THE USE OF FICOLL GRADIENT CENTRIFUGATION TO PRODUCE SYNCHRONOUS MOUSE LYMPHOMA CELLS

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This report describes the use of a 5 to 20% Ficoll gradient to produce synchronous P388 mouse lymphoma cells. Rate sedimentation of mammalian cells through Ficoll gradients has been used to resolve mixtures of cells according to their size and density (Boone, Harell and Bond, 1968). This criterion of size has also been used to separate cells into different stages of the cell cycle (Morris, Cramer and Reno, 1967; Sinclair and Bishop, 1965); G_1 cells are the smallest, G_2 the largest and S cells intermediate between these two. In the present experiments the position of the S cells after sedimentation on the Ficoll gradient was determined by pre-labelling the S cells with ^{125}I-IUdR and subsequent determination of radioactivity/cell/fraction. To confirm that the cells were synchronous, growth curves of the suspected G_1, S and G_2 cells were studied, together with the mitotic index of the G_1 and S cells after removal from the gradient. The viability of the cells after exposure to

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Ficoll was determined using a vital stain (Fox and Gilbert, 1966) and the reproductive integrity by plating in 0.4% agar. (Nias & Fox, 1968).

MATERIALS AND METHODS

The murine lymphoma cell line P388 F, isolated from a DBA/2 mouse bearing the transplantable lymphocytic leukaemia P388. Isolation and methods of culture of P388 have been described (Fox and Gilbert, 1966). Exponentially growing cultures (210ml; 8.4 x 10^4 cells/ml.) in Fischer's medium supplemented with 10% Horse serum were pulse labelled with ^{125}I - IUdR for 30 minutes at a concentration of 1μg/10^4 cells (specific activity 6.25 Ci/mM). The cells were harvested at 1,000 r.p.m. and washed twice in physiological saline. The washed cell pellet was suspended in 1 ml. of Fischer's medium and 0.5 ml. layered onto a cold 20 ml. linear 5-20% Ficoll gradient made up in Fischer's medium. The gradient was centrifuged at 800 r.p.m. for 7 minutes at 4^oC, on a swing out head on the MSE major centrifuge. 1 ml. fractions were collected from the bottom of the gradient by means of a fine hypodermic needle lowered slowly to the bottom of the gradient down the side of the tube mixing of the gradient. 0.2ml. of each fraction was counted on the Gamma Guard counter for γ -irradiation, and 0.5ml diluted in 9.5ml of formal saline and counted on a Coulter Particle Counter. The radioactivity per fraction, number of cells per fraction and the radioactivity per cell were determined to find the position of the S cells in the gradient.

The mitotic index of the suspected S and G_1 cells was determined as follows:- 1ml. fractions from the bottom of the gradient were collected into culture tubes containing

5ml. of Fischer's medium supplemented with 10% Horse serum. Fractions 5 to 7 (Group I) were pooled and made up to 25 ml. with Fischer's medium containing 10% Horse serum. Fractions 10 to 14 (Group II) were also pooled and made up to 40 ml. with whole culture medium. The pooled samples were gassed with 5% CO_2 in air to adjust the pH to 7.2, and 2.5ml. samples dispensed into culture tubes. A sample was taken from each group at hourly intervals and pulse-labelled with 1μc/ml of ^3H - TdR (specific activity 27.0 ci/mM) for 15 minutes. Each sample was centrifuged at 1,000 r.p.m., the cell pellet washed twice in 0.9% saline and then suspended in hypotonic 1% sodium citrate for 15 minutes. After a further centrifugation the cells were resuspended and fixed in 1:3 glacial acetic acid/ methanol and smears prepared by the blaze drying technique (Scherz, 1962). At least two smears were prepared from each sample. The preparations were stained in 1% Acetic-Orcein stain. The mitotic index of each group was then determined by counting at least 500 cells per slide for 10 hours after removal from the gradient. The growth curves of the suspected G_2, S and G_1 cells were examined as follows:- Fractions 3; 5; 6 and 7 and 13; 14 and 15 were collected into culture tubes containing 4 ml. of Fischer's medium supplemented with 10% Horse serum. Fractions 5; 6 and 7 and 13; 14 and 15 were pooled to give groups I and II respectively, (the suspected sand G_1 cells) and each made up to 30ml. Fraction 3 was taken as the suspected G_2 fraction even though the possibility of obtaining pure G_2 cells is small using this method, partly due to contamination by polyploid cells. This fraction was diluted as described above. All the cultures were gassed with 5% CO_2 in air and

incubated at 37°C. The cell count of each group was recorded at hourly intervals using a haemocytometer. As a control a duplicate gradient on which cells had been sedimented under the same conditions was mixed, and 3ml. of this made up to 30 ml. with full Fischer's medium.

The viability of the cells in each fraction after sedimentation on a 5-20% Ficoll gradient was determined using a vital stain (1% Nigrosin). The reproductive integrity of the cells in differing concentrations of Ficoll after 1 and 5 hours exposure at 37°C was determined.

