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Cultured Renal Epithelial Cells From Birds and Mice: Enhanced Resistance of Avian Cells to Oxidative Stress and DNA Damage

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Current mechanistic theories of aging would predict that many species of birds, given their unusually high metabolic rates, body temperatures, and blood sugar levels, should age more rapidly than mammals of comparable size. On the contrary, many avian species display unusually long life spans. This finding suggests that cells and tissues from some avian species may enjoy unusually robust and/or unique protective mechanisms against fundamental aging processes, including a relatively high resistance to oxidative stress. We therefore compared the sensitivities of presumptively homologous epithelial somatic cells derived from bird and mouse kidneys to various forms of oxidative stress. When compared to murine cells, we found enhanced resistance of avian cells from three species (budgerigars, starlings, canaries) to 95% oxygen, hydrogen peroxide, paraquat, and γ-radiation. Differential resistance to 95% oxygen was demonstrated with both replicating and quiescent cultures. Hydrogen peroxide was shown to induce DNA single-strand breaks. There were fewer breaks in avian cells than in mouse cells when similarly challenged.

AS a group, birds are remarkably long-lived for their body size (1,2). This has long been of interest to gerontologists because birds have much higher metabolic rates and body temperatures, on average, than mammals. Birds also maintain higher levels of blood glucose. Thus, on theoretical grounds, they should sustain proportionately greater damage from processes such as macromolecular damage mediated by free radicals and the glycation and glycoxidation of proteins and nucleic acids (2–5), which are hypothesized to be responsible for much of the physiological deteriorations that characterize senescence. The exceptional longevity of birds as a group suggests that they have evolved special mechanisms to protect against these processes. For instance, a 20-g mouse that lives 3 years experiences about one-twentieth the oxidative burden of a 20-g canary that lives 20 years (1,2). This result suggests that birds have mechanisms either to reduce the rate of production of oxidants per unit of energy expended and/or that they have enhanced antioxidant defenses.

Work presented here reports on the results of oxidative challenges to kidney epithelial cells from laboratory mice (C57Bl/6NNia) and three species of birds: budgerigars (*Melopsittacus undulatus*) and European starlings (*Sturnus vulgaris*), which both exhibit captive longevity up to 20 years (6,7), and canaries (*Serinus canarius*) with captive longevity as high as 24 years (7). DNA damage resulting from this oxidative challenge was measured by using a flow cytometric assay of single-strand breaks and alkaline labile sites based upon the metachromasia of acridine orange.

Materials and Methods

Animals

Three- to 4-month-old C57Bl/6NNia mice were obtained from a specific pathogen-free mouse colony at the University of Washington. Seven young (< 1 year) budgerigars and four young canaries were purchased from commercial suppliers and housed under conventional laboratory conditions, eating commercially available bird food, for approximately 2 days before being sacrificed for this study. Seven free-living starlings were live-trapped in the vicinity of Seattle, WA. The time of year at which they were trapped, combined with their juvenile plumage and the remnants of a yellow gape, led to the estimation that these birds were subadults, probably 4–5 months old. Starlings were sacrificed within 24 h of capture. All cell cultures used in these experiments were from animals that had expended from 3 to 6% of their maximum life span.

Because of limited numbers of animals available, we were not able to use all bird species in all assays. However, because body size (25–70 g), maximum longevity (20–24 years), and therefore oxygen consumption per gram of tissue (2000–3200 kcal/g/lifetime) varied over a relatively narrow range, we did not consider this a major complication in interpreting our results.

Cell Cultures

Fresh kidney tissue from birds and mice was aseptically dissected, and tissue samples transported to the cell culture facility in Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100

