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Caloric Restriction: Conservation of Cellular Replicative Capacity *in Vitro* Accompanies Life-Span Extension in Mice

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We have tested whether life-long caloric restriction (CR) slows or delays the age-related loss of cellular replicative potential that occurs during normal aging in *ad libitum* (AL) fed mice. Both mean and maximum life spans of the restricted animals (60% of AL intake) were significantly extended 30–40% by CR treatment. Proliferative potential, measured by determining the fraction of cells capable of forming large clones *in vitro*, was compared in five cell types from six tissue sites from two strains of mice (Male (C57BL/6 × DBA/2)F1("B6D2F1") and female (C57B1/6 × C3H)F1 ("B6C3F1")). This included four nonhematopoietic organ sites: fibroblast cells from ear skin, tail skin, and subdermal connective tissue and epithelial cells from the medullary part of the kidney and two cell types, myofibroblasts and endothelial-like cells, from spleen and bone marrow. The proliferative potential of cells from AL mice decreased progressively with age in all tissues sites of both mouse strains. CR delayed or decreased the loss of proliferative potential in all situations, but the timing of this was tissue specific. For cells from the four nonhematopoietic tissue sites from female B6C3F1 female mice, CR delayed the onset of proliferative loss, such that the fraction of large clones was significantly greater for the CR 18- to 24-month-old mice than in AL controls at three of four sites (as determined by the fraction of large clones after 1 week of clonal growth). The proliferative loss in CR tissues then accelerated from 24 to 30 months, so that both CR and AL mice had similar fractions of large clones after 30 months of age. CR was also seen to delay loss of proliferative potential in cells from skin and kidney of B6D2F1 male mice at 23–24 months of age when cloned for 2 weeks. For fibroblast and endothelial-like cells from bone marrow and spleen stromal sites from both strains of mice, CR also significantly decreased loss of proliferative potential; furthermore, in these tissues the proliferative advantages remained or increased from 24 to over 30 months of age. In companion studies (N. S. Wolf *et al.*, 1995. *Exp. Cell. Res.* 217, 000–000), CR was seen to decrease age-related losses in the maximal rates of cell replication *in vivo* in a panel of tissues from B6D2F1 male mice. The preservation of replicative potential by CR mice in all tissues tested, both *in vitro* and *in vivo*, indicates that CR preserves proliferative capacities in the cells and tissues of chronically restricted mice and may permit CR mice to better respond to proliferative stresses in old age. © 1995 Academic Press, Inc.

INTRODUCTION

Loss of proliferative potential with age is one of the phenotypic expressions of aging in mammals and perhaps all vertebrates [see reviews in Refs. 1–4]. The cellular proliferative potential of skin fibroblasts, measured as the maximum population doublings attained by cells established from a particular donor, is inversely proportional to the age in humans [1], and other species [3, 5], and is reduced in cells from patients with several progeroid diseases which shorten life span, such as Hutchinson–Gilford syndrome and Werner's syndrome [1, 6]. The maximum doublings that a particular cell lineage can undergo can be influenced by a number of extrinsic factors such as serum type or concentration [7], media composition [8], presence of specific mitogens [9–11], or temporary arrest in late G1 [12], but under standard conditions is reproducible [2]. The mechanism of loss of replicative potential with age is unknown. Hypotheses have been presented implicating both genetic factors and stochastic damage [4, 13–16].

Long-term caloric restriction (CR) has been shown to significantly extend life span in mice and rats and is, therefore, of interest not only as a possible intervention in human aging, but also as a means to test which putative "biomarkers" of aging may be more directly related to the physiological rate and, thus, the underlying mechanism(s) of aging [17–21].

In order to determine if CR decreases age-related losses in proliferative parameters, cells from several tissue sites of two well-characterized mouse strains, aged under both AL and CR conditions, were compared for

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their relative ability to form large clones *in vitro* (clone size analysis), rather than cumulative population doublings. Originally developed by Smith *et al.* [22], clone size analysis has since been used by several others as a sensitive measure of proliferative potential that better represents the original heterogeneity of a cell population than does life span in doublings achieved by mass cultures, which is dominated by the proliferative potential of the best clones [23–25]. The culture is then analyzed on the basis of the fraction of clones which are able to attain a given minimum size in 1- to 2-week periods. Clone size analysis is also less influenced by transformation events that produce frequent immortalization of mouse and rat cell populations during aging [3, 5].

