

The Loss of Growth Vitality of Human Lymphoid Cell Lines Derived from Healthy Adults¹ (35021)

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It is well known that the vast majority of human peripheral lymphoid cells, after appropriate stimulation, can undergo no more than several serial divisions within the first 3 weeks of *in vitro* cultivation (1). It is also well known that continuous lines of lymphoid cells with apparently unlimited *in vitro* life span can be established from the blood of human subjects (2-10). Lines of human lymphoid cells which lose growth vitality after varying numbers of serial subcultivations have not, however, been reported previously and are described in this report.

Materials and Method. Materials. The culture medium was 20% fetal calf serum in Eagle's minimal essential medium, supplemented with nonessential amino acids. Penicillin and streptomycin were added in amounts of 50 units and 50 $\mu\text{g}/\text{ml}$, respectively. The pH of the medium was adjusted to 7.0-7.4 by adding NaHCO_3 or gassing with 5% CO_2 in air. Citrate buffer was prepared by dissolving citric acid (8 g) and sodium citrate (22 g) in 1 liter of water; the solution was autoclaved at 10 lb for 15 min. The dextran solution (6% w/v) was purchased from Abbott Laboratory; the average molecular weight of dextran particles was said to be 75,000 (range 20,000 to 200,000).

Preparation and care of leukocyte culture. Five to 50 ml of venous blood were mixed with 0.1 vol of citrate buffer and 0.3 vol of dextran solution. The mixture was incubated at 37° for 30 min and the plasma fraction, rich in leukocytes, was collected. The mixture was incubated for 30 min more, and the

plasma fraction was again harvested. Plasma fractions from both harvests were pooled and centrifuged at 1500 rpm in a centrifuge with swing-out buckets for 10 min. The sedimented cells were washed once with 40 ml of saline and suspended in 1 to 10 ml of culture medium. The number of white cells and the percentage of lymphocytes was determined by routine white cell and differential counts. The cell suspension was then adjusted with culture medium to a final concentration of approximately 2 million lymphocytes/ml. This cell suspension was distributed in 1-ml aliquots into culture tubes (150 \times 15 mm) with round bottoms. Cultures were gassed with 5% CO_2 in air, closed with rubber stoppers to minimize the escape of CO_2 and incubated at 37° in upright position. One ml of fresh culture medium was added to each culture on the third or fourth day, and 1 ml of medium was replaced twice weekly for the next 2 weeks and at weekly intervals thereafter until the culture degenerated completely or until large clumps of cells began to appear. Cultures were examined at weekly intervals with an inverted microscope. Cultures which contained many large cell clumps and at least 0.5 million viable cells were divided 1 to 2 serially at intervals of 3 to 7 days. At each division of culture, 2 ml of medium were added, cell clumps were dispersed, and 2 ml of cell suspension were discarded. The procedure was generally similar to that described by others (4, 5, 10), except for three modifications: (a) the lymphocytes were cultivated as many individual units, each containing about 2 million lymphocytes; (b) the cultures were not combined to maintain a minimum cell population; and (c) culture tubes with round bottoms were used, which

¹Supported by Research Grants (HD04144 and FR05684-01 from NIH and from Cancer Research Co-ordinating Committee of the University of California.

facilitated sliding the lymphocytes toward the center to form a button of cells, permitting microscopic examination with little disturbance of the topographic relationship of cells in the culture, and expediting the changing of medium since centrifugation of culture, to sediment the lymphoid cells, was unnecessary.

Test for mycoplasma. Frey's medium (11), kindly furnished by Dr. Henry E. Adler, was plated with 0.05 ml of cell suspension. After 5 to 7 days at 37° in a closed chamber gassed with 5% CO₂ in air, inoculated plates were examined for mycoplasma colonies. A strain of mycoplasma previously isolated from the HeLa cell was included as a positive control in each test. Cultures were also tested by Dr. Henry E. Adler (12).

Test for cytopathic agents. 0.2 ml of culture fluid, not centrifuged or filtered, was inoculated into a culture of human embryonic fibroblasts. The inoculated fibroblast culture was examined periodically for 21 days for progressive cytopathic changes, and if negative, the culture was challenged with 30 infectious doses of vesicular stomatitis virus for the purpose of detecting interferons (13).

Results. Growth characteristics. Since the growth characteristics of human peripheral leukocytes under the prescribed cultural conditions have not been reported previously, they are described here in considerable detail. Cultures were examined at weekly intervals without dispersing the sedimented cells. On day 7, there was a central red button of erythrocytes surrounded by a white rim of leukocytes. Peripheral to the white rim were many cells, each less than 12 μ in diameter and irregular in shape. These cells were apparently adherent to glass and did not slide toward the center of the culture tube. On day 14, varying numbers of spindle, fusiform, or oval cells, all longer than 20 μ , were seen in the peripheral zone. Small cell aggregates of about 50–100 μ in diameter were sometimes seen along the white rim of leukocytes. On day 21, the peripheral zone was occupied by a confluent monolayer of spindle, fusiform, and oval cells, some of which exceeded 100 μ in length. The central button of cells

was smaller than it was on day 14. The small cell aggregates seen on day 14 also disappeared. Each culture contained from 20 to 60 thousand viable cells that were not adherent to glass. From the fifth week onward, the spindle, fusiform, and oval cells underwent progressive degeneration. The central button of free-floating cells became smaller and contained debris. By weeks 8 to 14, most of the culture contained no viable cells that were not adherent to glass. In approximately 1 per 10 cultures, cell aggregates were seen once again in weeks 5 to 13. The aggregates increased in number and in size, and a few reached 0.5 mm in diameter. Cultures with large aggregates invariably contained more than 0.5 million cells. Such cultures were divided 1 to 2 serially at intervals of 3 to 7 days.