μg/ml streptomycin, and 30 mM HEPES buffer (pH 7.4). The methods were those of Martin et al. (8). Those authors provided immunohistochemical evidence for an origin of cultured human epithelial cells from renal tubular epithelium. However, no comparable evidence is currently available for the case of murine and avian renal cell cultures, although their morphologies are consistent with origins from tubular epithelium. Cultures were initiated from cell suspensions prepared by enzymatic dissociation of kidney cortical tissue. Cortical tissue samples were aseptically dissected to remove as much adventitious tissue as possible. One to 2 g of tissue were used (for both mice and birds, this required pooling of the kidneys from three or four animals). The tissues were minced to obtain ≈1 mm^3 tissue fragments. These were washed two to three times in Ca- and Mg-free phosphate-buffered normal saline (PBS; pH 7.1) (Grand Island/BRL, Gaithersburg, MD) prior to enzymatic dissociation. Type 1 collagenase (Sigma, St. Louis, MO) was used to dissociate tissue fragments into cell suspensions. Minced tissue fragments were resuspended in 50 ml of a 1:1 mixture of type 1 collagenase (1 mg/ml in PBS) and DMEM with penicillin (100 units/ml) and streptomycin (100 μg/ml) (without the addition of serum). Digestion was performed in a sterile 50-ml polypropylene conical centrifuge tube with a small sterile stirring bar on a magnetic stir plate at moderate speed for 1 h in a 37°C incubator. After letting the tissue fragments settle, the supernatant was collected by pipetting into 50-ml tubes with 5 ml of fetal bovine serum (FBS) to stop the collagenase action and placed on ice. The remaining tissue fragments were digested with another 50 ml of the collagenase DMEM mixture as above for an additional 1 h at 37°C. At the end of this digest there were few if any tissue fragments remaining. The resulting cell suspension was combined with that previously collected and centrifuged at 2600 × *g* for 5 min. The resulting cell pellet was resuspended in 20 ml of DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS that was heat-inactivated at 56°C for 30 min. This suspension was split among three to six 75-cm^2 flasks containing 10 ml DMEM with penicillin and streptomycin and 10% heat-inactivated FBS. Flasks were incubated at 37°C in an humidified atmosphere of 5% CO_2/95% air. Cells were fed the next day by replacing the medium with fresh medium to remove unattached debris. The cultures typically became confluent after 2–3 days. Such primary cultures from murine kidneys appeared to achieve confluence more rapidly than the avian cultures. After the first passage, however, the growth of cells appeared to be comparable. Flow cytometric measurements showed no significant difference in the percentage of S-phase cells (10.7 ± 0.9% *SD* for avian cells and 10.9 ± 3.6% *SD* for murine cells) in subconfluent cultures. For the experiments to be described, cells were obtained from early passage (first or second) cultures.

Challenge with Oxidative Agents

Flasks of near-confluent cells were rinsed two times with 5 ml of .53 mM EDTA in PBS (without Ca^{2+} and Mg^{2+}), followed by the addition of 5 ml of .05% trypsin in this same EDTA/PBS solution for 5 min at 37°C. The resulting cell suspension was mixed with 5 ml of medium containing serum, counted, and plated in multiple 25-cm^2 flasks at 10^5 cells/flask. The next day (after cells had attached) they were treated with the agent of interest. To challenge with 95% oxygen, the cells were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA) and gassed with a mixture of 95% O_2/5% CO_2 for 10 min, sealed, and incubated at 37°C for the period indicated in the *Results*, with regassing every 24 h for the duration of the exposure. For H_2O_2 challenge, the flasks were fed with freshly prepared medium containing the indicated concentration of H_2O_2 and were refed every 24 h with fresh H_2O_2. Paraquat, which is thought to interact with oxygen to produce the superoxide anion (9), was added to serum containing freshly prepared medium at the indicated concentrations. The treated cultures were not refed during the experiment. To challenge with γ-radiation, cultures were placed in the chamber of a Gammacell 40 (Nordion International, Ontario, Canada) for a period of time sufficient to deliver the indicated dosage and then returned to the incubator for 48 h prior to counting. To obtain cell counts, cells were again released by using trypsin as described at the beginning of this paragraph and counted by using a hemocytometer.

Flow Cytometric Alkaline Unwinding Assay

The methods were adapted from Rydberg (10) and Affentranger and Burkart (11), with modifications. Hydrogen peroxide was added to individual cell suspensions (200 μl medium with 10^5 primary renal epithelial cells from birds or mice) on ice (at concentrations indicated in the *Results*) for exactly 5 min. "Control" samples were collected at this point, whereas "repair" samples were collected after dilution with medium, pelleting by centrifugation, resuspending in medium with 10% serum at the original cell volume, and incubating at 37°C for the indicated times. These times included centrifugation and resuspension manipulations. Nuclei were obtained from cells suspended in 1.8 ml of ice-cold NST buffer (146 mM NaCl/10 mM Tris, pH 7.4/2 mM $CaCl_2$/0.1% Noridet P-40). This treatment was followed by the dropwise addition of 6 ml of ice-cold 95% ethanol while vortex mixing to ensure homogeneous fixation. Fixation occurred on ice for at least 15 min (1 h maximum). Each sample was then transferred into tubes prewetted with PBS with nuclease-free bovine serum albumin (2 mg/ml) and spun at 1300 rpm for 10 min. Individual pellets were mixed with 0.5 ml of 95% ethanol and resuspended by dropwise addition of 5 ml of 0.9% NaCl while vortex mixing. Each sample was spun at 1300 rpm for 10 min. Then, 100 μl of buffer (20 mM Tris-HCl, pH 7.5/100 mM NaCl/1 mM EDTA) was used to resuspend each pellet. Samples were mixed with 2.25% ultra-low gelling temperature SeaPrep agarose to prevent clumping and cell loss during treatment with basic solution (FMC Bioproducts, Rockledge, ME) and divided into triplicate samples. After agarose was solidified on ice, 1 ml of alkaline solution (0.9% NaCl/50 μM EDTA/.02 M NaOH at a concentration in the range of .025–.034 M) was added to induce variable degrees of unwinding. After 45 min, the alkaline solution was removed and 1 ml of PBS was added. After three washes of the agarose pellet with PBS to equilibrate the

