Cell Biology of Aging

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Despite the universality of biological aging, life scientists have paid scant attention to the phenomenon, a fact no doubt engendered by the apparent intractibility of the problem. Nevertheless, the relatively few biologists now in this field have uncovered leads that may provide some important insights into our understanding of the biology of aging.

There is little disagreement with the principle that, after reaching sexual maturity, individual members of a species eventually accumulate physiological decrements that lead to an increase in their likelihood of dying. In fact, for man the actuarial data, first analyzed by the English actuary Gompertz in 1825, reveal that the force of mortality doubles every seven years beyond the age of 30. That is, after maturity, the rate and probability of dying are exponential with increasing age (Gompertz 1825). Nevertheless, the decrements in a variety of human physiological functions, although subject to some individual variation, show a slow, nearly linear decline from the age of 30. The rate constants for this linear loss seem to occur at about 0.8 to 0.9% loss per year of the functional capacity present at age 30 (Strehler and Mildvan 1960).

The notion that aging occurs in animals that reach a fixed size after maturity is beyond dispute, but is the inevitability of the aging and death of individual cells composing that organism predetermined? A superficial consideration of this thought may provoke some incredulity since it is intuitively obvious that a dead or aging organism must consist of dead or aging cells. Nevertheless, whatever causes age changes and death in the whole organism undoubtedly does not produce similar changes, and at the same rate, in each cell composing that organism. If the rates of aging vary among organs, tissues, and their constituent cells, then the root causes of aging may occur as a consequence of decrements in some few cell types where the rate is fastest and the effects greatest. I will explore the notion that normal somatic cells are predestined to undergo irreversible functional decrements that can be interpreted to presage aging in the whole organism.

There are at least two ways in which this question has been put to the test. First, vertebrate cells have been grown and studied in cell culture, and second, eukaryotic cells containing specific markers allowing them to be distinguished from host cells have been serially transplanted in isogenic laboratory animals. The goals of such studies, as they pertain to the science of gerontology, have been directed toward answering this fundamental question: Can vertebrate cells, functioning and replicating under ideal conditions, escape from the inevitability of aging and death, which is universally characteristic of the whole animals from which they were derived?

BACKGROUND

In respect to those studies undertaken in cell culture, one investigation stands out as the classic response to this intriguing question. In the early part of this century Alexis Carrel, a noted cell culturist, described experiments purporting to show that the fibroblast cells derived from chick heart tissue could be cultured ad seriatum indefinitely. The culture was voluntarily terminated after 34 years (Ebeling 1913, Parker 1961). This experiment is important to gerontologists because it implied that, if cells released from in vivo control could divide and function normally for periods in excess of the lifespan of a species, then either the type of cells cultured play no role in the aging phenomenon or aging is the result of changes occurring at the supracellular level. That is, aging would be the result of decrements that occur only in organized tissue or whole organs as a result of the physiological interactions between those organized cell hierarchies. The inference would be that aging per se is not the result of events occurring at the cellular level.

In the years that followed Carrel's observations, support for his experimental results seemed to be forthcoming from the many laboratories in which it was observed that cultured cell populations, derived from many tissues of a variety of animal species and from man, had the striking ability to replicate apparently indefinitely. These cell populations, which arise spontaneously from cultures, number in the hundreds and are best known by the prototype cell lines HeLa (derived from a human cervical carcinoma in 1952) and L cells (derived from mouse mesenchyme in 1943). They continue to flourish even to this day in cell culture laboratories throughout the world. Nevertheless, what seemed to be incontrovertible evidence for the potential immortality of vertebrate cells soon fell to new insights and a preponderance of opposing evidence.

AGING UNDER GLASS

Of central importance to the question is whether the cell populations studied in vitro are composed of normal or abnormal cells. Clearly the aging of animals occurs in normal cell populations. If we are to equate the behavior of normal cells in vivo to similar cells in vitro, then the latter must be shown to be normal as well. For this reason the "immortal" cell lines described above, of which the HeLa and L cell populations are prototypes, must be excluded from consideration because they are composed of cells that are abnormal in one or more important properties. For example, all immortal cell lines vary in their chromosomal constitution; they do not reveal either the exact number or the precise morphology of chromosomes characteristic of the cells composing the tissue from which they

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originally descended. Many such cell lines give rise to tumors when inoculated into laboratory animals, and some reveal biochemical properties uncharacteristic of the cells composing the tissue of origin. The widespread use of these cell lines for a variety of research purposes in laboratories throughout the world is subject to the criticism that, in most cases, they are not characteristic of any cell type found in human or animal tissue. Much experimental data generated from the use of such cell populations cannot be extrapolated to apply to cells that characterize the animal species from which they originally descended. Consequently, the use of cell lines is questionable because such cells undoubtedly represent laboratory artifacts whose behavior may be unrelated to cells found in vivo.