We report here that, in all tissues tested, CR significantly delays and/or slows the age-related loss of proliferative potential as assayed by the ability to form large clones *in vitro*. Companion studies on cell replication *in vivo* also reported in this journal [26] presents evidence that the cell replication rates of cells in a wide variety of tissues is conserved *in vivo* by CR. Together these findings indicate that CR-treated animals have a greater reserve of proliferative potential to respond to age-related cell damage or loss, and this may contribute to the longevity of CR animals.

MATERIALS AND METHODS

Animals. Male (C57BL/6 × DBA/2)F1 (B6D2F1) and female (C57B1/6 × C3H)F1 (B6C3F1) mice of different ages and diet conditions were obtained from the National Center for Toxicological Research (NCTR, Jefferson, AR), as part of the NCTR/NIA Biomarkers of Aging colony. The male B6D2F1 mice on the *ad libitum* (AL) diet lived a mean of 32 months, which was extended 36% by CR to 43 months (see Fig. 1a). Similarly, the mean life span of female B6C3F1 mice was extended 36% by CR (see Fig. 1b). The CR diet was instituted incrementally beginning at 14 weeks and completed by 16 weeks of age. It consisted of a reduction of the intake volume of a balanced pelleted diet (NIH-31) fed to both study groups, so that total caloric intake equaled 60% of that of the AL fed controls of the same age. The CR diet was supplemented only with vitamins to insure equivalent vitamin uptake. The growth curves and pathogen-free maintenance methods for both AL and CR mice, as well as the feeding protocol, are available upon request from the NCTR. After being received from the NCTR, the mice were maintained in our facility under the same conditions including a 12-h diurnal light cycle and strict pathogen-free barrier conditions. The CR animals were fed 30 min after initiation of the dark cycle from the time of their arrival. This placed their chronologic period of maximum food intake on a par with that of the AL fed mice, thus reducing diurnal cycle differences since mice are nocturnal feeders. All the mice were allowed a 2-week acclimatizing period after arrival prior to use in any procedure. For these studies the animals were received at specified ages and were only maintained a few weeks in our facility prior to sacrifice; therefore, we have not presented separate life tables for mice kept in our facility. Preweighed pellets were fed to the CR mice based on their weights at shipment time. CR mice gain very little weight after 6 months of age. The mice on site were regularly tested and were free of all testable mouse pathogens [27]. Sacrifices were by cervical dislocations.

Isolation and cloning of nonhematopoietic organ cells. Following sacrifice, tissue samples of the medullary portion of the kidney, ear skin, tail skin, and tail subdermal connective tissue, were surgically removed from areas sterilized with 70% ethanol. The tissues were washed in phosphate-buffered saline (PBS) and cut into 2-mm pieces. Tissue pieces were washed in Dulbecco's modified Eagle's medium (DMEM) buffered with 20 m*M* *N*-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes) then resuspended in a digestion solution of DMEM + Hepes containing 0.1% collagenase/dispase (Boehringer Mannheim, Indianapolis, IN) and 10% fetal bovine serum (FBS) to reduce cell damage by the proteolytic enzymes. The digestion mixture was immediately transferred to a sterile plastic bag, sealed with heat, and then taped to the outside of a 1-liter roller bottle with 500 ml of water inside and allowed to roll over the bags containing tissue at 40 rev/h at 37°C. The optimum time of maceration was 45 min for ear and tail skin, 60 min for subdermal tail tissue, and 30 min for back skin. Kidney pieces were macerated 15 min without enzymes. The tissue digests were then washed with versene buffer (PBS containing 0.5 m*M* ethylenediaminetetraacetic acid (EDTA)) to inactivate the proteases and then, with the exception of kidney epithelial cells, were plated into 25-cm^2 flasks with 7 ml of cloning medium (F-12 medium supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin and 1.25 μg/ml fungizone). After 2 days, the cells were trypsinized (0.05% trypsin) and filtered through a 20-μ*M* nylon mesh (Nytex 20 μ*M* mesh from Research Nets Inc., Mountlake Terrace, WA) to isolate single cells and cloned into 25-cm^2 flasks with the cloning medium at 300–400 cells/flask. The plating efficiency of the cells from all tissues was 20–40%. Note that relative plating efficiency does not directly influence the fraction of good or poor growing clones within the plating densities used, since this measurement is made on the fraction of cells that plate and remain for 1–2 weeks; however, similar plating efficiencies were noted for tissues from AL and CR mice. Kidney epithelial cells, being more sensitive to passaging, were cloned directly following maceration for 15 min as above, but without any proteolytic enzymes, filtered through 20 μ*M* nylon mesh, and directly cloned at 500 cells/25-cm^2 dish in the cloning medium supplemented with 20 ng/ml epidermal growth factor (UBI, Lake Placid, New York). After allowing clones to grow for 7 or 14 days, they were fixed with 95% ethanol and stained with crystal violet and the number of cells/clone enumerated by eye with a dissecting microscope.

