obtained from this source varied in resistance to *M. sexta*. Resistant plant material used in our study consisted of a clone, propagated by cuttings, obtained from a single plant of *L. hirsutum* f. *glabratum* (plant 134417) selected as highly resistant to *M. sexta*. Potted plants were grown in a greenhouse under natural light between April and October and were fertilized as needed to maintain vigorous growth.

8. One hundred percent mortality of both species occurred when first instar larvae were confined for 2 hours on filter paper treated with crude trichome exudate (210 $\mu g/cm^2$) in a covered petri dish. As controls larvae were confined on filter paper treated with 1 ml of chloroform. No mortality was observed among controls.
9. Bio-Rad silicic acid (100 to 200 mesh) at a ratio of 100:1 (by weight) relative to the sample weight. The column (2.5 by 27 cm) was eluted with a step gradient from 100 percent heptane to 100 percent chloroform. Each collected fraction corresponded to a 77-ml addition of solvent. All fractions were combined for further purification.
10. The column (2 by 26 cm) was eluted with a step gradient from 100 percent heptane to 100 percent chloroform in 10 percent increments. Each fraction from the column corresponded to a 55-ml addition of solvent.
11. Only fractions 3 and 4, corresponding to 20 and 30 percent chloroform, showed activity. Bioassays were conducted by confining 20 newly hatched, first-instar *M. sexta* larvae on a filter paper disk (5.5 cm in diameter) treated with 5 mg of each fraction dissolved in 1 ml of chloroform. The chloroform was evaporated prior to addition of the larvae. Larval mortality was recorded after 6 hours. As controls, larvae were confined in an identical manner on filter paper previously treated with 1 ml of chloroform. No mortality was observed among controls.
12. 2-Tridecanone has been identified as one of many essential oils present in *L. hirsutum* f. *glabratum* (*4*), but no function was ascribed to it.
13. Pfaltz & Bauer, Inc., 375 Fairfield Avenue, Stamford, Conn.).
14. Bioassays were done as in (*11*), except that after 6 hours on treated filter paper larvae were transferred to *L. esculentum* foliage for 18 hours, after which mortality was recorded. The crude chlorofrom extract contained an average of 51 percent 2-tridecanone.
15. As bioassayed in (*11*), 63 μg per square centimeter of surface produced 100 percent mortality in both species.
16. The commercial *L. esculentum* cultivar 'Manapal.' Plants were grown in the Southeastern Plant Environment Laboratory at North Carolina State University, under 9 hours of photosynthetically active radiation from a combination of cool white fluorescent and incandescent lamps providing an illuminance of 451 hectolux at 95 cm from the lamps. Days were kept effectively long by interrupting the dark period from 11:00 p.m. to 2:00 a.m. with 12 W/m^2 of photomorphogenic radiation (41 hectolux) from incandescent lamps. Temperatures from day to night were 26° to 22°C, respectively. Extracts were prepared by steeping foliage in chloroform for 1 hour. The extract was then passed through Whatman phase separating filter paper (three chloroform rinses) onto Na_2SO_4 for drying and subsequently filtered to remove the Na_2SO_4 (Whatman No. 2).
17. Varian 3700 gas chromatograph with a flame ionization detector and a Varian CDS-111 processor.
18. Based upon eight foliage samples ($\bar{X}$, 360 mg fresh weight) *L. hirsutum* f. *glabratum* and ten samples ($\bar{X}$, 365 mg fresh weight) *L. esculentum*.
19. Trichome exudate was collected by brushing the abaxial surface of each of 100 *L. hirsutum* f. *glabratum* leaflets with a camel's hair brush and thoroughly rinsing the brush in chloroform. The resulting solution was analyzed for 2-tridecanone content by GC.
20. Based on analysis of extract from 100 leaflets collected at the same time as the trichome exudate and prepared as in (*16*).
21. Most of the insect pests that damage the fruit spend a portion of their life feeding on the foliage, for example, *H. zea*, *M. sexta*, and *Spodoptera exigua* (Hubner). *Lycopersicon hirsutum* appears in the pedigree of some newly released tomato cultivars [J. R. Baggett and W. A. Frazier, *HortScience* **13**, 598 (1978); *ibid.*, p. 599].
22. B. C. Campbell and S. S. Duffey, *Science* **205**, 700 (1979).
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Cellular Senescence in a Cloned Strain of Bovine Fetal Aortic Endothelial Cells