sample at a neutral pH, samples could be held for up to 3 days before analysis. Immediately prior to flow analysis, each vial containing the agar/cell suspension was heated at 65°C until the gel melted (about 1 min). Fifty microliters of this sample, 2–5 µl of chicken red blood cells (CEN singlet cytometry control, BioSure, Riese Enterprises, Inc., Grass Valley, CA) diluted 1:5 with PBS, and 100 µl of solution A (0.1% Triton X-100/0.08 M HCl/0.15 M NaCl) were mixed in a round-bottomed polystyrene tube for 45 sec. Three hundred microliters of working solution B (6.7 µg acridine orange per ml [CalBiochem-Behring Corp., La Jolla, CA], .126 M Na_2HPO_4/.037M citric acid, pH 6/.15 M NaCl/1 mM Na_4EDTA) were then added giving flow samples a final dye concentration of 4.5 µg/ml. A 15-mW argon ion laser was used to excite acridine orange at 488 nm (binding to single-stranded DNA yields red fluorescence, whereas binding to double-stranded DNA yields green fluorescence). An ELITE flow cytometer (Coulter, Miami, FL) registered red fluorescence by using a long pass filter at 645 nm and green fluorescence from 510 to 540 nm. Forward scatter and right angle parameters were used to "gate-out" debris. Data were stored as FCS listmode files that were analyzed by using Listview (Palo Verde Software, Inc., Tucson, AZ). A calculated parameter of the ratio of red/green (single-stranded DNA relative to double-stranded DNA) was plotted against a calculated parameter of red + green (total DNA). Mean red/green values of the sample populations and of chicken red blood cells (CRBC standard) were calculated from these cytograms. Relative unwinding was measured as a ratio of sample mean/CRBC mean.

Statistical Methods

Each dose-response experiment was analyzed using two-factor analysis of variance (ANOVA). Two nominal or categorical variables (species; dose) and one continuous dependent variable (cell survival) were employed. The null hypothesis that the effect of doses of oxygen, hydrogen peroxide, paraquat, or γ-irradiation are the same regardless of species was tested by using the species by dose interaction term. Paired *t*-tests were used to compare the levels of alkaline unwinding of bird and mouse cells before and after hydrogen peroxide treatments. The species by repair time interaction term of the ANOVA was used to test whether the rate of repair on unwinding was the same regardless of species. These analyses were done by using Statview 4.5 software from Abacus Concepts, Inc. (Berkeley, CA). It is understood that the pooling of individual animals required by our protocol limits the power of the analyses if individual animals were significantly divergent in their sensitivities to particular agents tested.

Results

Experiments using 95% oxygen to challenge bird and mouse cells showed significantly better replication and survival of starling and budgerigar cells at all times of exposure in both replicating and quiescent cultures (Figure 1). A subset of bird cells continued to replicate despite exposure to 95% oxygen for at least 48 h, whereas the hyper-oxygenated mouse cultures rapidly lost cells (see Figure 1A: Experiment 1, $p = .0016$; Experiment 2, $p = .0025$). The quiescent mouse cell cultures suffered substantial cell loss with increasing times of exposure to oxygen, whereas the numbers of challenged quiescent avian cells remained relatively constant as compared to the unchallenged controls (see Figure 1B: Experiment 1, $p = .0025$; Experiment 2, $p < .001$).