This fundamental flaw in interpreting normal cell behavior in vitro can, in fact, be circumvented: Cell populations entirely typical of normal cells found in vivo can be cultured and, in respect to gerontological inquiry, the findings are profoundly different from the behavior of abnormal cell lines.

Some 15 years ago, Moorhead and I found that cultured normal human embryonic fibroblasts underwent a finite number of serial subcultivations or population doublings and then died (Hayflick and Moorhead 1961). We demonstrated that when such cells were grown under the most favorable conditions, death was inevitable after about 50 population doublings (the Phase III phenomenon). We also showed that the death of the normal cells was not due to some trivial explanation involving medium components or cultural conditions but that the death of cultured normal cells was an inherent property of the cells themselves (Hayflick 1965, Hayflick and Moorhead 1961). That observation has now been confirmed in hundreds of laboratories in which variations in medium components and cultural conditions have been as numerous as the laboratories themselves (see, for example, Castor et al. 1962, Cristofalo 1970, Goldstein et al. 1969, Macieira-Coelho 1970, Macieira-Coelho and Pontén 1969, Macieira-Coelho et al. 1966, Martin et al. 1970, Miles 1964, Robbins et al. 1970, Simons 1970).

Since normal diploid cell strains have a limited doubling potential in vitro, studies on any single strain would be severely curtailed were it not possible to preserve these cells at subzero temperatures for apparently indefinite periods of time. The reconstitution of frozen human fetal diploid cell strains has revealed that, regardless of the doubling level reached by the population at the time it is preserved, the total number of doublings that can be expected is about 50 when those made before and after preservation are combined (Hayflick 1965, Hayflick and Moorhead 1961). Storage of human diploid cell strains merely arrests the cells at a particular population doubling level but does not influence the total number of expected doublings.

As of this date, we have now reconstituted a total of 130 ampules of our human diploid cell strain WI-38, which was placed in liquid nitrogen storage 13 years ago. Since 1962 one ampule has been reconstituted approximately each month, and all have yielded cell populations that have undergone 50 ± 10 cumulative population doublings. This represents the longest period of time that viable normal human cells have been arrested at subzero temperatures (Hayflick 1972).

Since normal human embryo fibroblasts are able to undergo only a fixed number of reproductive cycles in vitro, we postulated that this observation might be interpreted as representing aging at the cellular level. Although we and others were skeptical of this interpretation at first, subsequent experimental data have tended to support the validity of this notion. However, before considering the newer developments in this field, it is necessary to reconsider Carrel's experiment, in which a presumptive normal chicken cell population was cultured for 34 years and then voluntarily terminated. The cells cultured by Carrel are presumed to have been normal simply because abnormal immortal cell lines have never been known to arise from chick tissue. In the years following Carrel's observation, and even in very recent times when more sophisticated cell culture techniques have been used, no one has been able to culture chick fibroblasts serially in a state of continuous, rapid proliferation beyond one year (see Table 1). Thus, there is serious doubt that the original interpretation of Carrel's work can be accepted because it has never been confirmed. We have proposed one explanation for Carrel's findings that was subsequently supported by personal communications from one of his laboratory technicians. The method of preparation of chick embryo extract, used as a source of nutrients for his cultures and prepared daily under conditions of low-speed centrifugation, allowed for the survival and introduction of new, viable fibroblasts into the so-called "immortal" culture at each feeding (Hayflick 1970, 1972).

One possible exception to the lack of confirmation of Carrel's study is the recent finding that chick embryo muscle explants have survived successive transplants in vitro up to 44 months on a collagen substrate but not on commonly used glass surfaces (Gey et al. 1974). However, because of the difficulty in determining population doublings in explant cultures, it is probable that this longevous culture represents a considerable amount of cell maintenance time and not necessarily an unusually large number of population doublings. Maintenance of slowly dividing cell populations can occur over much longer periods of time than will cultures of similar cells allowed to proliferate at their maximum rate. We and others have found this to be so when culturing normal human and chick cells in which cell replication was slowed by diminishing serum concentrations or reducing the

TABLE 1. The Phase III phenomenon as expressed in white leghorn chick embryo fibroblasts.

