

## THE LIMITED IN VITRO LIFETIME OF HUMAN DIPLOID CELL STRAINS<sup>1,2</sup>

L. HAYFLICK

*The Wistar Institute of Anatomy and Biology, Philadelphia, Pa., U.S.A.*

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PREVIOUS reports from this laboratory have emphasized the fact that serially cultured human diploid cell strains have a finite lifetime *in vitro* [2, 12, 14, 15]. After a period of active multiplication, generally less than one year, these cells demonstrate an increased generation time, gradual cessation of mitotic activity, accumulation of cellular debris and, ultimately, total degeneration of the culture. The limited *in vitro* multiplication of many kinds of cultured cells has been a common observation of cell culturists. Until recently [4], technical difficulties were invoked as an explanation for this event. This phenomenon in the course of *in vitro* cultivation of human fetal diploid cell strains, which we refer to as Phase III, has been shown to occur after  $50 \pm 10$  serial passages *in vitro* using a 2:1 subcultivation ratio [14]. This event has now been confirmed in other laboratories [36, 41] and appears to be causally unrelated to conditions of cell culture, the media composition used, presence of mycoplasma or latent viruses, or the depletion of some non-replicating intracellular metabolic pool [14]. Consequently, we advanced the hypothesis that the finite lifetime of diploid cell strains *in vitro* may be an expression of aging or senescence at the cellular level. Experiments to be described extend the studies of this phenomenon and have further bearing on an interpretation based on a theory of senescence.

### MATERIALS AND METHODS

Medium.—The medium used was Eagle's Basal Medium [7] supplemented with 10 per cent calf serum. Sufficient  $\text{NaHCO}_3$  was added so that the medium, upon equilibration to 37°C, reached a pH of 7.4. For prevention of microbial (including

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mycoplasma) contamination, 50  $\mu\text{g/ml}$  of Aureomycin (Lederle product no. 4691-96, intravenous) was used. The material, packaged in 500 mg amounts, was reconstituted, with agitation, in 50 ml of sterile distilled water at 37°C. Five ml aliquots of this stock concentrate were stored at -20°C. Each liter of warm medium was supplemented before use with 5 ml of stock concentrate. Mycoplasma determinations made over a three-year period on approximately 2000 cell cultures have revealed a total absence of mycoplasma where Aureomycin has been used. Subsequent growth of treated cultures in antibiotic-free medium for extended periods of time has also been proven negative for the presence of these microorganisms. Trypsin was prepared as previously described [14], pre-warmed to 37°C, and raised to pH 7.5 before use.

*Human diploid cell strains.*—Strains WI-26, IVI-38 and WI-44 were used. WI-26 was derived from male fetal human lung and WI-38 and WI-44 from female human fetal lung. All embryos were obtained from surgical abortions and were of approximately three months' gestation.

*Subcultivation of confluent cultures.*—The method of subcultivation was a modification of that previously described [14]. The medium from confluent cell sheets was removed and pre-warmed (37°C) trypsin solution (Difco 1:250) was added to each culture for 1 min. All except 1 or 2 ml of the trypsin was then decanted and the bottle culture allowed to stand at room temperature for about 30 min. A small amount of Eagle's medium was added and splashed over the loosened cell sheet. The suspension was then vigorously aspirated with a narrow-bore 5 or 10 ml pipette to obtain discrete single cells. Sufficient additional medium was added for the total volume of the suspension to cover twice the surface area from which it was obtained. This is referred to as a 2:1 split ratio. Cultures were incubated at 37°C.

Initiation of the new strains, chromosome analysis, preservation in liquid nitrogen and reconstitution were performed as previously described [12, 14, 40]. These strains have characteristics similar to others previously reported [14]. Chromosome analyses have shown the human diploid cell strains WI-26, WI-38 and WI-44 to be normal or classic diploid [30]. Preliminary studies on the human cell strains of adult lung origin also indicate classic diploidg [24].

## EXPERIMENTAL RESULTS

*Reconstitution of frozen cells.*—As previously shown, human fetal diploid cell strains, preserved at sub-zero temperatures and subsequently reconstituted, enter Phase III at a total number of passages (2:1 split ratio) of  $50 \pm 10$  [12, 14, 15]. This compares favorably with the passage level at which Phase III occurs in the original passage series of the strain which had never been frozen [12, 14, 15]. Further experiments with human diploid cell strains WI-26 and WI-38 have confirmed and extended these results. As indicated in Fig. 1, the average passage level at which mitoses ceased (Phase III) in 20 ampules of WI-26, reconstituted from various passage levels and preserved for periods of time up to 19 months, was 47 passages. The

related to such anomalies occurring in the cells of older animals, including man. The survival curves obtained with human diploid cell strains are comparable to "multiple-hit" or "multiple-target" curves obtained with other biological systems where an initial threshold dose is required before an exponential form of the curve is established. Whatever cell component(s) may be involved in the finite lifetime of human diploid cell strains, the ultimate accumulation of nondividing cells could be the result of accumulated damage to a single cellular target or to inactivation of many targets.

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