Isolation and cloning of stromal cells from hematopoietic organs. After sacrifice of mice, the femurs and spleens were taken by sterile technique. Femur medullary content was flushed repeatedly with DMEM supplemented with 10^{-4} *M* 2-mercaptoethanol, antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin, and 0.5 mg/ml fungizone), and with 15% FBS (DMEM-F), using needles of decreasing calibers (18–22 gauge). Spleens were finely minced with scissors in the same medium, passaged repeatedly through 20- and 22-gauge needles and the cells were then passed through a 100 mesh/square inch stainless steel screen. The single cell suspensions from either organ were counted with a ZBI Coulter cell counter (Kirkland, WA). For both organs, cultures were carried out in MEM-F at 37°C in humidified 7% CO_2, 5% O_2, and 88% N_2. Myofibroblast clones [28] were obtained as described by Fei *et al.* [29]. A primary culture of 10^7 bone marrow or spleen cells was prepared in a 60-mm culture dish with 5 ml of MEM-F and allowed to attach for 24 h. Nonadherent cells were washed away with two washes of PBS containing 0.1% EDTA. Adherent cells were trypsinized (0.05% trypsin, GIBCO), washed with PBS, and resuspended in MEM-F. The cells were counted on a hemocytometer and cloned at 3000 cells/35-mm dish. An operational identification of cell types was by morphology, histology, specific antigen presence, and attachment requirements. Myofibroblasts were identified by shape and size and confirmed by alkaline phosphatase staining and positivity for HHF-35 a-actin smooth muscle fiber antigen [28, 30, 31]. Prior to final plating, endothelial-like cells were isolated by their extreme phagocytic properties; these phagocytic clone formers were separated by exposure to magnetic beads (Dynal M-450) for 2 h at 37°C in a ratio of three beads per cell. Endothelial-like colonies grew only in the presence of 50 units/ml of granulocyte