Number of different embryo cultures studied	Range of population doublings observed (average = 22)	Reference
6	15 - 25	Hay and Strehler (1967)
16	20 - 27	Pontén (1970)
8	30 - 35	Lima and Macieira-Coelho (1972)
2	16 - 20*	Harris (1957)
3	12 - 15	Volkmann and Morgan (1974)

*Chicken strain not given. Doublings not given but calculated from subcultivation ratios and inoculation density of 1 x 10^6 per T-60 flask.

incubation temperature (Hay and Strehler 1967, Dell'Orco et al. 1973, Goldstein and Singal 1974). The essential point is that, although such cultures may survive for longer periods of time, the maximum number of population doublings achieved is comparable to that for similar populations cultured under conditions allowing a maximum rate of replication. However, it still remains to be seen whether this longevous culture (Gey et al. 1974) ultimately terminates.

DONOR AGE VS. CELL DOUBLING POTENTIAL

Because cultured normal human cells derived from embryonic tissue have a finite proliferative capacity of about 50 population doublings and because this may represent cellular aging, it is important to determine the proliferative capacity of normal cells derived from human adults of varying ages. Our first report of such studies did, indeed, show a diminished proliferative capacity for cultured normal human adult fibroblasts in which 14 to 29 doublings occurred in cells derived from eight adult donors (Hayflick 1965). This compared to a range of 35 to 63 doublings found in cells cultured from 13 human embryos. Although it was clear that fibroblasts cultured from human adults as a group had a greatly diminished in vitro proliferative capacity compared with cells derived from embryos, we were unable to detect a direct correspondence between donor age and population doubling potential. If a precise relationship did exist between donor age and population doubling potential, then our inability to detect it might be attributed to the lack of precision by which population doublings were measured at that time.

Subsequent to these studies, reports by Martin et al. (1970) and Le Guilly et al. (1973) not only confirmed the principle we observed but extended it significantly. The former investigators cultured fibroblasts derived from biopsies taken from the upper arm of human donors ranging from fetal to 90 years of age. They found the regression coefficient, from the first to the ninth decade, to be -0.20 population doublings per year of life with a standard deviation of 0.05 and a correlation coefficient of -0.50. The regression coefficient was significantly different from zero ($p < 0.01$). Le Guilley et al. (1973), studying human liver cells, obtained results similar to these. Thus, it is now generally believed that there is an inverse relationship between the age of a human donor and the in vitro proliferative capacity of at least two cell types—fibroblasts derived from skin and lung and cells derived from liver.

Progeria and Werner's Syndrome

Progeria (Hutchinson-Gilford syndrome) is a human condition leading to a severe deceleration of growth in patients as young as nine years of age (Reichel et al. 1971). A very rare disease, it is thought by many to represent a model for precocious aging in which individuals, at the end of the first decade of life, manifest the physical signs of aging typical of their normal counterparts in the seventh decade of life. Werner's syndrome is like progeria in many ways although its salient manifestations occur in later years. The full clinical picture shows early greying and loss of hair, short stature, juvenile cataracts, proneness to diabetes, atherosclerosis and calcification of the blood vessels, osteoporosis, and a high incidence of malignancy (Epstein et al. 1966).

If Werner's syndrome and progeria are examples of accelerated aging, when does senescence of cultured fibroblasts taken from these patients occur? From 2 to 10 doublings were found to occur (Goldstein 1969, 1971, Martin et al. 1970), whereas normal values would be between 20 and 40. Others have reported decreased mitotic activity, DNA synthesis, and cloning efficiency of cultured progeria cells (Danes 1971, Nienhaus et al. 1971).

Possible Correlation between Population Doubling Potential of Cultured Normal Fibroblasts and Mean Maximum Species Lifespan

Although the data are fragmentary, it is interesting to explore the possibility that the population doubling potential of normal fibroblasts derived from the embryonic tissue of a variety of animal species might be proportional to the mean maximum lifespan for those species (see Table 2). Some of the data need confirmation, and many more exhaustive studies should be done. But if such a correlation is ultimately found to exist, a significant finding will have been made. It would then be possible to determine the mean maximum lifespan for any animal species simply by assessing the number of population doublings of which their cultured normal embryonic cells are capable. Results would include (a) more precise figures for the longevity of particular animal species; (b) the determination of species lifespans without observing animals for many years; and (c) predicting lifespans for hybrid animal species and for species whose records for longevity are scant or nonexistent.

In addition, it would be valuable to determine the longevity of cultured normal fibroblasts from a variety of animal species, cultured at intervals throughout their lifespan. That is, several animal species could be studied by obtaining tissue biopsies from the same tissue throughout the lifespan of the same animal. It is possible to undertake these studies on man as well. Such longitudinal studies might reveal a greater reliability upon population doubling measurements than is now possible.

TABLE 2. The finite lifetime of cultured normal embryonic human and animal fibroblasts.

