

The Kinetics of Cell Proliferation in Cultures of Human Peripheral Blood

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PROLIFERATION of cells of the peripheral blood in vitro was first demonstrated in 1915.¹ Recently a modification of Osgood's technic² by Hungerford³ focused attention on the rapidity with which an actively growing cell population can be obtained. In cultures of peripheral blood, mitotic indices of 1 per cent are observed by the third day. Two mechanisms could explain the rapid development of mitotable cells. They may originate either from the small number of cells in DNA synthesis present in the freshly drawn blood,⁴ or from other mononuclear cells, possibly lymphocytes, which manifest their capacity for DNA synthesis and division only during culture. The first possibility implies a very short generation time. The second possibility was of particular interest because of the divergent views of the potential of small lymphocytes. The present studies were undertaken to distinguish between these alternatives.

METHODS

Heparinized normal human peripheral blood was cultured by the method of Hungerford et al.³ Three to six separate 20 cc. aliquots of cultured cells were studied at 12–24 hour intervals; 2 μ c./cc. of tritiated thymidine (H^3Th)^{*} was added during the final two hours of incubation. At each time interval, white cells were counted using duplicate pipettes and hemocytometers. Coverslip smears were stained with Giemsa and 200–400 cell differentials were done. Other coverslips were used for autoradiography with NTB 3 or AR-10 film.† Metaphase preparations were made at the end of three days using 1×10^{-6} M colchicine for one hour, and Ford's⁵ or Moorhead's⁶ technic. These procedures enabled us to follow the changes in cell count, cell morphology, and per cent of H^3Th labeled cells at frequent intervals during the growth of the culture.

Further studies required modifications of the above time sequence. When we wished to follow a cohort of labeled cells, H^3Th was added to the culture for 2–4 hours and further uptake of H^3Th was suppressed by substituting medium containing 20 mg. of unlabeled thymidine. Studies in which colchicine and H^3Th were added to the same aliquot are described in the text.

RESULTS

The total number of white cells decreased to ~ 45 per cent of the initial value during the first 36 hours and remained relatively stable until 60 hours (fig. 1). The morphologic changes which occurred during culture are shown in figure 2. During the first 24 hours two types of mononuclear cells were seen. Cells of the first type resembled the small lymphocytes of the peripheral

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†Eastman Kodak, Rochester, N. Y.

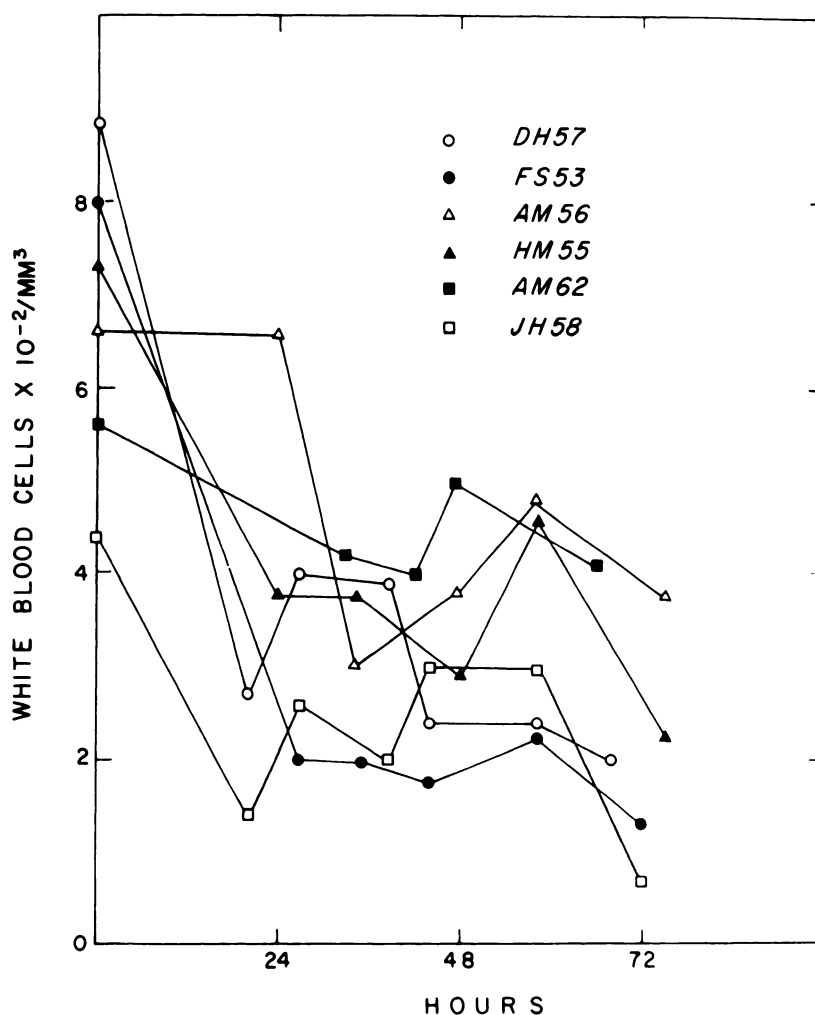


Fig. 1.—The changes in total white cells during culture.

