Lymphocyte Survival and Macrophage Growth in Long Term in vitro Leucocyte Cultures

Little work has been done on the long-term maintenance of normal blood lymphocytes in culture. However, it is known that phytohaemagglutinin (PHA) increases the in-vitro survival of rabbit[1] and human[2] lymphocytes; also that low concentrations[3] of PHA (subthreshold doses) are able to maintain lymphocytes viable for up to 6 weeks.

The factors involved in the enhanced survival of lymphocytes by subthreshold doses of PHA have been investigated.

Leucocyte suspensions were prepared from heparinized normal blood by free sedimentation at 37 °C. 3×10^6 lymphocytes were cultured in 5 ml bottles in 2.5 ml of culture medium TC 199 (Glaxo) and 0.5 ml normal plasma. 3 types of duplicate cultures were set up. a) Short-term cultures using an optimum mitogenic dose of PHA (0.03 ml of reconstituted PHA (Wellcome) per bottle); these were harvested after 3 days. b) Long-term cultures using subthreshold doses of PHA (1/30 and 1/150 of the mitogenic dose). Medium, plasma and PHA were renewed weekly without centrifugation by pipetting off the supernatant. After 28 days the subthreshold dose of PHA was replaced by a mitogenic dose and the cells were harvested 3 days later. c) Long-term control cultures; as in b) but without subthreshold doses of PHA. Blast transformation and DNA synthesis were assessed morphologically, by ^{3}H-thymidine incorporation[4], and by autoradiography[5].

The results in 10 short- and long-term cultures are shown in Figure 1. Blast transformation was greater in the long-term cultures, but ^{3}H-thymidine uptake was similar in cultures a) and b), and the rate of DNA synthesis was the same. In the control cultures c) the radioactive counts were 40–90% lower, although the proportion of blast cells was the same as in a) and b). 50–80% of the lymphocytes were present at the end of long-term incubation when subthreshold doses of PHA were used; in the long-term control cultures only 20–40% of the lymphocytes remained. Some loss of lymphocytes probably occurs during the manipulation of long-term cultures.

The lymphocyte populations at the end of 28 days' culture consisted mainly of small lymphocytes with a few blastoid and intermediate lymphocytes. Macrophages were also present, in larger numbers in the controls than with subthreshold PHA; their number decreased after stimulation with mitogenic PHA, especially when good blast transformation occurred. Blast cell morphology was identical in the short- and long-term cultures (Figure 3, A); the cells were heavily labelled in the autoradiographs confirming their morphology and the scintillation counts.

Pokeweed mitogen (PKW) in subthreshold concentration (1/30 the mitogenic dose) also was able to maintain many lymphocytes alive during 4-week cultures. Antilymphocytic serum (ALS), purified protein derivative (PPD) and staphylococcal filtrate in subthreshold doses (1/100 the mitogenic doses) failed to do this, the only cells present after 4 weeks being macrophages. When PKW or ALS was used terminally to re-stimulate successful long-term cultures, a similar degree of blastoid transformation and DNA synthesis occurred as when the same mitogens were used in short-term cultures; the morphology of the blast cells was the same. Even with a specific antigen (e.g., PPD) some response was observed in terminal stimulation.

Further experiments were carried out to observe in more detail events during long-term culture (Figure 2). With subthreshold doses of PHA, especially the 1/30 dilution, a small proportion of lymphocytes became blast cells; the majority of the blasts were large cells, with a few of smaller type. In autoradiographs the larger blasts were predominantly labelled, less frequently the smaller cells which probably derived from earlier divisions and were about to become small lymphocytes again[6–8]. The ^{3}H-thymidine measurements confirmed a small degree of

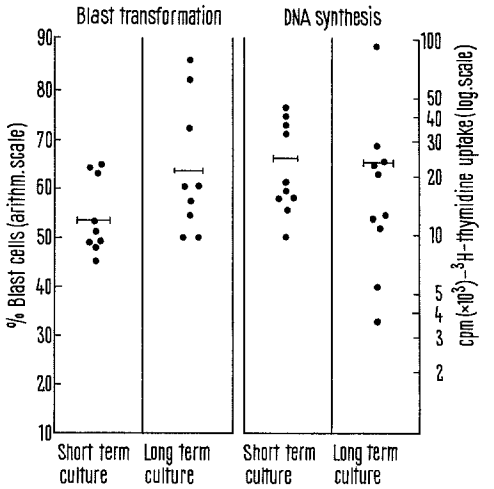

Fig. 1. Comparison between 10 short- and 10 long-term cultures after re-stimulation with a mitogenic dose of PHA.