Both starling cells (Figure 2, Experiment 1, $p = .0025$) and budgerigar cells (Figure 2, Experiment 2, $p = .0013$) were more resistant to hydrogen peroxide. Survival differences between Experiment 1 and Experiment 2 most likely reflect the difficulty of precisely controlling the effective concentrations of hydrogen peroxide because of a number of experimental variables. Small pipetting errors in making stock solutions and changes in effective concentrations during storage are likely explanations. Variations in residual traces of active catalase derived from the heat-inactivated bovine fetal sera added to the growth medium is another theoretical possibility. The relative heat stability of catalase may vary as functions of tissue origin and fetal age (12).

Figures 3 and 4 summarize our experiments with two additional agents known to generate oxidative stress: a chemical agent (Figure 3, paraquat, $p < .001$ for both experiments) and a physical agent (Figure 4, ionizing radiation, $p = .0045$). Once again, we find consistent evidence of superior resistance of the cultured avian cells to oxidative stress.

The levels of alkaline labile sites (which include single-strand breaks, double-strand breaks, and abasic sites) and the kinetics of repair of such damage in bird and mouse cells following challenge with hydrogen peroxide was examined using an adaptation of a previously described flow cytometric technique (10,11), as described under *Materials and Methods*. Figure 5 shows that the levels of damage sustained from such challenge and the prechallenge levels of such damage were significantly lower in the bird cells relative to the mouse cells ($p < .001$ in both cases). We were unable to detect a difference in the rate of repair of the strand breaks for mice and birds, perhaps largely because of the very low levels of breaks induced in the bird cells when using comparable doses of hydrogen peroxide.

Discussion

Gerontologists have appreciated the unusual longevity of a number of avian species for at least two decades (13), yet surprisingly little research addressing underlying mechanisms has been carried out. Most of the potentially relevant biochemical and cell biological research that has been carried out with avian materials has utilized poultry because of their easy availability and obvious economic importance. But such species are only weakly flying birds and are relatively short lived, given such parameters as body size, in comparison with a number of other avian species (2). A recent review has particularly emphasized the potential value, for gerontology, of research using passeriformes (e.g., *S. canarius*, the canary), psittaciformes (e.g., *M. undulatus*, also known as a budgerigar, budgie or parakeet), and columbiformes (e.g., *Columba livia*, the pigeon) (1). For example, the maximum life span of the canary is about 24 years, whereas that of the laboratory mouse, a mammal of comparable mass, is about 4 years. The fact that mice