blood. They had round nuclei with clumped chromatin and a small amount of cytoplasm. The cells of the second type had nuclei with an irregular outline, often a little larger than in the cells of the first type. The cells of the second type had a more diffuse pattern of less densely staining chromatin, more abundant pale staining cytoplasm, and resembled monocytes. They were, however, two numerous in the freshly separated samples to be derived solely from monocytes and were believed to represent, in part, distorted lymphocytes. The two types were therefore classified together as "mature lymphocytes and monocytes." Neutrophilic and eosinophilic granulocytes were the only other well defined groups in early samples. They had degenerated by 24 hours. At that time, a small number of cells were seen which were the size of small lymphocytes, but differed in that they had a more abundant, deeply baso-

philic cytoplasm which included distinct vacuoles. The nuclei were round and nucleoli were visible in some. These cells were classified as young lymphocytes. They become more numerous in later samples. In addition, large mononuclear cells were seen in the samples taken at 26 to 72 hours. These cells were larger than the small lymphocytes, and reached diameters two or three times that of mature lymphocytes. They had round, oval or indented nuclei, and abundant cytoplasm which frequently contained numerous distinct vacuoles. Both nuclear and cytoplasmic basophilia varied greatly in intensity between different groups of cells, and even from cell to cell. These cells were quite dissimilar from any normal hemic or lymphoid cells. Smudges, naked nuclei and grossly distorted cells varied between cultures and even between areas of the same slide, and were omitted in the final differential counts.

The classification of the cells in culture into mature lymphocytes, young lymphocytes and large mononuclear cells is largely arbitrary since "transitional" forms between any of the mononuclear cells described could be readily found. The division merely serves to characterize the changes in the appearance of the predominant cell types during the three days of culture and to relate them to the morphology of familiar cell types. Multinucleated giant cells were not seen although rarely a large mononuclear cell contained a micronucleus. Fibroblasts were not seen. A sample differential count is given in table 1.

At the beginning of the culture 0.1–0.5 per cent of the cells were in DNA synthesis, as estimated by labeling with H^3Th . The addition of H^3Th to the cultures at intervals thereafter permitted estimates of the proportion of cells entering DNA synthesis at various times. There was no increase in the percentage of labeled cells between 0 and 24 hours but thereafter the percentage of cells in DNA synthesis increased rapidly (fig. 3). When H^3Th was added at 48 hours 10 per cent of the cells incorporated label; at 72 hours 40–45 per cent of the cells incorporated label. There was a parallel increase in the absolute numbers of cells in DNA synthesis.

Two studies were done to determine if cells entering DNA synthesis between 24 and 48 hours were the progeny of those cells in DNA synthesis at time zero. In the first study H^3Th was added at time zero; after 2–4 hours the medium was changed and 20 mg. of non-radioactive thymidine was added to block further uptake of any residual H^3Th . Aliquots were studied up to 45 hours. Two thousand cells were examined from each aliquot. During this time the percentage of labeled cells increased from an average of 0.2 per cent to ~ 0.4 per cent. All labeled cells had more than 100 grains. In similar cultures, when H^3Th was added at 42 or 46 hours and the samples examined at 44 or 48 hours, 6–12 per cent of the cells were labeled.