Proportion of cultures which yielded cell lines and the number of serial divisions at which a decrease in growth vitality was noted. The results are summarized in Table I. Of the 186 cultures from 8 healthy adults, 20 developed cell clumps on weeks 5 to 14 of cultivation. Of these 20 cultures, 15 showed a decrease in growth vitality after the 3rd to 48th serial divisions, while four have attained the 85th to 107th serial divisions and are still growing vigorously. A decrease in growth vitality was characterized by the gradual decrease both in the amount of cell sediment and in metabolic activity. Further serial division of the culture resulted in its loss. When the culture was maintained by nutrient renewal at weekly intervals and by abandonment of further division of the culture, 9 of the 15 cultures degenerated in 3 to 15 more weeks, while the remaining 6 still contained 20 to 80 thousand viable cells after 2 to 4 months.

Morphology. Giemsa stained smears were prepared from all 20 lines before the 20th serial division and from the 4 lines which have undergone more than 85 serial divisions. Cells morphologically similar to the lymphoid series in various stages of maturity were seen in all smears.

Failure to demonstrate mycoplasmas and transmissible cytopathic or interferon-

TABLE I. Lymphoid Cell Lines from the Blood of 8 Healthy Adults and the Number of Serial Division at Which Loss in Growth Vitality was Noted.

Donor	Sample	Line/ total ^a	No. of serial division ^b
C	1-5	0/35	8, ^c 18, 23 ^d
	6	3/20	
R	1	1/10	(85)
	2	0/5	
B	1	0/10	
F	1	2/8	12, 26 ^d
Mc	1, 3	0/15	5 (97), 48 ^d
	2	1/7	
	4	2/10	
Me	1	5/7	(107), (107), 21, ^d 28, 44
	2	0/5	
W	1-3	0/20	10, 20 ^d 5
	4	2/5	
	5	1/10	
H	1	3/19	3, ^d 3, ^d 5 ^d

^a Line/total = number of cultures which yielded cell lines/total number of cultures observed.

^b No. = number of serial division at which loss in growth vitality was noted; number of serial division of a line which continued to grow actively at the time the manuscript was prepared is given in parentheses.

^c Line contaminated by fungi at the eighth serial division.

^d Cultures had been tested and found negative for mycoplasma and viruses.

inducing agents. In the latter portion of this study, mycoplasma or viral contamination came to my attention as two possible explanations for the decrease in growth vitality in previously actively growing lines of lymphoid cells. Eight of the 15 cultures listed in Table I were available for testing. Mycoplasmas, transmissible cytopathic agents or interferon-inducing agents were not detected by the methods used in this study. Dr. Henry E. Adler, a specialist in mycoplasma, was also unable to detect mycoplasmas by a more elaborate method used in his laboratory (12).

Loss of growth vitality of cultures initiated from frozen cells. One line preserved for 3 months in liquid nitrogen refrigeration at the

10th serial division was revived. After 12 more serial divisions the revived culture lost growth vitality. A sister culture, not interrupted by subzero preservation, lost growth vitality at the 26th serial division. A second line, similarly preserved for 2 months at the time a decrease in growth vitality was noted, was also revived. Viable cell population decreased from 0.48 to 0.31 million in 6 weeks with no division of the culture.

Discussion. The loss in growth vitality in 15 of 20 lymphoid cell lines remained unexplained. The possibilities of accidental contamination by mycoplasmas and cytotoxic viruses or of activation of the cytomegalovirus which might exist in the occult form in the lymphocyte (14) were unsubstantiated by the tests employed in this study. Technical errors were also considered unlikely because: (a) all cell lines were kept together in one incubator, fed with one batch of medium and subdivided by one investigator; (b) only one or two cultures among many actively growing cultures showed a loss in growth vitality at any one time; and (c) at least two lines revived from a frozen state lost growth vitality at roughly the same number of serial division as sister cultures not subjected to subzero preservation. This loss in growth vitality of lymphoid cell lines from healthy adults has also been repeatedly observed in experiments designed for other purposes, but they were not included in this report. It should be emphasized that human fibroblasts and amnion cells also showed an unexplained loss in growth vitality after varying numbers of serial passages *in vitro* (15-17).

This finding may be helpful to investigators interested in studying the enhancing effect of viruses and chemicals on the establishment of lymphoid cell lines from the blood of healthy individuals (also referred to as transformation). It seems important, in such a study, to distinguish between cell lines which can multiply indefinitely and cell lines which lose growth vitality after varying extents of multiplication. None of the previous reports (18-22) on this important subject contain this information, and the reader is not certain if those transformed cell lines

are truly established cell lines as defined by the Nomenclature Committee of the Tissue Culture Association (23). Data presented above suggest that a lymphoid cell line from a healthy adult should undergo at least 50 to 60 serial 1 to 2 divisions at 3-7-day intervals before it can be called an established line.

Summary. Under the culture conditions described in this report, 20 lines of cells morphologically similar to the lymphoid cells were derived from the blood of eight healthy adults. Four of the 20 lines have undergone 85 to 107 serial divisions and are still growing vigorously. Fifteen lines showed an unexplained loss in growth vitality after the 3rd to 48th serial division. One line was contaminated by fungi in the 8th serial division.

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Received Mar. 13, 1970. P.S.E.B.M., 1970, Vol. 135.