Species	Range of population doublings for cultured normal embryo fibroblasts	Mean maximum lifespan (years)	Reference
Galapagos tortoise	90 - 125*	175 (?)	Goldstein (1974)
Man	40 - 60	110	Hayflick and Moorhead (1961)
Mink+	30 - 34	10	Porter (1974)‡
Chicken	12 - 35	30 (?)	(See Table 1)
Mouse	14 - 28	3.5	Rothfels et al. (1963) Todaro and Green (1963)

* Young donors, not embryos.
+Data from 20 embryos.
‡Personal communication, D. D. Porter, Pathology Department, University of California, Los Angeles.

THE FINITE LIFETIME OF NORMAL CELLS IN VIVO

If normal human embryonic cells grown in vitro have a finite lifetime of 50 ± 10 doublings, how does one account for the lifelong multiplication of bone marrow cells and intestinal and skin epithelium in vivo? Presumably these cell populations undergo far more than 50 population doublings during an individual's lifetime. There are several answers to this question, all of which are based on the number of cells produced by a primary cell population that undergoes the maximum 50 doublings. That number, as we reported some years ago, is 20 million metric tons of cells (Hayflick and Moorhead 1961), certainly a quantity sufficient to account for all cells generated during the lifetime of an individual.

In a more elegant consideration of this problem, Kay (1965) promoted consideration of the following explanation: Cells can divide in several ways, the two extremes of which are (a) tangential division, in which a stem cell multiplies to produce another stem cell and one differentiated cell, and (b) logarithmic division, in which—if all mitoses are synchronous—a single cell divides yielding a doubled number of cells at each division (similar to our hypothetical model for the ultimate yield of 20 million tons of WI-38 cells if all cells remain after 50 population doublings). Kay pointed out that a maintained cell output in such rapidly dividing tissue as bone marrow could be maintained by an asynchronous division of cells within the logarithmic model. The essential factor is a variation in the rate of primitive stem cell division so as to produce a continuous release of mature differentiated cells. The advantage of this system, called "clonal succession," compared to the tangential model would be a reduction in the number of cell generations required to yield a given population of mature cells, thus allowing a much closer adherence to the original genetic message (Kay 1965).

Only 54 population doublings would be required for a single cell to produce, by the logarithmic pattern, the total output of human erythrocytes and leukocytes during 60 years of life. This figure is surprisingly close to the 50 ± 10 doubling limit that we found for normal human fibroblasts grown in vitro. The tangential system would require about 12,000 doublings. Thus, the asynchronous logarithmic division model can account for all cells produced during an individual's lifetime because it would contain several generations of dormant ancestral cells lingering, for example, at the 25th population doubling and which would be successively promoted to form clones of maturing stem cells.

If all of the multitude of animal cell types were continually renewed, without loss of function or capacity for self-renewal, we would expect that the organs composed of such cells would function normally indefinitely and that their host would live forever. Unhappily, however, renewal cell populations do not occur in most tissues, and when they do, a proliferative finitude is often manifest. The important question then is: Is it possible to circumvent experimentally the aging and death of normal animal cells that result from the aging and death of the host by transferring marked cells to younger animals ad seriatum? If such experiments could be conducted, then an in vivo counterpart of in vitro experiments would be available, and we would predict that normal cells transplanted serially to proper inbred hosts would, like their in vitro counterparts, age and die. Such experiments would largely rule out those objections to in vitro findings that are based on the artificiality of cell replication in vitro. The question could be answered by serial orthotopic transplantation of normal somatic tissue to new, young, inbred hosts each time the recipient approaches old age. Under these conditions, do transplanted normal cells of age-chimeras proliferate indefinitely?

Data from seven different laboratories, in which mammary tissues (Daniel 1973, Daniel and Young 1971, Daniel et al. 1968), skin (Krohn 1962), hematopoietic cells (Cudkowicz et al. 1964, Ford et al. 1959, Harrison 1973, Siminovitch et al. 1964), and leukocytes (Williamson and Askonas 1972) were employed, indicate that normal cells, serially transplanted to isogenic hosts, do not survive indefinitely. Furthermore, the trauma of transplantation does not appear to influence the results (Krohn 1962). And finally, in heterochronic transplants, survival time is related to the age of the grafted tissue (Krohn 1962). It is well known that under similar conditions of tissue transplantation, cancer cell populations, like immortal cell lines, can be serially passed indefinitely (Daniel et al. 1975, Stewart et al. 1959, Till et al. 1964). The implications of this may be that acquisition of the potential for unlimited cell division or escape from senescent changes by mammalian cells in vitro or in vivo can only be achieved by cells that have acquired some or all the properties of cancer cells. Paradoxically, this leads to the conclusion that, in order for mammalian somatic cells to become biologically "immortal," they first must be induced to an abnormal or neoplastic state either in vivo or in vitro, whereupon they can then be subcultivated or transplanted indefinitely.