It seemed clear from the above experiment that the rapid increase in DNA synthesizing cells between 24 and 48 hours could not be due to proliferation of those cells in DNA synthesis at time zero. This was further documented by a different approach. If the cells in DNA synthesis at 48 hours were the progeny of the mononuclear cells labeled at zero time an average of ~ 5 divisions in a 24 hour period would be necessary to account for the observed ~ 30 fold in-

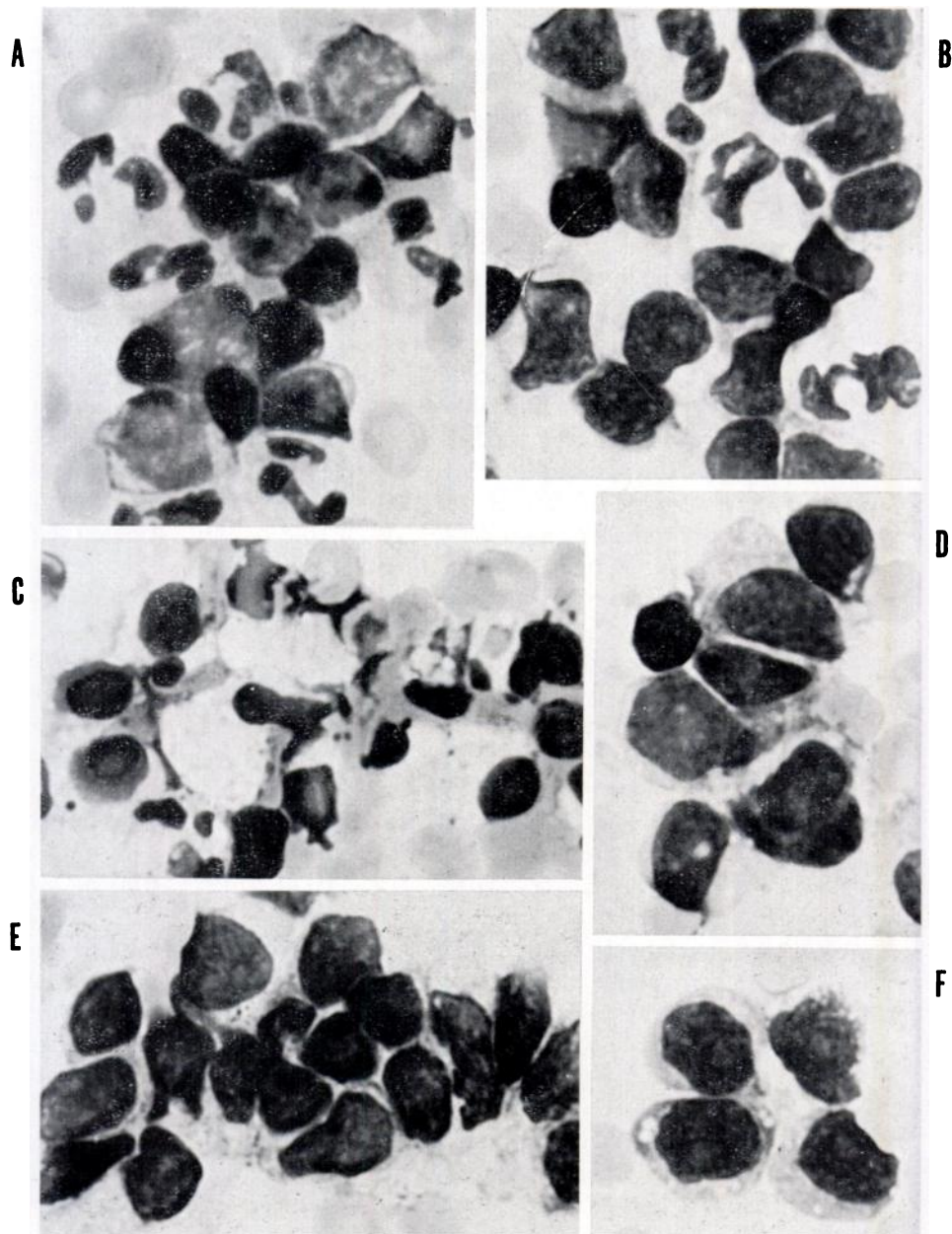


Fig. 2.—Cells from peripheral blood culture at various time intervals. **a.** 0 hour. Polymorphonuclear leukocytes and mature lymphocytes with some distortion. **b.** 2 hours. No change. **c-f.** 24–26 hours. **c.** Degenerating polymorphonuclear cells and mature lymphocytes. **d & f.** Young lymphocytes. **e.** Persistent mature lymphocytes.