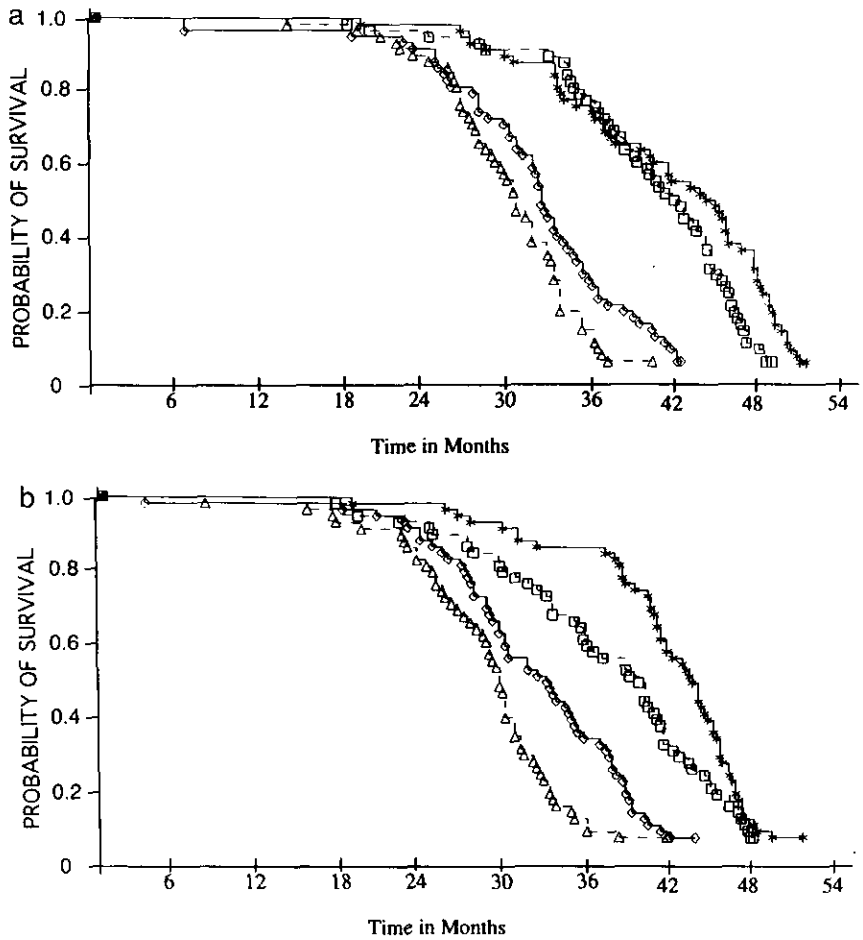

FIG. 1. Male B6D26F1 and female B6C3F1 mice were used in these experiments. The survival curves for both sexes of these strains are depicted in Fig. 1a (B6D2F1) and Fig. 1b (B6C3F1). The diets and maintenance conditions are described under Materials and Methods. Male and female "NIH31 R" refers to male and female mice maintained on the restricted diet, and "NIH31 A" refers to mice maintained on an *ad libitum* diet. These curves were prepared by the National Center for Toxicological Research (NCTR, Jefferson, AR) as part of the NCTR/NIA "Biomarkers of Aging" project and are presented with the permission of the NCTR. *, Male NIH31 R; ◇, male NIH31 A; □, female NIH31 R; △, female NIH31 A.

macrophage stimulating factor added to the MEM-F. Endothelial-like cells had their identity confirmed by size and shape and positivity for vWf [29, 32, 33], for BSL I-B4 lectin [28, 34], and for rat anti-mouse endothelium monoclonal antibody MECA 32 [35]. Myofibroblast colonies were read at 9 days and endothelial colonies at 10 days.

Statistical analysis. The number of cells per clone were counted and grouped into clones that attained 0, 1, 2, 3, 4, 5, 6, 7, or more doublings and displayed as a cumulative clone size distribution [22]. The animals were assayed two to three at a time, and at least two different aging cohorts were analyzed. Differences in the cumulative clone size distribution for the different conditions were analyzed by comparing the fraction of large well-growing clones that grew to over four or five population doubling levels (see figure legends) using a Student's two-tailed t test. Analysis of small clones yielded similar results (data not shown).

RESULTS

In order to analyze the proliferative potential of each cell type, "cumulative" clone size distributions were prepared for each cell type under each condition and age and the fraction of relatively "large" clones determined as well. In Fig. 2, a sample cumulative clone size distribution is depicted for kidney epithelial cells from 18- to 24-month-old AL and CR animals and is compared to the 6-month AL values. This form of presentation compares the accumulated fraction of cells able to divide (y-axis) to the size of clone formed (x-axis). Note that when analyzing kidney cell clones, only epithelial-like cells were scored, and only skin or connective tissue cells that formed fibroblast clones were scored in analysis of those tissues. The few ($\leq$10%) transformed-like clones seen (clones lacking proper morphology or lacking contact inhibition) were not scored. Hematopoietic stromal fibroblast and endothelial-like cells were scored as described under Materials and Methods. For statistical comparisons between age and diet conditions, the fraction of cells able to form relatively large clones [22,

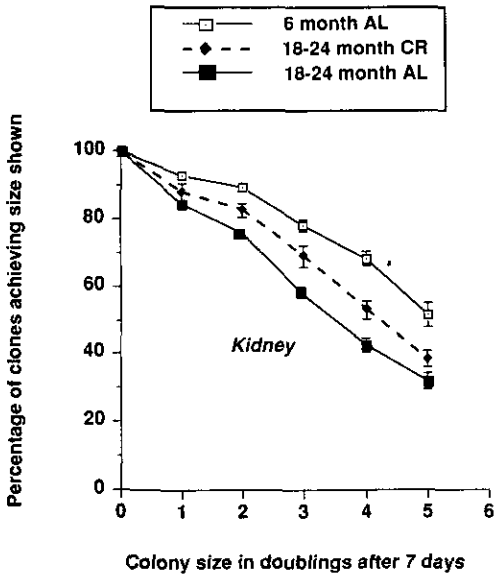

FIG. 2. Sample cumulative clone size distributions comparing AL fed and CR mice. The tissues for clone size analysis were obtained following sacrifice of B6C3F1 mice at two ages: 6 months and 18–24 months. For this example the clone forming ability of cells from kidney medullary tissue from middle-aged AL and CR mice (18–24 months age group) are compared to cells from young AL mice (3–6 month). The tissues were isolated and cloned for 7 days as described under Materials and Methods. Clone size distribution from 7 to 12 animals were combined to make each point on the graph. The error bars represent standard errors of the means.