Abstract. *The life-span in vitro and other proliferative characteristics of a strain of endothelial cells cloned from the aorta of a fetal calf were examined. Cultures of these cells had a replicative life-span of approximately 80 cumulative population doublings. Growth rates in the logarithmic phase and plateau densities decreased as the cumulative population-doubling level increased. After approximately 65 percent of the life-span of a culture was completed, the percentage of cells that incorporated [^{3}H]thymidine during a 24-hour labeling period began to decrease rapidly. The cells expressed factor VIII antigen and their intercellular borders were stainable with silver nitrate throughout the life-span of each culture. Average cellular attachment size increased more than threefold between cumulative population-doubling levels 41 and 80. The facility with which cloned strains of endothelial cells can be isolated should encourage further exploitation of this important cell culture model.*

The vascular endothelium occupies a histologically unique and strategic location, is critical in normal physiological functioning, and figures importantly in such age-related diseases as atherosclerosis and neoplasia (*1*–*4*). Recent improvements in culture techniques have yielded endothelial cultures that retain many differentiated properties of the endothelium in vivo (*1*, *3*, *4*). The possibility that primary age-associated cellular changes take place in the endothelium in vivo makes the study of cellular senescence in cultured endothelial cells particularly relevant (*5*). Cellular senescence has been intensively investigated in human and chick fibroblast-like diploid cells that have a finite life-span in vitro