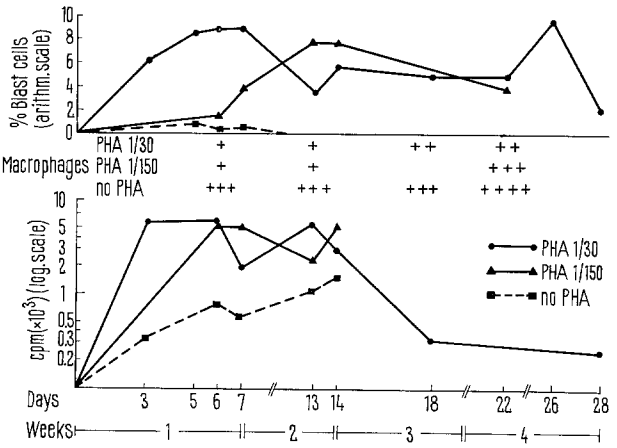

Fig. 2. Observations during the 4-weeks maintenance of long-term cultures. Top graphs: % blast cells. Lower graphs: ^{3}H-thymidine uptake (added 24 h before each determination). Each + represents 4–5% of macrophages.

[1] J. W. Foft and P. A. Romero, Lab. Invest. *13*, 575 (1964).
[2] B. P. MacLaurin, Lancet *1*, 816 (1965).
[3] Ch. K. Naspitz and M. Richter, Blood *30*, 381 (1967).
[4] L. J. Holt, N. R. Ling and D. R. Stanworth, Immunochemistry *3*, 359 (1966).
[5] B. M. Kopriwa and C. P. Leblond, J. Histochem. Cytochem. *10*, 269 (1962).
[6] H. Yamomoto, Nature *212*, 997 (1966).
[7] P. R. Polgar, S. Kibrick and J. M. Foster, Nature *218*, 596 (1968).
[8] G. A. Caron, Nature *218*, 1053 (1968).

DNA synthesis with both concentrations of subthreshold PHA. Macrophages were present in higher numbers in the controls from the end of the first week (Figure 3). Macrophage-lymphocyte interaction (Figure 3, B–C), and the phagocytosis of lymphocytes by macrophages (Figure 3, D–E), were striking findings.

Two further sets of long-term cultures were established: in one, leucocytes were cultured as described above; in the other most of the phagocytic cells were removed using a magnet after incubation with iron particles[9]. The lymphocyte concentration was adjusted to be the same in both sets of cultures. In the unseparated leucocyte culture the response to re-stimulation with mitogenic PHA was as before; in the pure lymphocyte culture the response was very good and equal whether or not subthreshold PHA was used during the maintenance period (Table), and only very few macrophages appeared in the cultures.