The study of single antibody-forming cell clones in vivo has shown that these cells are also capable of only a finite ability to replicate after serial transfer in vivo (Williamson 1972, Williamson and Askonas 1972). Harrison (1972, 1973), however, reported that when marrow cell transplants from young and old normal donors are made to a genetically anemic recipient mouse strain, the old as well as young transplants populate the recipients, curing the anemia. He further reported that such transplants to anemic mice can be made to function normally over a period of 73 months (Harrison 1973). After five successive transplantations, most expired, once again exhibiting the finitude of normal cell proliferation in vivo (Harrison 1975). The fact that this normal hematopoietic cell population produced red blood cells far beyond the normal lifespan of the mouse is in keeping with speculations that aging is not necessarily timed at the same rate in all tissues. Furthermore, aging may not be due to the loss of cell division capacity but is probably due to other functional decrements that are known to occur prior to the loss of ability to replicate. The loss of capacity to divide is presumably an extreme limit, capable only of demonstration in vitro and by serial transplantation in vivo.

ORGAN CLOCKS?

Is it possible that a limit on cell proliferation or function in some strategic organ could orchestrate the entire phenomenon of senescence? Burnet (1970, 1971, 1974) speculated that, if this is so, the most likely organ is the thymus and its dependent tissues. Burnet reasoned that aging is largely the result of somatic mutations which are mediated by autoimmune processes and

which are influenced by progressive weakening of the function of immunological surveillance. He further argued that weakening of immunological surveillance may be related to weakness of the thymus-dependent immune system. He concluded that the thymus and its dependent tissues are subject to a proliferative limit similar to the Phase III phenomenon or senescence in vitro described by us for human cells. Whether the role played by the thymus and its dependent tissues as the pacemaker in senescence is important or not still remains to be established.

LATENT PERIOD OF EXPLANTED CELLS VS. DONOR AGE

Since 1925 it has been known that the time elapsing between introduction of embryo tissue in culture and cell migration from the explant increases with embryo age (Cohn and Murray 1925, Suzuki 1926). More recently, investigators have shown again that the time necessary for the first cells to emigrate from rat tissue explants grown in vitro (the latent period) correlates inversely with the age of the donor (Michl et al. 1968, Soukupová and Holečková 1964, Soukupová et al. 1965, 1968). Similar observations have been reported for chicken cells (Chaytor 1962, Lefford 1964), and a linear increase in the latent period has been found to occur in explants cultured from human donors ranging in age from newborn to 80 years (Soukupová and Hnêvkovský 1972, Waters and Walford 1970).

DECREMENTS OCCURRING IN CULTURED NORMAL HUMAN CELLS

The likelihood that animals age because one or more important cell populations lose their proliferative capacity is unlikely. It is more probable that, as we have shown, normal cells have a finite capacity for replication and that this finite limit is rarely, if ever, reached by cells in vivo but is, of course, demonstrable in vitro. We would, therefore, suggest that other functional losses that occur in cells prior to the cessation of division capacity produce physiological decrements in animals much before their normal cells have reached their maximum proliferative capacity. Indeed, we are now becoming more aware of many functional changes taking place in normal human cells grown in vitro and expressed well before they lose their capacity to replicate (see Table 3). It is more likely that these changes, which herald the approaching loss of division capacity, play the central role in the expression of aging and result in the death of the individual animal well before its cells fail to divide.

To be sure, those several classes of cells that are incapable of division in mature animals (e.g., neurons, muscle cells) may play a greater role in the expression of age changes than those cell classes in which division commonly occurs. It is important, therefore, to indicate that the cessation of mitotic activity is only one functional decrement whose genetic basis may be similar to those functional decrements known to occur in nondividing cells. It is supposed, therefore, that the same kind of gene action resulting in physiological decrements in aging nondividing cells also occurs in aging cells that can divide. It is not our contention, therefore, that age changes result necessarily from losses in cell division capacity but simply in loss of function in any class of cells. That function might be measured as reduced division capacity or any number of the myriad functional decrements characteristic of aging cells. The genetic changes leading to these decrements is postulated to be the common denominator, so that the measurement of loss of population doubling potential in vitro may have the same basis as the

TABLE 3. Metabolic and cell parameters that increase, decrease, or do not change as normal human fibroblasts age in vitro.*