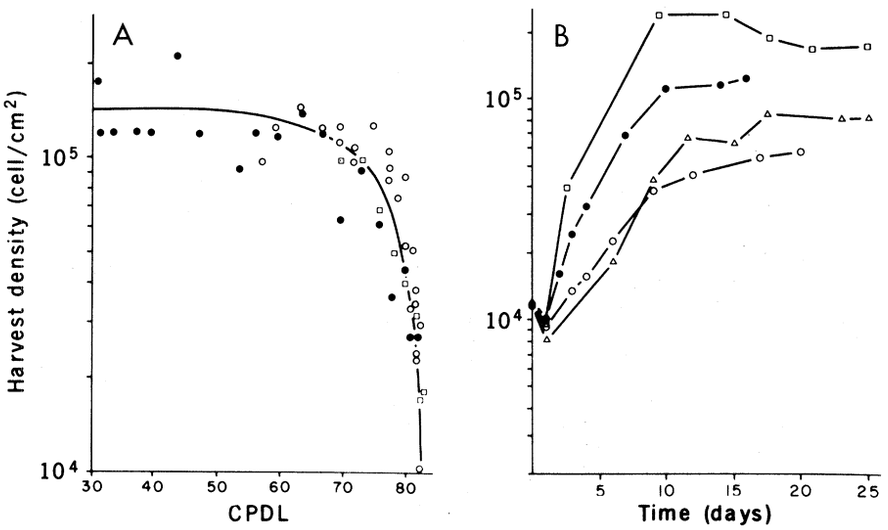

Fig. 1. (A) Cell density at subculture versus CPDL for endothelial cell clone BFA-1c. Data were obtained from (○) individual A (inoculation density, 2.5×10^4 cell/cm²); (●) individual B (1.0×10^4 cell/cm²); and (□) individual C (1.0×10^4 cell/cm²). The number of population doublings (PD's) that occurred in each subculture was calculated by using the formula: PD = $\log_2$ [cell density at subculture/(cell density at inoculation × attachment efficiency)], where attachment efficiency was the number of cells attached to the flask 24 hours after inoculation expressed as a fraction of the total number of cells inoculated (it varied from 0.70 to 0.95). The CPDL at any time, therefore, was the sum of all previously determined PD's. Weekly subculture cell density data could not be obtained until CPDL 30, because approximately 30 PD's occurred during the cloning procedure. Subcultivation was continued until inoculation cell density did not double after 2 weeks with weekly refeeding. The BFA-1c cells were frozen and stored in liquid nitrogen for various periods of time; when thawed, these cells proliferated similarly to cells that had not been frozen. All cultures remained free of detectable *Mycoplasma* infection, as demonstrated by agar plate cultivation, fluorescent staining for cytoplasmic DNA, and immunofluorescent staining for *M. hyorhinis* antigen (*28*). (B) Growth curves as a function of the CPDL for endothelial cell clone BFA-1c. Changes in proliferative rates and plateau densities associated with cellular senescence were monitored by comparing growth curves of cell density with time for cells taken from cultures at different CPDL's. Stock 75-cm² cultures at the desired CPDL were trypsinized and the cells were inoculated into numbered 25-cm² flasks at densities of 1.0×10^4 to 1.2×10^4 cell/cm² (8 ml of medium per flask). Flasks were incubated under standard conditions and given fresh culture medium every 6 or 7 days. At various times after inoculation, duplicate cultures were selected according to a random number table and counted with a Coulter counter to determine density. Replicate cell density determinations agreed to within 10 percent. The CPDL's studied were 55 (□), 59 (●), 70 (△), and 79 (○).

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(*6–9*). However, the use of these cells in culture has been complicated because the type of cell of origin in vivo is uncertain and few specialized functions are retained in vitro. We describe here our investigations of a cloned strain of bovine fetal aortic endothelial cells that has a finite life-span and retains characteristically endothelial properties.

In general, the primary isolation of endothelial cells from vascular tissue yields cultures that are contaminated by small numbers of smooth muscle cells and perhaps vascular fibroblasts that, upon subcultivation, eventually overgrow the endothelial cells (*1, 4*). To avoid this, we isolated clones and verified their endothelial identity immunologically, histochemically, and morphologically. The primary endothelial cell culture was obtained (*5*) from the thoracic aorta of a male fetal calf in the sixth month of gestation. Collagenase treatment of the vessel's luminal surface (*4*), followed by gentle scraping with a policeman, was used to dislodge the endothelial cells, which were suspended in Eagle's minimal essential medium (MEM) (*10*) plus 10 percent fetal calf serum (FCS) and inoculated into a 25-cm^2 tissue culture flask. Clones were derived from a secondary culture by inoculating cells (suspended in the same nutrient medium) into 2-cm^2 tissue culture wells at a density of one cell per well. Although cloning efficiencies were low (~ 1 percent) under these conditions, several clones exhibiting the typical "cobblestone" endothelial morphology described by others (*4*) were obtained, and one, designated BFA-1c, was used for these studies.

The proliferative life-span of BFA-1c cells was determined by subcultivating the cultures at weekly intervals by trypsinization (0.25 percent trypsin and 0.09 percent EDTA in phosphate-buffered saline), determination of population densities with a Coulter counter, and inoculation into 75-cm^2 plastic tissue culture flasks containing 40 ml of Ham's F-12 medium (*11*) plus 20 percent FCS without antibiotics. Figure 1A shows the relation between the cell density at the time of subculture (usually after 1 week's growth) and the cumulative population-doubling level (CPDL) of the culture. During most of the life-span of strain BFA-1c [phase II in Hayflick's terminology (*7*)], weekly cell densities were 1.2×10^5 to 2.3×10^5 cell/cm^2. After about 70 population doublings (PD's), a rapid decrease in subculture cell density occurred (phase III). Cultures were considered senescent when the inoculation cell density did not double after 2 weeks with weekly refeeding. By this definition, the proliferative life-span of BFA-1c was approximately 80 CPDL. To date, weekly refeeding of senescent BFA-1c cultures has not resulted in the reappearance of rapidly proliferating cells. The proliferative life-spans of human diploid fibroblast cell lines maintained under similar conditions in this laboratory resembled those determined previously (*6–8*). Figure 1B shows the effects of serial subcultivation on growth rate and plateau density of BFA-1c cells. As the CPDL of the culture increased, a small but progressive decrease in the initial growth rate and a significant drop in the plateau density were noted. Similar results were obtained for cultures of a strain cloned from a different fetal calf;