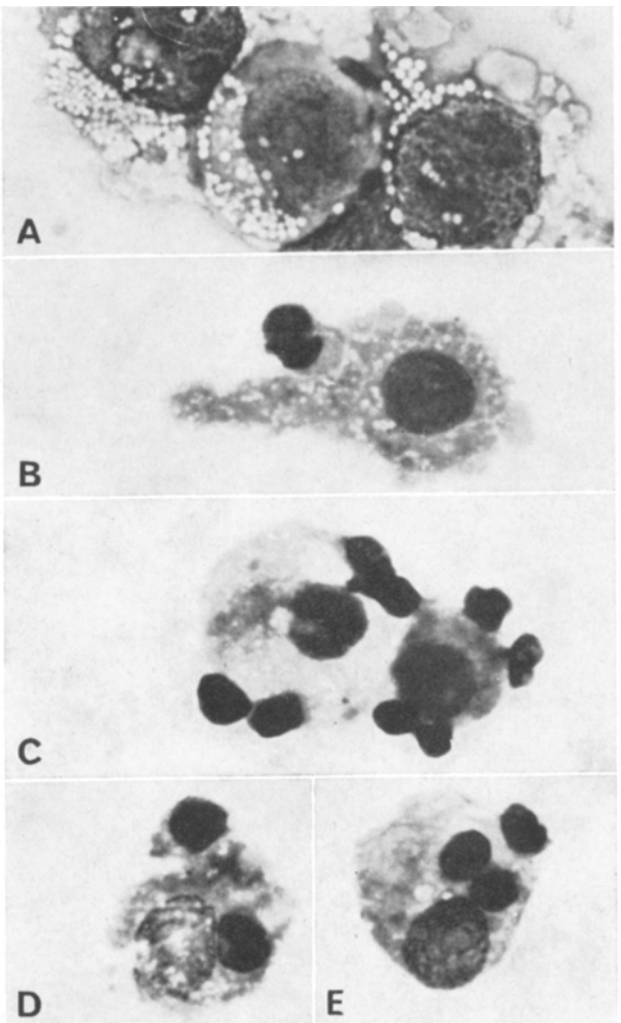

Fig. 3. Photomicrographs of long-term cultures (×1000). A, blast cells after re-stimulation with PHA (31-day culture). B and C, macrophage-lymphocyte interaction (14-day culture). D and E, macrophages phagocytosing lymphocytes (7-day culture).

Response to re-stimulation with a mitogenic dose of PHA in 2 long-term cultures, one with and one without weekly PHA (1/150)

	PHA re-stimulation of lymphocytes	
	Phagocytic cells not removed	Phagocytic cells removed
Control (no PHA)	1,020	100,547
Subthreshold PHA (1/150)	61,664	122,216

Our experiments show that subthreshold stimulation with PHA during long-term culture produces enhanced lymphocyte survival, mitotic division in a small proportion of lymphocytes, reduction in the number of macrophages in the culture, and increase in the proportion of PHA-responsive lymphocytes at the end of the culture.

Survival of lymphocytes in long-term culture seems to be related to the fate of macrophages in possibly three ways: a) macrophages can be derived from lymphocytes[10] and subthreshold PHA may suppress this by continual stimulation of lymphocytes; b) lymphocytes stimulated with subthreshold PHA become cytotoxic against macrophages as has been shown against other types of target cells[11,12]; c) macrophages phagocytose lymphocytes (Figure 3, D–E). Mechanism a) seems unlikely from our observations on unstimulated pure lymphocyte cultures. Also, there is other evidence that monocytes are the main source of macrophages in tissue culture[13,14]. The combination of mechanisms b) and c) provides a better explanation. The good survival of lymphocytes in unstimulated cultures in the absence of macrophages (Table) supports the concept that subthreshold PHA and PKW are only indirectly responsible for enhanced lymphocyte survival; they act probably by protecting lymphocytes from an excess number of macrophages.

Long-term culture with subthreshold PHA may prove to be useful in the study of the immunological capacities of lymphocyte populations[15].

Zusammenfassung. Unterschwellige Dosen von Phytohämagglutinin verlängern die Lebensdauer von Lymphozyten in menschlichen Zellkulturen, vermindern die Zahl der Makrophagen in der Kultur und erhöhen die Zahl der transformationsfähigen Lymphozyten.

D. CATOVSKY and P. J. L. HOLT

Departments of Haematology and Medicine, Royal Postgraduate Medical School, London W.12 (England), 14 January 1970.

[9] K. CARSTAIRS, Lancet *1*, 829 (1962).
[10] J. G. HOWARD, J. L. BOAK and G. H. CHRISTIE, in *The Lymphocyte in Immunology and Haemopoyesis* (Ed. J. M. YOFFEY; E. Arnold Publishers Ltd., London 1967), p. 216.
[11] G. HOLM, P. PERLMANN and B. WERNER, Nature *203*, 841 (1964).
[12] W. ROSENAU, Fedn. Proc. *27*, 39 (1968).
[13] H. BRÜCHER, A. DILL and M. GRÄBER, Acta haemat. *41*, 76 (1969).
[14] F. SCHMALZL and H. BRAUNSTEINER, Acta haemat. *38*, 281 (1967).
[15] We acknowledge the technical assistance of MRS. R. EVANS, and the encouragement of Professor J. V. DACIE and Dr. D. A. G. GALTON. This work was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas de la Republica Argentina.