Increase	Decrease	Do Not Change
Glycogen content	Glycolytic enzymes	Glycolysis
Lipid content	Pentose phosphate shunt	Permeability to glucose
Lipid synthesis	Mucopolysaccharide synthesis	Respiration
Protein content	Transaminases	Respiratory enzymes
RNA content	Collagen synthesis	Permeability to amino acids
RNA turnover	DNA content	Glutamic dehydrogenase
Lysosomes and lysosomal enzymes	Nucleic acid synthesis	Nucleohistone content
Heat lability of G6PD and 6 phospho-gluconate dehydrogenase	Collagen synthesis and collagenolytic activity	Alkaline phosphatase
Proportion of RNA and histone in chromatin	Lactic dehydrogenase isoenzyme pattern	Soluble RNAase, soluble DNAase, soluble seryl T-RNA synthetase, soluble and chromatin-associated DNA polymerase
Activity of "chromatin-associated enzymes" (RNAase, DNAase, protease, nucleoside triphosphatase, DPN pyrophosphorylase)	Ribosomal RNA content	Mean temperature of denaturation of DNA and chromatin
5' MNase activity	Incorporation of tritiated thymidine	Numbers of mitochondria
Esterase activity	RNA synthesizing activity of chromatin	HL-A specificities (mass cultures)
Acid phosphatase band 3	Alkaline phosphatase	Virus susceptibility
β-glucuronidase activity	Specific activity of lactic dehydrogenase	Poliovirus and herpesvirus titer, mutation rate and protein chemistry
Membrane associated ATPase activity	Rate of histone acetylation	Cell viability at subzero temperatures
Cell size and volume	Numbers of cells in proliferating pool	Diploidy (only in Phase III)
Number and size of lysosomes	Population doubling potential as a function of donor age	Histone/DNA ratio
Number of residual bodies	Proportion of mitochrondria with completely transverse cristae	
Cytoplasmic microfibrils, constricted and "empty"	HL-A specificities (cloned cells)	
Endoplasmic reticulum	Adherence to polymerizing fibrin and influence on fibrin retraction	
Cyclic AMP level/mg protein	Cyclic AMP level (molar values)	

*See Hayflick, L. 1976. The cellular basis for biological aging. In C. Finch and L. Hayflick, eds. *The Handbook of the Biology of Aging.* Van Nostrand Reinhold, New York, in press.

loss of other cell functions characteristic of nondividing cells. It follows, therefore, that an understanding of the mechanism by which cultured normal cells lose their capacity to replicate could provide insights into the causes of decrements in other functional properties which are characteristic of nondividing cells and which may be even more direct causes of biological aging.

PROTOZOA AND CULTURED CELLS COMPARED

The concept that normal dividing cells never have an opportunity to age because they periodically yield new daughter cells before age changes take place bears reconsideration in light of the finite lifetime of normal cultured vertebrate cells. The question here is whether the product of a cell division is always a pair of daughter cells, each having the same age status. This notion makes the very important assumption that dividing cells yield daughters that are "separate but equal." There is little, if any, evidence that bears on this important point in cultured mammalian cells, and there are no data that oppose the possibility that one daughter cell may receive one or more old organelles and the other only new organelles. In addition, information-containing molecules with different error levels may be unequally distributed. Thus, to assume that each daughter mammalian cell is equivalent in age status may be spurious. Some recent cell culture studies bear on this question.

When individual normal human cells are isolated from young (low population doubling level) mass populations, only about 50% of the developing clones are capable of more than eight population doublings. This percentage is further reduced when clones are isolated from older mass cultures. Nevertheless, the highest doubling potential observed in clones isolated from any culture was about the same as the doubling potential of the mass culture from which the cells were taken (Smith and Hayflick 1974). Genealogical trees have been described recently for several clones of the normal human embryonic cell strain WI-38, derived at different population doubling levels of the mass cultures. Using phase-contrast cinemicrophotography, a gradual lengthening of average interdivision time with successive generations has been observed in all genealogies regardless of the doubling level of the mass population from which the clones were derived. Cell pedigrees derived from older mass cultures generally exhibited greater variation in interdivision time; they were less synchronous; and their individual cells were found to be less motile (Absher et al. 1974).

Since populations of vertebrate cells in culture have an independent existence and can be manipulated like microorganisms, it is useful to compare the replicative capacity of both. Studies with protozoa do not unequivocally demonstrate the "immortality" of all unicellular organisms or that the outcome of a protozoan cell division is a pair of rejuvenated infant cells instead of mother and daughter cells of different seniorities.