23] was determined from the clone size distributions (clones growing to over 16 or 32 cells in 7–14 days, see Materials and Methods). Similar statistical results were also obtained by comparing the fraction of relatively small "senescent-like" clones formed in the same period of time (less than 1 or 2 doublings, not shown). By 7–14 days, the large clones were generally full of small cells with frequent mitotic figures, and the small clones contained large flattened "senescent" looking cells with no mitotic figures.

A potential complication of clone size analysis may occur if cell migration from primary clones is rapid enough to seed secondary subclones that become confused with primary clones. Figure 4 indicates that this is not a significant problem with skin fibroblasts, since the total number of clones is not significantly affected whether counted on Days 3, 5, or 7. The total number of kidney epithelial cell clones were also similar at 7 and 14 days (data not shown).

In Fig. 3, the proportion of large clones (≥16 cells) formed by cultures from nonhematopoietic organs (tail and ear dermal fibroblast cells, tail tendon fibroblast cells, and medullary kidney epithelium) of AL and CR mice (female, B6C3F1) of different ages is compared. Multiple groups of two to three animals were assayed per cohort of mice and the results accumulated to produce each age point; this was done in part to avoid any errors attributable to a particular aging cohort. In all tissues, the fraction of large clones decreased with age by 36 to 51%. By 22 months CR had delayed the loss of proliferative potential significantly in three of the four tissue sites relative to AL controls. However, by 30 months of age the proliferative advantages of these CR tissues were lost. Note that proliferative loss in the AL animals is especially rapid from 12 to 22 months and then slows, whereas, the proliferative loss in the CR animals is relatively slow until after 22 months and then accelerates from 22 to 30 months forming parallelogram-like patterns. This may indicate that a "minimum" proliferative potential is reached first by the AL animals and later by the CR animals, or alternatively that a selection for the heartier animals among the AL group had occurred by mortality attrition by 30 months of age. In studies on cells from hematopoietic stromal tissues, however, the differences between AL and CR animals was maximal at the oldest ages examined, i.e., over 30 months of age (see below). In the results depicted in Fig. 3 we have selected to display the fraction of clones growing to ≥4 doublings as best representing

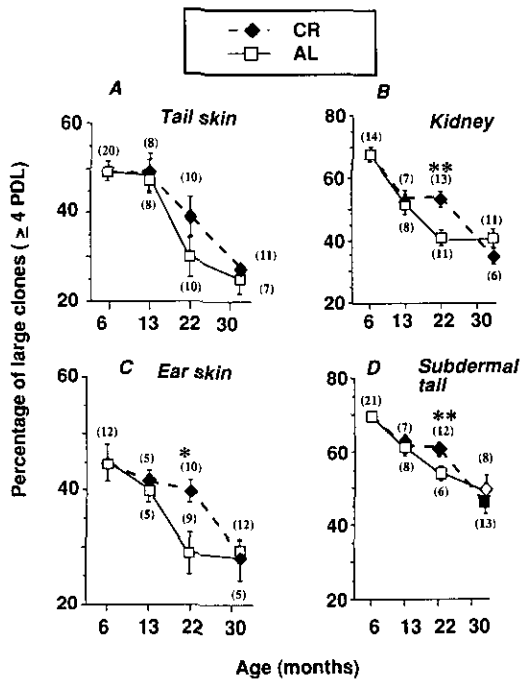

FIG. 3. Percentage of large clones (over 16 cells) as a function of age from cells derived from nonhematopoietic organs of mice on CR and AL diets. Cells from tail skin, ear skin, subdermal connective tissue, and kidney from female B6C3F1 mice were cloned from the primary cultures as described under Materials and Methods. Age groups correspond to mean of 6, 11–13, 18–24, and 30–32 months. The fraction of large clones (over 16 cells) is plotted versus diet condition and age. The significance levels refer to comparisons made using a Student's two-tailed t test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. The error bars represent the standard errors of the means.