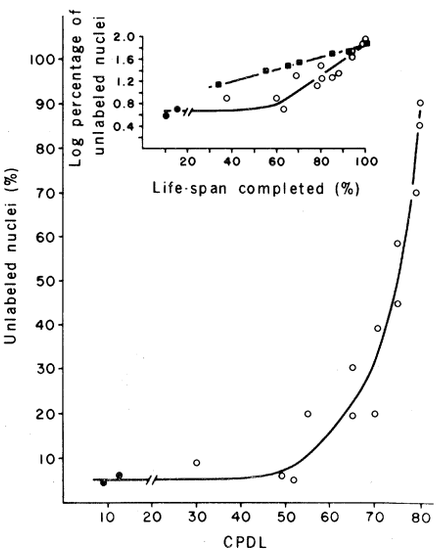

Fig. 2. Percentage of unlabeled nuclei versus CPDL for endothelial cell clone BFA-1c. Cells from stock cultures at different CPDL's were inoculated at densities of 1.1×10^4 to 1.2×10^4 cell/cm^2 into petri dishes containing glass cover slips. Cultures were incubated for 24 hours in 5 ml of medium; then [^{3}H]-thymidine (specific activity, 2 Ci/mmole) was added (final concentration, 0.1 μCi/ml) and the cultures were incubated for another 24 hours. Cover slips were removed, washed three times in phosphate-buffered saline, fixed in methanol, and autoradiographed according to a standard protocol (*14*). Slides were stained with Papanicolaou hematoxylin and examined with a Zeiss compound microscope (×500) to determine the percentage of cells with nuclei containing grains above background (five or more silver grains per nucleus). At least 400 cells in randomly selected fields were counted for each cover slip. Each symbol represents data from a single cover slip: (○) data for strain BFA-1c; (●) data for uncloned endothelial cultures at low CPDL. Inset: Data for bovine endothelial cells (○ and ●) and data for WI-38 human diploid fibroblasts (■) taken from the results of Cristofalo and Sharf (*14*), replotted, and expressed as a function of the logarithm of the percentage of unlabeled nuclei versus the percentage of life-span completed.

phase III cultures, inoculated at a density of 10^4 cell/cm^2, reached only 2×10^4 cell/cm^2 (one PD) after 11 days.

Karyotypic analyses of BFA-1c cultures with Giemsa-banding techniques gave results that were consistent with those of other cell systems that exhibit senescence in vitro (*12*). A normal bovine male karyotype (modal chromosome number, 60) (*13*) with a low level of polyploidy (7 percent) was found in cultures at CPDL 31; a similar level of polyploidy (11 percent) was detected in an uncloned culture from a different fetal calf at CPDL 5. By CPDL 43, chromosomal translocations were detected in 12 percent of the BFA-1c cells examined, and this value increased to 20 percent by CPDL 66. As in other models of cellular senescence in vitro, this increase in chromosomal aberrations accompanied the loss of cellular proliferative capacity (*12*).

In human diploid fibroblasts, such as WI-38 cells, the progressive decline in cell proliferation as the cultures senesce is a reflection of a progressive decrease in the fraction of rapidly dividing cells (*14, 15*). However, unlike WI-38 cells, for which there is a linear relation between the logarithm of the percentage of noncycling cells and the percentage of life-span completed (*14*), the BFA-1c strain had a fairly constant low percentage of noncycling cells until about 65 percent of its life-span was completed, after which the proportion of these cells increased dramatically (Fig. 2 and inset). This difference in culture growth kinetics may have been due to the difference between an uncloned culture (WI-38, comprised of a great number of "clones" with different life-spans) and a cloned culture (BFA-1c), or it may have reflected differences in the tissue (lung versus endothelium) or species (human versus bovine) of origin.