For example, it has been shown (Danielli and Muggleton 1959, Muggleton and Danielli 1968) that amoebae will multiply indefinitely if kept on a food supply permitting logarithmic vegetative multiplication, but if kept on a limited food supply and then transferred to the optimum diet they have a variable lifespan. This span (from 30 days to 30 weeks) is dependent on the conditions of exposure to the deficient diet. Since it is likely that amoebae in the natural state do not always have an optimum food supply, their usual fate is probably one in which senescence occurs.

A number of other investigators have also concluded that many clones of protozoa do not propagate asexually indefinitely. Such observations have been made with *Uroleptus* (Calkins 1919), *Paramecium* (Nanney 1974, Smith-Sonneborn et al. 1974, Sonneborn 1938, 1954), and with an ascomycete (Rizet 1953). Other clones of protozoa apparently do reproduce asexually and indefinitely. The extensive studies of Jennings (1945) bear directly on this question and on clonal rejuvenation by conjugation. It was found that the viability of the progeny *Paramecium bursaria* produced by conjugation varies greatly even when the conjugants are young and that a high proportion of ex-conjugants normally die. The rate appears to be highest in those clones that are most closely related. Fifty-three percent of ex-conjugants die before undergoing five cell divisions, and 30% die without dividing at all. Conjugation produced nonviable clones, clones of limited survival, and some vigorous clones apparently capable of unlimited asexual reproduction. It is suggested that it is from these last clones that laboratory cultures are normally obtained. Jennings concluded that death is not a consequence of multicellularity and that it occurs on a vast scale in the protozoa "from causes which are intrinsic to the organism." He claimed that "most if not all clones ultimately die if they do not undergo some form of sexual reproduction. . . . Rejuvenation through sexual reproduction is a fact. . .yet conjugation produces, in addition to rejuvenated clones, vast numbers of weak, pathological or abnormal clones whose predestined fate is early death." He added that some very vigorous clones may be produced "that may continue vegetatively for an indefinite period, without decline or death."

It is, however, interesting to note that a similar kind of clonal variation occurs with human diploid cell strains. Some isolated single embryonic cells give rise to progeny capable of about 50 doublings (Hayflick 1965), and others yield colonies composed of varying numbers of cells or no clones at all (Merz and Ross 1969, Smith and Hayflick 1974). However, the uncloned or wild embryonic cell population always undergoes about 50 doublings.

Of equal importance in this context is the intriguing possibility that cultures of bacteria in which exchange of genetic information between organisms is prevented might also reveal a senescence phenomenon. In order to test this hypothesis, daughter microorganisms would have to be kept isolated from other members of the culture as they replicate serially. Is it possible that immortal cell populations can only occur after periodic exchange of genetic information which somehow recycles the genetic clock?

LOCATION OF THE "CLOCK"

In an effort to locate and understand the mechanism controlling the finite replicative capacity of cultured normal cells, we have recently completed a series of experiments that bear on this important question. When cultured cells are treated with cytochalasin B and then centrifuged, it is possible to obtain millions of the anucleate cells, cytoplasts (Prescott et al. 1972, Wright 1973, Wright and Hayflick 1972, 1973, 1975a). Cytoplasts remain viable for several days, allowing sufficient time to fuse them with inactivated Sendai virus to whole cells, producing hetero-

plasmons (as distinguished from heterokaryons, which result from fusions made between whole cells only). Using these techniques, it is possible to determine whether the "clock" which dictates a cell's replicative capacity is located in the nucleus or in the cytoplasm. This question is approached by fusing cytoplasts derived from young cells to whole old cells, and the reverse. By determining the remaining number of population doublings traversed by these heteroplasmons, it is possible to determine the influence of young or old human cytoplasm on opposite-aged whole human cells. Data derived from such studies have been interpreted to mean that the "clock" is located in the nucleus (Wright and Hayflick 1975b, c). The more recent development of techniques by which viable eukaryotic nuclei can be isolated and themselves reinserted into cytoplasts facilitates an even more direct answer to this question because nuclei from old cells can now be inserted into young cytoplasm and the reverse (Ege and Ringertz 1974, Ege et al. 1974).

CAN CELL DEATH BE NORMAL?

The death of cells, as well as the destruction of tissues and organs, is, indeed, a normal part of morphogenic or developmental sequences in animals. It is the common method of eliminating organs and tissues that are useful only in the larval or embryonic stages of many animals; for example, the pronephros and mesonephros of higher vertebrates, the tail and gills of tadpoles, larval insect organs, and in many cases, the thymus. The degeneration of cells is an important part of development, and it is a widespread occurrence in mammalian cells (Saunders 1966). During the development of vertebrate limbs, cell death and cell resorption model not only digits but also thigh and upper arm contours (Whitten 1969). In the limbs of vertebrates, the death clocks function on schedule even when heterochronic tissue grafts are made (Saunders and Fallon 1966).