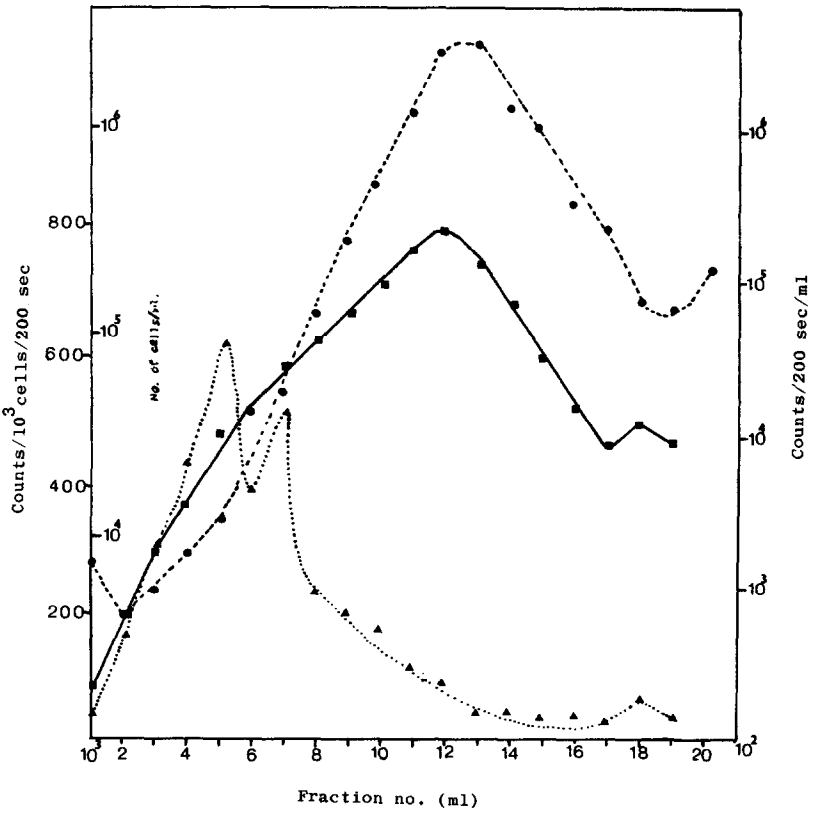

Fig. 1. Rate sedimentation of P388 mouse lymphoma cells using 5-20% Ficoll gradient. ●-----● number of cells/ml.; ■——■ counts/200 sec./ml.; ▲·····▲ counts/10³ cells/200 sec.

RESULTS AND DISCUSSION

The peak of cell number per fraction was found in fractions 11 to 12 (Fig. 1). The radioactivity per fraction was located in fraction 11. The S cells were found on the heavy side of the gradient. The curve of cell number markedly skewed towards the lighter side of the gradient, where the smaller cells in the population sediment (i.e. G_1 cells). The relative positions of the peaks corresponded closely to those obtained by Morris, Cramer and Reno, (1967) for P815Y cells on a 5 to 20% sucrose gradient. In replicate gradients the peak of cell count and radioactivity occurred in the same fraction. However, there was a shift in the location of this fraction by two fractions on comparing the two gradients. This slight difference in the two gradients could be overcome by locating the peak of radioactivity/cell as a function of the density of Ficoll. The S cells formed a sharp peak in fraction 5 in one gradient, and a broader peak between fractions 5 to 7 in the duplicate gradient.

From the study of mitotic index with time (Fig. 2) it was established that Group I contained a high proportion of cells in the middle and early stages of S; and that group II contained cells in G_1 - probably at the end of G_1. It would be expected that the cells at the beginning of G_1 would tend to occur at a lighter part of the gradient.

The suspected G_1 cells of group II came into division after about 14 to 15 hours. Cells in early G_1 would be expected to be dividing after 10 to 12 hours (Fig. 3). This inferrs that although these cells are synchronous there is some division delay or elongation of the cell cycle. The suspected S cells increase in number in steps beginning at

