA to diminish with time. In the experiment where thymidine and its degradation products were measured, the loss due to degradation meant that 1·16 μg of thymidine added to 5-day mixed leucocyte cultures decreased to 0·53 μg after 24 hr. The total uptake after 24 hr was only about half of what it would have been had the rate during the first 4 hr been maintained. However, the thymidine dose *versus* uptake curves for up to 8 hr exposure (Fig. 2b) demonstrated that a decrease of available thymidine in this range should not have limited so severely the amount incorporated into DNA, and that DNA synthesis was relatively insensitive to changes in thymidine concentration. The contrastingly steep curve for 24 hr exposure suggests mechanisms other than lack of thymidine. One idea which was not proven in the present experiments, but which fits the observations, is that the cell nuclei might have been damaged by prolonged exposure to radioactivity when the specific activity was high. Although the amount of radioactivity added to the cultures was the same in all cases, the total thymidine concentration, and specific activity, varied over a 100-fold range. Thus, for a given amount of thymidine incorporated into DNA, the amount of radioactivity incorporated into the nuclei varied with the specific activity. Such an effect is illustrated in Table 1. Another possibility is the inhibition of thymidine uptake by one of the degradation products, but no experiments were done to test the existence of such a mechanism.

The values for thymidine uptake shown in Fig. 2(a) and (b) were calculated on the basis of the amount of exogenous thymidine added to the cultures. The contribution of an endogenous pool of thymidine or its metabolic equivalents, within the cells or in the culture medium, was not taken into account. Precursors of DNA thymine have been detected in blood cells and plasma, mainly in the form of deoxycytidine (Schneider, 1954). The products of DNA breakdown in cultures might add to the pool. Table 1 indicates that the endogenous pool was small in our experiments, since the incorporation of radioactivity into DNA (counts/min) was almost inversely proportional to the amount of thymidine added, even at the lowest concentrations used (0·194 and 0·582 μg/culture). Therefore the pool must have been appreciably diluted by the added thymidine even in this low concentration range.

Relatively short periods of exposure to radioactive thymidine can generally be used to quantitate blast cell transformation. The rate of ^{3}H-thymidine uptake remains linear for 4 hr even when the thymidine concentration is low (0·11 μg/culture; Fig. 1), except for a

slower rate during the first hour. This initial lag is quite consistently seen and is probably due to the cells being disturbed and cooled when ^{3}H-thymidine is added. This should not give rise to an appreciable error if the total exposure time is as long as 4 hr. Some kinetic studies of DNA synthesis require longer exposure times, and the results may be misinterpreted if thymidine breakdown is not taken into consideration. Increasing the thymidine concentration can offset the degradation effect to some extent, and if the specific activity is decreased at the same time radiation damage can be minimized. Another possibility is to use a more long-lasting source of radioactive DNA precursors, such as ^{3}H-deoxycytidine-5′-monophosphate (Cooper & Milton, 1964).

Our results suggest that there is no precisely 'optimum' technique for measuring radioactive thymidine uptake *in vitro*, but that conditions can be varied considerably to suit individual requirements. We have selected our present method on the basis of these experimental findings. ^{3}H-thymidine is now added to the cultures for 4 hr instead of the 1 hr used previously (Bain & Lowenstein, 1965a). The amount of radioactivity per culture has been decreased to 2 μCi from 4 μCi, and the specific activity has been standardized at 2 μCi/μg (0·48 Ci/mM). Under these conditions, the rate of thymidine uptake is constant for the entire exposure period, so loss of thymidine by degradation does not become a limiting factor. The effect of the lag during the 1st hour is minimized. The decrease in radioactivity and specific activity reduces the possibility of effects arising from radiation damage to the nuclei. The method usually gives 10,000–50,000 counts/min for mixed cultures, and 500–2000 counts/min for unmixed controls, so prolonged counting times are not required.

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