Three characteristics identified the BFA-1c cells as endothelial. The first was the expression by the cultures of factor VIII antigen throughout their life-spans. Although factor VIII antigen is not antihemophilic, it can be detected in endothelial cells in vivo and in vitro but not in vascular smooth muscle cells or fibroblasts (*16*). Factor VIII antigen was detected by indirect immunofluorescence with rabbit antibody to bovine factor VIII antigen at $1:10^4$ dilution and with goat antibody to rabbit immunoglobulin G–fluorescein isothiocyanate conjugate at 1:40 dilution (*16*). The conjugate was first absorbed against acetone-treated BFA-1c cells to reduce nonspecific fluorescence. Cells containing factor VIII antigen exhibited bright

green-yellow fluorescent granules that were absent in control cells stained with serum from unimmunized rabbits at $1:10^3$ dilution. The second endothelial characteristic of the BFA-1c cells was their absorption of silver nitrate stain at cell-cell borders that formed black "cement" lines in vivo and in vitro (*17, 18*) throughout the culture life-span. We measured more than 200 cells with a calibrated ocular grid and found that the average cell attachment area increased from approximately 620 μm^2 at CPDL 41 to 2000 μm^2 at CPDL 80. A similar increase in size occurs in human diploid fibroblasts as they senesce in vitro (*19*). Even though cell attachment area increased more than threefold, the typical endothelial morphology (close apposition of cell borders) was maintained. The third identifying characteristic was that the cultures were not stimulated to overgrow monolayer densities by repeated refeeding with fresh medium and serum [(*5, 20*) and Fig. 1B]. Like others (*21, 22*), however, we observed the undergrowth or "sprouting" peculiar to some endothelial cultures: islands of factor VIII antigen-positive cells appeared beneath the intact cell sheet. In addition, we observed that these sprouting cells were less obvious with increasing CPDL and decreased in number if the culture was refed and held at confluency beyond 2 to 3 weeks. Because a dilution cloning procedure was used to isolate strain BFA-1c, it is conceivable, but unlikely, that it arose from more than one cell. However, on the basis of the above criteria, there is no question that the BFA-1c cultures were comprised of endothelial cells throughout their life-spans. We extended our studies to include 15 endothelial cell strains definitely known to be derived from single cells; experiments with four such clones yielded data in close agreement with those presented here for BFA-1c cells.

We have thus demonstrated, in a cloned strain of bovine fetal aortic endothelial cells, a finite life-span in vitro of approximately 80 CPDL. Our documentation of a finite life-span in cloned strains is in agreement with more limited data from other laboratories (*23, 24*), our data on the finite life-span of mass cultures of bovine endothelial cells, and the observation of a finite life-span of cultured bovine endothelial cells selected by means of a [^{3}H]thymidine labeling technique (*21*). A number of reports on human umbilical vein endothelial cells and smooth muscle cells isolated from human vascular medial tissue also indicate that these cultures have a finite life-span (*4, 13, 25*). However, Gospodarowicz and co-workers (*22*) reported (i) that the life-span of human endothelial cell cultures was extended to at least 100 PD's when fibroblast growth factor (FGF) and thrombin were added to the culture medium and (ii) that fetal bovine endothelial cells cloned in the presence of FGF developed into lines that could be subcultured for at least 390 PD's if FGF were added to the medium. In contrast, in preliminary experiments with strain BFA-1c, which was isolated in medium without added FGF, cultures seeded at densities from 1 to 10^4 cell/cm^2 did not require FGF for proliferation, and cultures in phase III were not rescued from senescence by the addition of FGF (100 ng/ml) to the medium. Under our culture conditions, then, added growth factors such as FGF were not required for the development and propagation of endothelial clones. Our observation is consistent with the findings of others (*21, 24*) and suggests that the culture conditions used by Gospodarowicz and coworkers (*22*) may have favored a variant cell type dependent on FGF and possessing an infinite life-span. Interestingly, continuous bovine cell lines have been isolated in a few instances (*26*), indicating that bovine cells do possess a limited capacity to "transform" under certain environmental conditions. In general, however, the frequency of such spontaneous events is low in bovine cells and has not been documented in human fibroblast-like cells (*6, 8, 9, 15, 27*).

Further exploitation of the endothelial cell culture model should be encouraged by the facility with which cloned strains such as BFA-1c can be isolated. Our finding that these cloned strains exhibit growth characteristics typical of other normal diploid cells in culture (restricted proliferation at monolayer densities and a finite life-span) affords the opportunity for many studies on normal endothelial cell proliferation. Finally, the fact that at least some specialized endothelial characteristics are detectable throughout the culture life-span will encourage investigation of specialized functions in a metabolically active and physiologically important cell type in vitro.

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