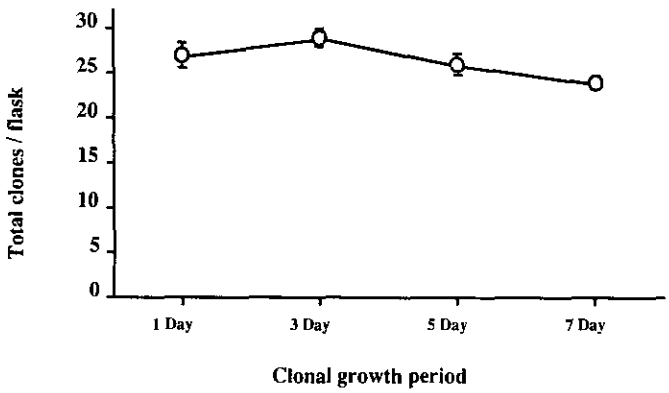

FIG. 4. Total number of skin fibroblast clones of any size from female B6C3F1 mice at 6 months of age on Days 1, 3, and 7 after plating. Tail skin cells from a single female B6C3F1 mouse were isolated and cloned as described under Materials and Methods in multiple flasks. The three flasks were fixed on each day shown and the total clones of any size counted. The error bars represent the standard errors of the means.

the fraction of highly proliferative cells in the culture. In Table 1 we compare the proliferative potential of CR and AL cultures at 22 months of age using different clone size criteria. The fraction of clones ⩾5 doublings was the least sensitive criteria showing significance for kidney cells and strong trends for the other three tissues. The fraction of small clones ⩽2 doublings demonstrated significant differences for kidney and ear skin and near significance for the other two tissues. The 3 doubling clones are not depicted because they represent a swing point between the good growing and poor growing clones and show no changes with age or diet. Not enough clones grow over 6 doublings or less than 1 doubling for good statistics. We also analyzed the clone sizes (⩾4 doublings) of cells from tail skin and kidney of B6D2F1 male mice cloned for 2 weeks rather than 1 week (Fig. 5). The "protection" against proliferative loss by CR was significant in this 2-week analysis in both tissues. Finally, we analyzed the fraction of large clones derived from stromal tissues from bone marrow and spleen from both female B6C3F1 and male B6D2F1 mice (Fig. 6). In all of these tissues, CR also delayed or decreased the age-related losses of proliferative potential. Furthermore, in these two hematopoietic stromal cell types, the CR-induced proliferative advantages were maintained, or increased, into advanced old age (30–32 months). Note that clones ⩾5 doublings are displayed here because these tissues were grown longer, but that similar differences exist with clones ⩾4 doublings or clones ⩽2 doublings (data not shown). In conclusion, in 11 of 12 studies in both sexes and mouse strains studied, there were statistically significant increases in the proportions of large clones for cells from CR animals at one or more time points above 18 months of age.

DISCUSSION

We have presented evidence that CR preserves the replicative potential of cells from six different tissue sites during aging of male B6D2F1 and female B6C3F1 mice. Since CR is the most successful method known for extending the mean and maximal life spans of mice and rats [see reviews in Refs. 17, 21, 36], it is important to determine whether CR protects the proliferative potential of cells and tissues capable of division during aging. Two previous papers that examined the effects of CR on proliferative potential have reported ambiguous or no effect of CR on proliferative potential. Volicer *et al.* [37] examined saturation densities of aortic smooth muscle cells form AL and CR rats of various ages. Higher cell densities were noted for 29-month-old rats, but not 24-month-old CR rats versus age-matched AL controls.

TABLE 1

Statistics for Alternate Clone Size Classes from Female B6C3F1 Mice from Nonhematopoietic Tissues at 22 Months

Tissues	[1] Diet (age, months)	*N*	% of clones 0–2 PDL ± SD (%)	*P*	% of clones ⩾ 4 PDL ± SD (%)	*P*	% of clones ⩾ 5 PDL ± SD (%)	*P*
Tail skin	Res (18–22)	10	46 ± 16	$P = 0.09$	38 ± 16	$P = 0.11$	24 ± 13	$P = 0.12$
Tail skin	AL (18–22)	10	57 ± 17		30 ± 13		18 ± 11	
Kidney	Res (18–22)	13	31 ± 12**	$P = 0.005$	53 ± 10**	$P = 0.003$	39 ± 9**	$P = 0.004$
Kidney	AL (18–22)	11	43 ± 5**		42 ± 7**		30 ± 6**	
Ear skin	Res (18–22)	10	46 ± 10**	$P = 0.004$	39 ± 8*	$P = 0.01$	22 ± 9	$P = 0.10$
Ear skin	AL (18–22)	9	58 ± 9**		29 ± 10*		19 ± 7	
Tail subderm.	Res (18–22)	12	25 ± 6	$P = 0.09$	61 ± 5**	$P = 0.002$	51 ± 9	$P = 0.15$
Tail subderm.	AL (18–22)	6	32 ± 6		55 ± 3**		48 ± 5	

Note. The values in the middle column (⩾4PDL) are the same as in Fig. 4. Diet "Res" indicates restricted, and "AL" indicates *ad libitum* fed with age in months. The percentage of clones falling within the size grouping is indicated ± standard deviation. The *P* values are from a two-tailed Student's *t* test comparing restricted and *ad libitum* fed mice at each age. * Indicates *P* value less than 0.05; ** indicates *P* value less than 0.01.