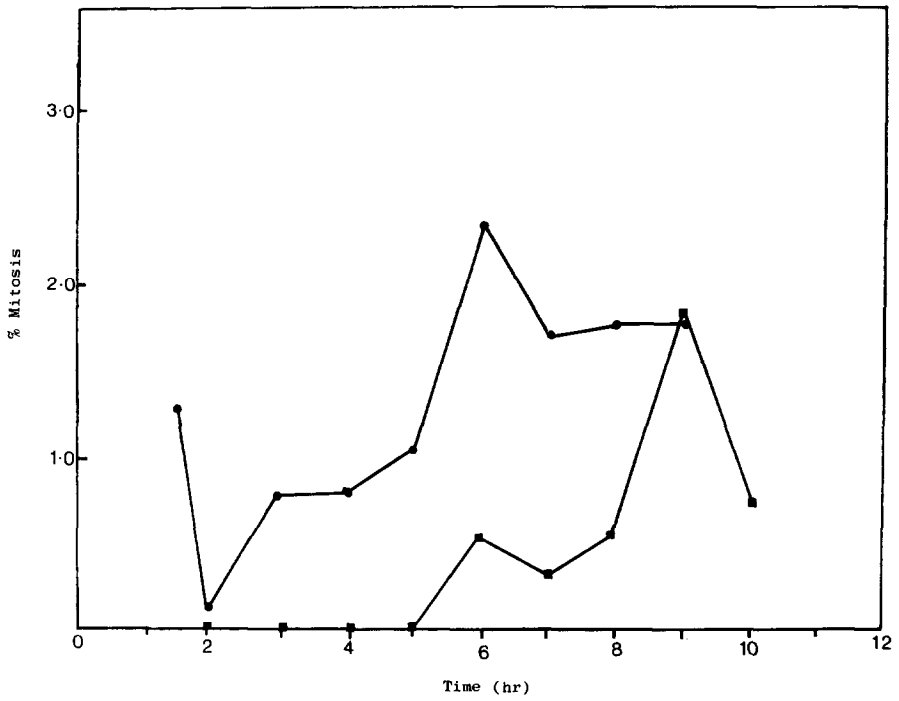

Fig. 2. The mitotic index of P388 mouse lymphoma cells after sedimentation on Ficoll gradient. ●——●Group I, fractions 5-7 (suspected S cells); ■——■Group II, fractions 10-14 (suspected G_1 cells).

2 hours, this indicates that these cells are at the end of S. The G_2 cells showed no stepping indicating that resolution in this part of the gradient was rather poor.

Using Nigrosin staining the viability of the cells in the suspected G_2, S and G_1 fractions was almost 100%. However, the viability towards the light side of the gradient i.e. after fraction 16 dropped. This reduction was probably due to a high proportion of dead cells at the light end of the gradient as observed by Sinclair and Bishop (1965) using sucrose gradients. On exposure of the P388 cells to 5,10, 15 and 20% Ficoll in Fischer's medium for 1 hour the plating efficiency was that of the control cells in Fischer's medium

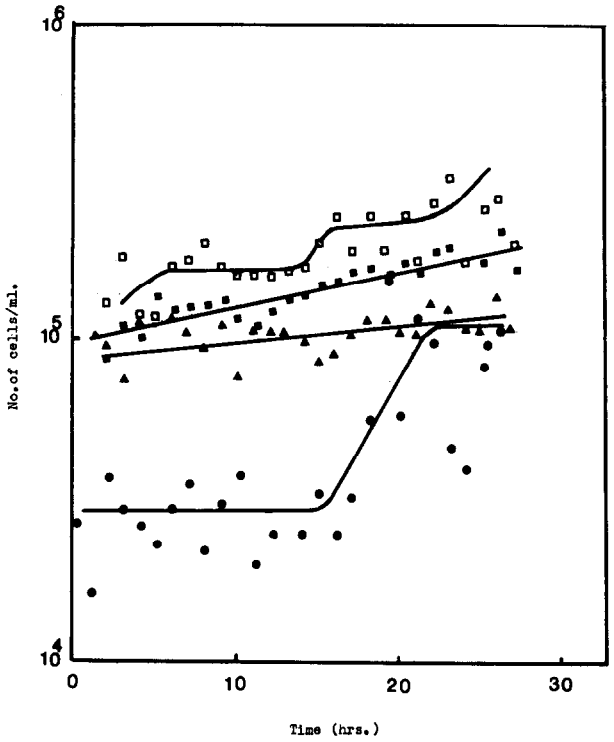

Fig. 3. Growth curves of P388 mouse lymphoma cells after sedimentation on Ficoll gradient. Group I fraction 5-7 (suspected S cells); control (cells from mixed gradient); Fraction 13 (suspected G_2 cells; Group II, fraction 13-15 (suspected G_1 cells).

supplemented with Horse serum. However, after 4 to 5 hours in Ficoll the plating efficiency decreased as a function of the concentration of Ficoll. During the sedimentation procedure the cells were only exposed to Ficoll for a maximum of 1 hour and therefore the plating efficiency was not affected. Boone, Harell and Bond (1968) found that the plating effieiency of Chinese Hamster cells on exposure to 10% and 25% Ficoll for 1 hour at 20°C was essentially that of the control cells in medium. They also showed that exposure of the cells to Ficoll for periods of up to 4 hours does not

affect the viability.

The method of synchrony described in this report therefore appears to produce fractions enriched with cells at a certain stage of the cell cycle and of high viability.

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REFERENCES

Boone, W.C., Harell, G.S. and Bond, H.E. J. Cell. Biol., 36, 369 (1968)
Morris, N.R., Cramer, J.W. and Reno, Donna, Exp. Cell. Res., 4, 216 (1967)
Sinclair, R. and Bishop, D.H.L., Nature, 205, 1272 (1965)
Nias, A.H.W. and Fox, M., Brit. J. Radiol., 41, 468 (1968)
Fox, M. and Gilbert, C.W. Int. J. Rad. Biol. 11, 339 (1966)
Scherz, R.C., Stain Tech. 37, 386 (1962)