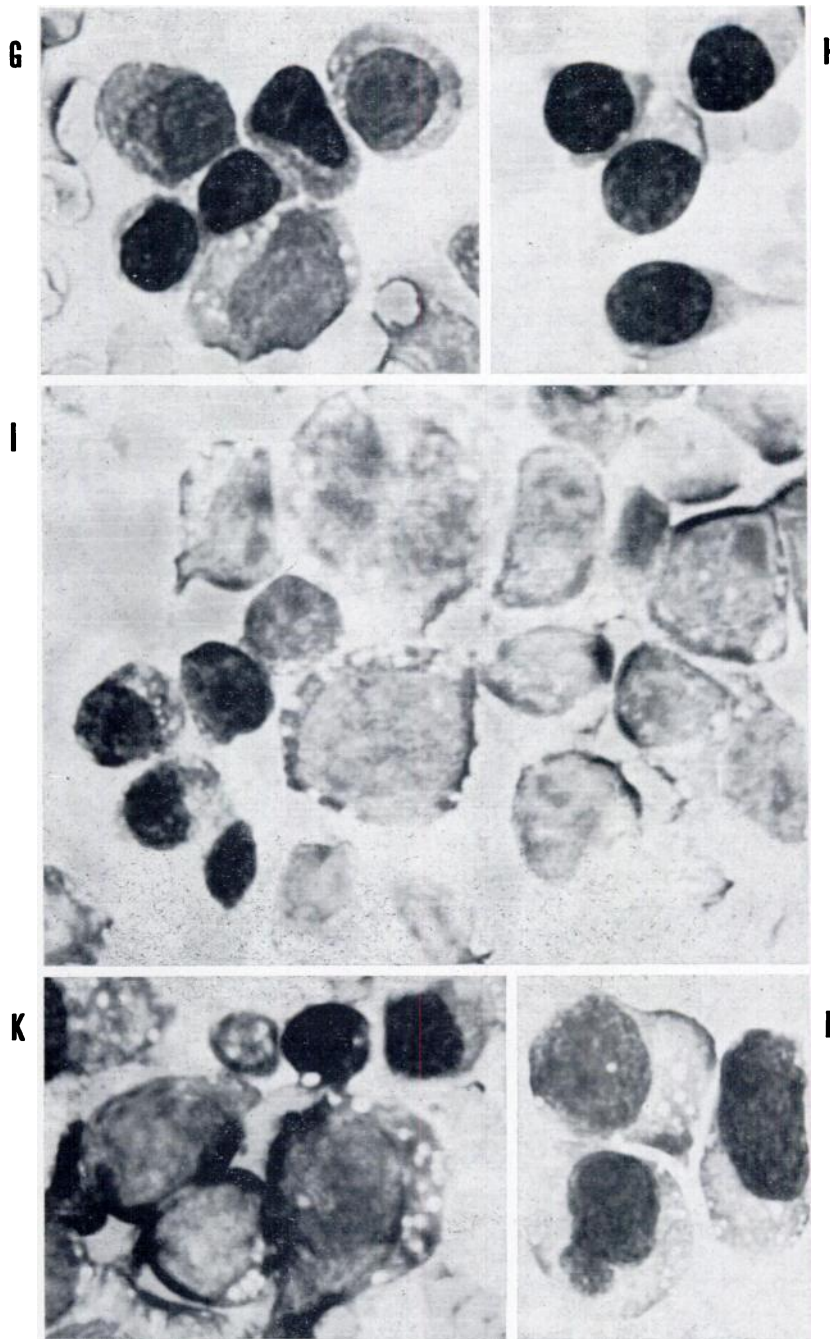


Fig. 2. (cont.)—g, h. 32–34 hours. Predominantly young lymphocytes. i. 48 hours. k. 68 hours. l. 75 hours. i, k, l. Predominance of large mononuclear cells, with a few young and mature lymphocytes.

Table 1.—*Differential Counts of In Vitro Peripheral Blood Cell Cultures*

Time*	Neutrophilic Granulocytes	Eosinophilic Granulocytes	Mature Lymphocytes & Monocytes	Young Lymphocytes	Large Mononuclear Cells
0	59	3	38	—	—
12	3	1	95	1	—
24	1	1	73	21	4
34	—	—	56	36	8
48	—	—	54	23	23
58	—	—	31	21	48
75	—	—	15	32	53

* Hours after start of culture.

crease in DNA synthesizing cells. This implies an average generation time of ~ 5 hours. The addition of colchicine to cultures during the period of rapid increase in DNA synthesizing cells should result in (a) the collection of a substantial number of metaphases, and (b) a reduction in the number of labeled cells when H^3Th was added after the colchicine—i.e., by blocking mitosis, the number of cells dividing and again entering division would be reduced. In one experiment, colchicine was added to aliquots at 27 or 30 hours and H^3Th at 31 hours. These specimens and parallel controls were examined at 33 hours. In a second experiment, the colchicine was added at 33 or 36 hours and the H^3Th at 37 hours; the cultures were studied at 39 hours. In neither experiment were mitoses observed amongst 3000 cells counted in each aliquot. The percentage of labeled cells in the control and colchicine treated cultures were not significantly different (table 2).