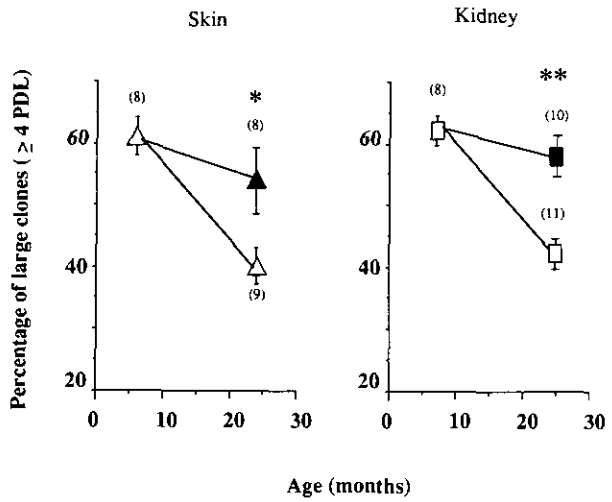

FIG. 5. Fourteen-day clone size studies on kidney epithelial cells and tail skin fibroblast cells from B6D2F1 male mice. Primary skin and kidney epithelial clones were prepared as described under Materials and Methods from 6- and 18- to 24-month-old mice on AL or CR diets and allowed to grow for 2 weeks. Other conditions are as described in the legend to Fig. 3.

Pignolo *et al.* [5] reported that skin fibroblasts from CR animals attained the same number of population doublings as age-matched AL controls before senescence or transformation occurred. We believe that our positive findings are the result of: (1) using clone size analysis, rather than the total population doubling level method of analyzing proliferative potential, and (2) examining multiple tissue types from many animals. Clone size analysis tends to reflect the original heterogeneity of individual proliferative potentials present in the primary cultures. Thus, our findings indicate that the fraction of senescent or near senescent cells unable to divide more than three times, increases with age *in vivo* more rapidly in AL animals than CR animals, but it does not speak to the ultimate proliferative potential of the best growing clones. The total population doublings method is more sensitive to the proliferative potential of the best clones in the population since these will eventually predominate [24]. It is further complicated by the presence of spontaneously transformed cells in the rodent cultures, which are not a significant problem when using clone size analysis since they remain as a few isolated large clones usually of distinct morphology. We do not believe that the differences we observed are a result of arbitrarily picking the four doubling cut off point for designating highly proliferative clones, since significant or near significant differences occur for clones greater than five doublings or by scoring the fraction of small clones less than two doublings (see Table 1). Other potentially important differences are the use of multiple tissue types in this study, and more individual animals (7–20) per time point rather than a maximum of four by Pignolo *et al.* [5], or the use of mice in these studies compared to rats in the other studies. The previous studies each examined cells from only one tissue site. At present we are not sure why the hematopoietic stromal cells are more sensitive to protection by CR, but note that *in vivo* cell replication in these tissues were also protected better by CR (see below).

In a related study [26], we report evidence that CR preserves the proliferative potential of cells *in vivo*. We found that cell replication rates (determined by *in vivo* labeling with BrdU delivered subcutaneously by miniosmotic pumps for 2 weeks) decreased with age in both AL and CR animals. Chronic CR-treated animals actually had lower cell replication rates prior to 10 months of age, but from 13 months of age onward cell replication rates were similar for all CR and AL tissues. However, if the old chronic CR mice were allowed to eat AL for 2–6