Thus, cell death is an intrinsic part of development. To the casual observer, the contemplation that normal human embryo cells grown in vitro will die after dividing vigorously for 50 population doublings is difficult to accept. Should we apply the same logic that accepts the aging and death of whole animals and their cells as being universal and inevitable, when the same phenomenon is observed in cells derived from these animals after they are grown in vitro?

Proliferating and Nonproliferating Cells

Most gerontologists agree that there is probably no single cause of aging. A phenomenon, which probably comes closer to a unifying theory, involves those concepts based on genetic instability as a cause of aging (Hayflick 1975). It also seems that the genetic contribution to the aging process is foremost in the determination of a lifespan that is characteristic of each species. This is so because the range of variation in the maximum lifespan among different species is obviously much greater than the range of individual lifespans within the species.

One fundamental problem in relating genetic processes to aging involves the attempt to separate the genetic basis for differentiation from a possible genetic basis for aging (the concept of "first we ripen, and then we rot"). In metazoan aging, we are concerned essentially with three types of cell populations: (a) fixed postmitotics, which are represented by neurons and muscle cells and are essentially unable to divide; (b) slowly dividing cells, such as those found in the liver; and (c) intermitotic cells that divide at a faster rate, that is, fibroblasts and blast cells generally.

There are essentially three types of proliferative cell populations that can be used to illustrate the processes by which the last two categories of dividing cells replicate. The first would be a population whose numbers increase with time, such as embryonic tissue or tumor tissue (the latter case would exemplify antisocial behavior). The second type of proliferation would be a steady-state renewal system, in which the rate of cell death equals the rate of cell birth, such as the hair follicles in the skin. Finally, there is a population that is decreasing in number. This situation is perhaps programmed as a part of differentiation; for example, the massive destruction of cells during vertebrate embryogenesis or in the thymus.

Genetic instability as a cause of age changes might include the progressive accumulation of faulty copying in dividing cells or the accumulation of errors in information-containing molecules. Another possibility involves the cumulative effects of cross linking in information-carrying molecules. Cross linkages occurring between proteins and DNA are known, and this concept of permanent gene repression may be responsible for the manifestations of aging, where repressors are irreversibly bound to structural genes.

Either the progressive accumulation of errors in the function of fixed postmitotics or actively dividing cells could act as a clock. This would initiate secondary types of mischief, which would ultimately be manifest as biological aging as we know it. Thus, aging could be a special case of morphogenesis: Cells may be programmed simply to run out of program.

Functional and Mitotic Failure

We may call the lapse of time during which these results become manifest as the "mean time to failure." The concept of mean time to failure has a precise relevance to the deterioration of mechanical as well as biological systems, and it can be simply illustrated by considering the mean time to failure of, for example, automobiles. The mean time to failure may be five to six years, which may be extended or decreased by the competence of repair processes. Barring total replacement of all vital elements, deterioration is inevitable. Similarly, the progressive decrease in the adequacy of the cellular transcription mechanism may ultimately result in an error catastrophe in which cell function or cell division is impaired or wrongly directed (Orgel 1973).

By virtue of the fact that biological activities (and repair mechanisms) are imperfect, we are led to the conclusion that the ultimate death of a cell, or loss or misdirection of functionality, could be a programmed event having a mean time to failure. Just as mechanical systems of different purpose have different mean times to failure, it is proposed that such differences have their counterpart in informational molecules. Consequently, the mean time to failure may be ultimately applicable to a single cell, clone, tissue, organ, or the intact animal itself. It is proposed that the genetic mechanism simply runs out of accurate program, which results in a mean time to failure of all the dependent biological systems. Thus, the existence of different average lifespans for each animal species and among the cells, tissues, and organs of different animals may be the manifestation of the evolution of more perfect repair mechanisms in those biological systems of greater longevity.

In what way can we fit this concept

to account for those biological systems that seemingly have escaped from the inevitability of aging? Specifically, we must consider the continuity of the germ plasm and continuously propagable or transplantable tumor cell populations. Many cancer cell populations can replicate indefinitely in vitro and in vivo. Is it possible that cancer cells, unlike normal cells, can exchange genetic information, thereby ensuring their apparent immortality? Gametes do not have an unlimited propensity to multiply unless they unite to form a zygote. Thus, the exchange of genetic material may serve to reprogram or to reset a more perfect biological clock. By this mechanism, species survival is guaranteed, but the individual animal is ultimately programmed to failure.

ACKNOWLEDGMENT

Studies reported from our laboratories are supported by Grant HD04004 from the National Institute of Child Health and Human Development, United States Public Health Service.

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