In a third group of experiments, the time of onset of mitosis was determined (table 3). Colchicine was added to aliquots at successive time intervals and metaphase preparations made after 3–4 hours incubation with colchicine. Mitoses were first seen between 40 and 45 hours and increased rapidly; at 65–70 hours the mitotic index was about 1 per cent/hour (range 0.5–1.9 per cent) of exposure to colchicine.

DISCUSSION

The lymphocytic origin of dividing cells in cultures of peripheral blood was postulated by Maximow⁷ on the basis of a smooth series of transitional cells from the small "mature" lymphocyte to the large mononuclear cells, which he termed polyblasts. Origin from monocytes or a small number of special "young" mononuclear cells, some of which are now known to be synthesizing DNA at the time the blood is drawn, was put forth as a possible alternative. Controversy on this point has now continued for half a century and persuaded many investigators that morphologic criteria alone cannot resolve the problem.^{8,9}

In the present study, the possibility that the large number of DNA synthesizing cells found at 48 hours were the progeny of cells already in DNA synthesis at time zero has been excluded by following the initially labeled cells. The intensity of labeling was high enough to preclude that cells were "lost" by dilution of the label. Moreover, the origin from cells that synthesize DNA

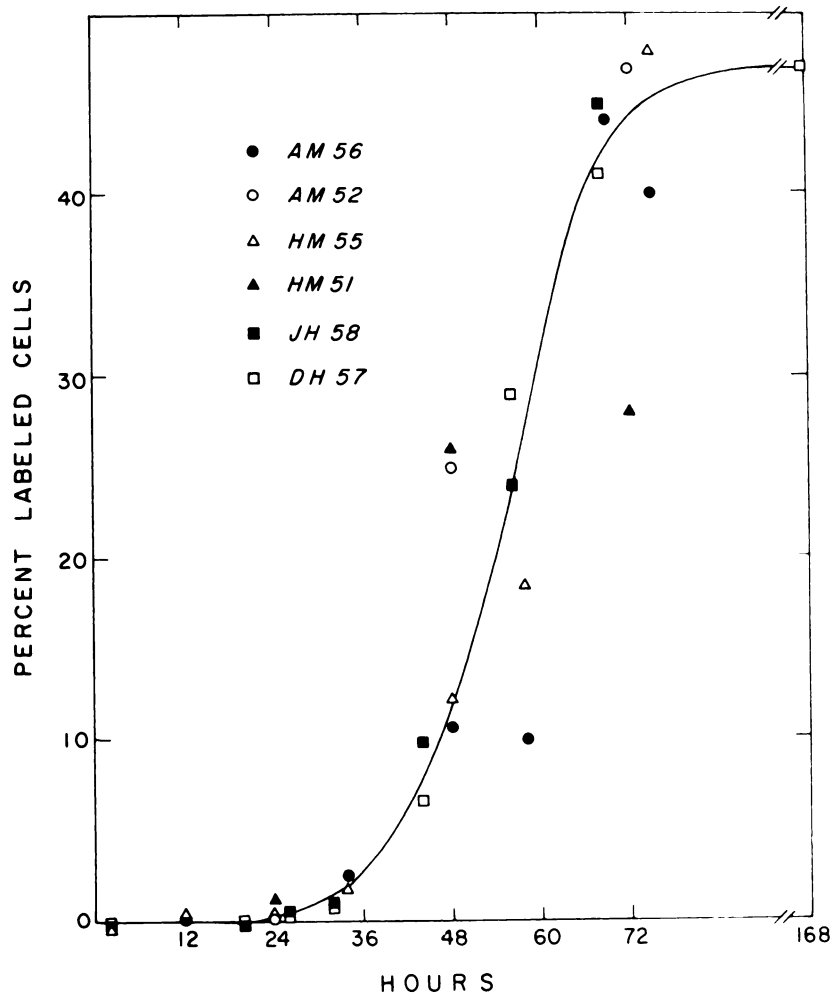


Fig. 3.—The percentage of cells in DNA synthesis, as measured by tritiated thymidine uptake, during culture. Tritiated thymidine was added 2 hours prior to examination of cultures.

initially or from any comparably small percentage of cells would imply a generation time of about five hours. Addition of colchicine produced neither the number of mitoses expected with such short generation times nor was there a reduction in the number of cells entering DNA synthesis which would necessarily result if a substantial number of cells were arrested in mitosis and failed to enter a subsequent period of DNA synthesis.