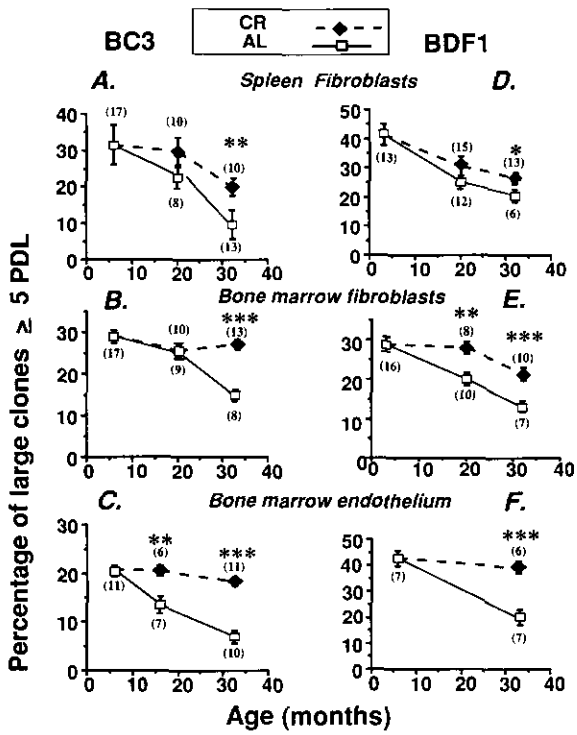

FIG. 6. Percentage of clones with ≥5 population doublings among stromal cells from femurs and spleens of AL and CR B6C3F1 female and B6D2F1 male mice. Hematopoietic organ fibroblast-like cells and endothelial-like cells were prepared from AL or CR female B6C3F1 mice and male B6D2F1 mice as described under Materials and Methods. Fibroblast cells were cloned for 9 days and endothelial-like cells for 10 days prior to fixation and counting. A, B, and C represent the % of large (≥5 doublings) clones found among the total in triplicate cultures for the ages shown on the abscissa for spleen fibroblasts, marrow fibroblasts, and marrow endothelial cells, respectively from B6C3F1 females. D, E, and F represent those values in the same order of B6D2F1 male mice. The number of mice in each group is indicated in parentheses on each line. Other conditions are as described in the legend to Fig. 3. Age groupings were the same as in the legend to Fig. 3, with the addition of a 15-month-old group.

weeks prior to beginning measurement of cell replication, the rates increased significantly above AL control values, and in some tissues, such as hematopoietic stromal cells, approached that of young controls. We interpret this as indicating that CR not only decreases cell replication in young animals, but also preserves replicative potential *in vivo* in old animals. Further evidence that CR preserves replicative potential *in vivo* has been found by a collaborator (M. Reed, unpublished observations), who demonstrated that the age-related decreases in the rate of wound healing are prevented by chronic CR, provided that the old CR animals are allowed to feed AL for 4 weeks prior to wounding.

These data suggest that CR may preserve replicative potential by delaying early expenditure of a finite number of population doublings until old age. Indeed, mitogenic stimulation by growth hormone and insulin-like growth factor *in vivo* has been associated with premature loss of replicative potential and a shortened life span. Tissues from short-lived transgenic growth hormone (GH) mice lose proliferative potential at a much earlier age (3–6 months) than in normal controls (18–24 months) [25]. This loss of proliferative potential begins just when receptors for IGF-1 and GH are expressed in the transgenic animal (3–4 weeks) [38]. However, this can only be a partial explanation for these findings, because *in vivo,* CR decreases cell replication only in animals younger than 10 months of age [26]; whereas, *in vitro,* losses of replicative potential are not detectable until after 12 months of age (Figs. 2–4); furthermore, CR instituted after 12 months of age still lengthens life [17]. Therefore, some other form of "damage" to the proliferative potential of the cells must accumulate beyond 10 months of age.

One possible explanation consistent with these findings is that mitogenic stimulation is qualitatively different in CR animals such that it is less "damaging" to the replicative potential of cells. Others have reported a number of extrinsic factors that can reduce the proliferative potential of mammalian diploid cells grown *in vitro;* some of these may act *in vivo.* These include serum type, or concentration [7], toxic substances in serum [39], media composition [8], presence or absence of specific mitogens [9–11], or temporary arrest in late G1 [12]. Evidence that *in vitro* senescence is directly influenced by factors other than the number of doublings has previously been published by one of the authors (W.R.P.), and others. They found that chronic exposure of subconfluent human diploid fibroblasts to low levels of serum stimulation *in vitro* for extended times can deplete them of proliferative potential in the absence of cell division [12, 23]. Note, however, that contact inhibited cells are protected from the effects of low serum and do not lose proliferative potential with time [23, 40, 41, 42]. CR may act in some other way to protect cells from extrinsic factors that reduce replicative potential. Of course other factors such as decreased oxidation, or glycation, or other mechanisms only indirectly related to mitogenic stimulation could also reduce damage to the proliferative potential in the CR animals. In summary, these findings indicate that CR delays or decreases age-related losses of cellular replicative potential *in vitro* and *in vivo* and that this may enable CR animals to better respond to age-related stresses. Further studies will be needed to differentiate the exact mechanism(s) by which CR preserves replicative potential *in vivo* and *in vitro.*

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