The present studies, therefore, indicated that the dividing cells in culture of peripheral blood must be derived from a relatively large population of cells. Since granulocytes disintegrated during the first 24 hours, it appears from numerical consideration alone that lymphocytes were responsible for the growth and that the sequence of morphologic patterns of mononuclear cells

Table 2.—Effect of Colchicine on H^3 Thymidine Labeling

Time (hours) from Start of Cultures			
Colchicine added	Thymidine added	Culture examined	% Labeled Cells
Experiment I			
—	22	24	0.1
—	31	33	1.2
30	31	33	0.8
27	31	33	0.9
—	70	72	40
Experiment II			
—	31	33	2.3
—	37	39	11.4
36	37	39	14.6
33	37	39	15.1
—	61	63	43

Table 3.—Mitotic Index

Experiment	Time in culture (hours) *							
	27*	30	41	42	45	47	64	66
A	0†	—	0.05	—	0.17	—	0.43	—
B	—	0	—	0.05	—	0.37	—	1.3

*Time at which colchicine was added to the cultures. Specimens were examined 3 hours after addition of colchicine except in the 41-hour study in which the culture was examined after 4 hours incubation with colchicine.

†The values given are the % mitoses hour and are based on examination of 1000–3000 cells.

represented developmental stages or transformation of the small lymphocytes, as originally suggested by Maximow.

It is tempting to assume that the transformation of small lymphocytes and the multiplication of transformed cells, demonstrated under the specific condition of culture used in our experiments, may have a bearing on some of the controversial points on the potentialities of the lymphocyte. It should be noted, however, that the transformation claimed to exist in vivo involved renewed differentiation of the rejuvenated "mature" lymphocyte. Maximow's school suggested that the circulating lymphocyte can serve as stem cell of both red cells and granulocytes.¹⁰ Rebeck and Crowley¹¹ reviewed the evidence suggesting that lymphocytes from the peripheral blood are transformed into histiocytes after entering the extravascular spaces. Observations of Cronkite et al.¹² in the Selye sterile inflammation pouch also suggest but do not prove that such transformation takes place extravascularly in vivo. Petrakis¹³ has shown that macrophages and fibroblasts develop from mononuclear cells of the peripheral blood in subcutaneously implanted diffusion chambers. The morphology of the predominant cell type in 72 hour old cultures does not indicate any differentiation toward fibroblasts or hemic cells, possibly because the time in culture was too short. Alternatively, it may be suggested that the small lymphocyte develops into larger antibody producing cells. This is strongly suggested by recent in vivo studies of Gowans,¹⁴ who injected labeled small

lymphocytes from the thoracic duct. In the spleen these cells underwent a series of transformations similar to those described here except for the absence of vacuoles. Antibody production by these cells was indicated by graft rejection or runt disease when suitable antigenic combinations of donor and recipient rats were used. Transformation of small lymphocytes into larger antibody producing cells has also been demonstrated in diffusion chambers implanted into new born rabbits by Holub.¹⁵

In general, while the present investigations establish the growth potential of small lymphocytes, they cannot provide any indication of the frequency or direction of lymphocyte transformation nor of the specific conditions or stimuli that may be necessary to induce such transformation and growth in vivo.

SUMMARY

The kinetics of growth of peripheral blood cells in tissue culture are described. There was a rapid increase of cells in DNA synthesis beginning at 24 hours and by 72 hours 40–45 per cent of the cells were in DNA synthesis. Mitotic rates of ~ 1 per cent/hour were seen after 72 hours in cultures. Studies with tritiated thymidine and colchicine indicated that these cells represent transformed lymphocytes and do not arise from the cells in DNA synthesis at time zero or from any other small population of cells.

SUMMARIO IN INTERLINGUA

Es describe le cinetica del crescentia de cellulas de sanguine peripheric in histoculturas. Occurreva un rapide augmento del cellulas in stato de synthese de acido disoxyribonucleic comenciante post 24 horas. Post 72 horas, 40 a 45 pro cento del cellulas esseva in stato de synthese de acido disoxyribonucleic. Un intensitate del mitotismo de circa un pro cento per hora esseva constatate post 72 horas in le culturas. Studios con tritiate thymidina e colchicina indicava que iste cellulas representa transformate lymphocytos e non emerge ab le cellulas in stato de synthese de acido disoxyribonucleic al tempore zero o ab un del altere micre populationes de cellulas.

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