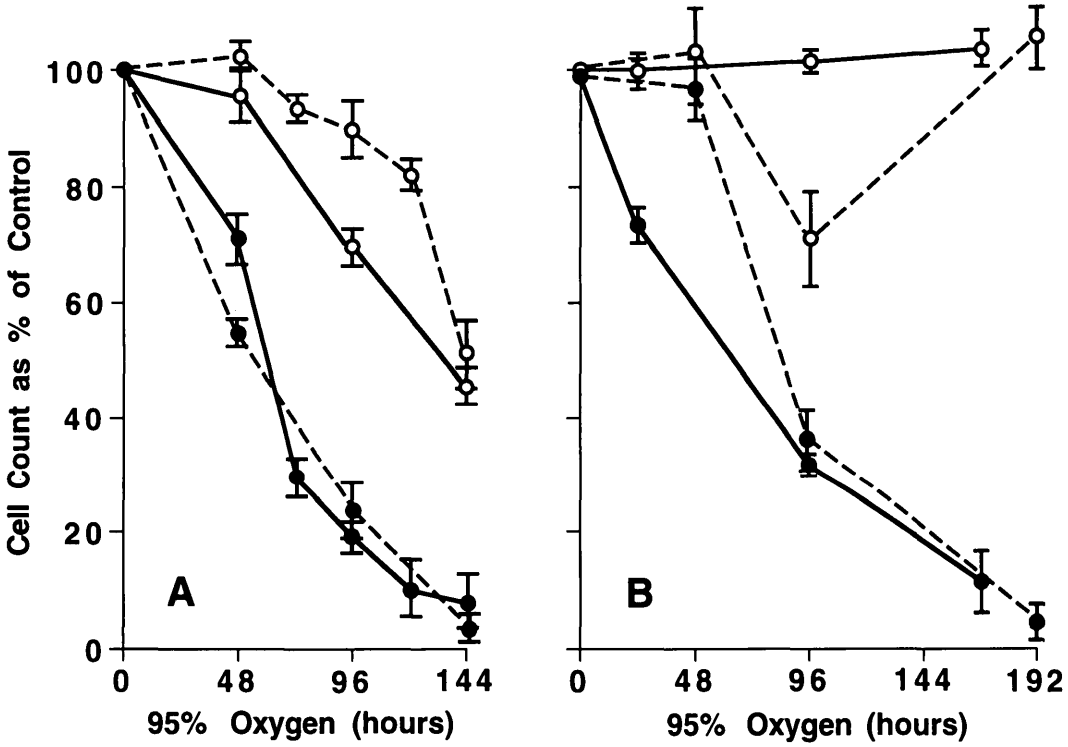

Figure 1. Survival (as a percent of control cell counts) of triplicate cultures of subconfluent renal tubular epithelial cells exposed to 95% oxygen: (A) Replicating cultures. Two experiments, in each case using replicating cell cultures independently derived from the pooled kidney tissue of three animals for each species (Experiment 1, dashed lines; Experiment 2, solid lines) with budgerigar (open circles) and mouse (closed circles) renal epithelial cells in DMEM with 10% fetal bovine serum. (B) Quiescent cultures. Two independent experiments with cells made quiescent in DMEM with 0.2% FBS prior to experiment. Experiment 1 employed canary (open circles, dashed line) and mouse (closed circles, dashed line) early passage renal epithelial cell pools made quiescent for 48 h, and Experiment 2 employed budgerigar (open circles, solid line) and mouse (closed circles, solid line) early passage renal epithelial cell pools (a different pool of three mice was used for each of the two experiments; three budgerigars and four canaries contributed to the bird cell pools) made quiescent for 5 days prior to experiment. Error bars are *SEMs*.

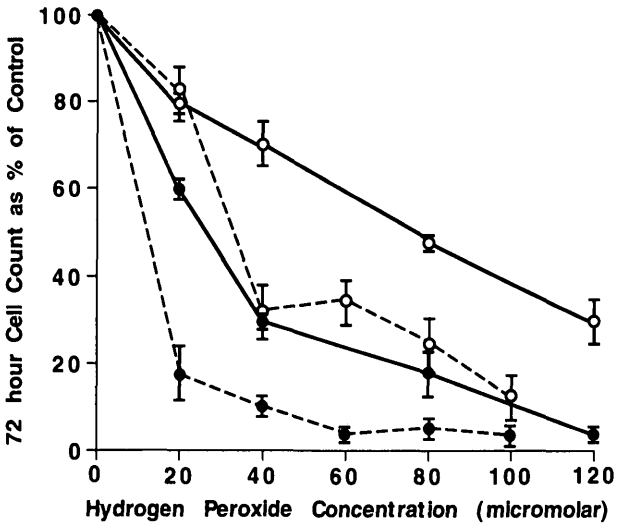

Figure 2. Two independent experiments measuring the survival (as a percent of control cell counts) of triplicate cultures of pooled early passage, replicating renal epithelial cells (in DMEM with 16% heat-inactivated serum) exposed to various concentrations of hydrogen peroxide for 72 h. Cells were refed with freshly prepared hydrogen peroxide containing medium every 24 h. Experiment 1 (dashed lines) compares starling (open circles; cells pooled from three birds) and mouse (closed circles; cells pooled from three mice) renal epithelial cells. Experiment 2 (solid lines) compares budgerigar (open circles; cells pooled from three birds) and a different pool of mouse (closed circles; cells pooled from three mice) renal epithelial cells. Error bars are *SEMs*.

have lifetime energy expenditures of around 8% of that of the canary would seem to argue against a theory of aging that considers variation in life span among homeothermic species to be a simple function of differences in metabolic rate (14,15). Holmes and Austad (2), however, argued that these observations are entirely consistent with the evolutionary theory of aging. That theory emphasizes the key role of environmental hazards in the evolutionary emergence of life history traits (16). High environmental hazards (predation, infectious disease, accidents, drought, and starvation) select for gene actions that result in relatively rapid sexual maturation and comparatively large numbers of progeny. Such rapid and substantial energetic investments in reproduction obviate the necessity for alternative strategies of evolving gene action that ensure longer life spans. There is indeed evidence that flightless or weakly flying avian species experience higher mortality rates in the wild than do avian species with highly developed capacities for flight (17).

In the present investigation, we carried out an initial set of experiments to test the hypothesis that unusually robust defenses against oxidative stress were among a number of potential types of gene actions that the evolution of "strong" flight eventually made possible. We compared the sensitivities of murine and avian somatic cells to each of four different types of oxidative stress. The results were

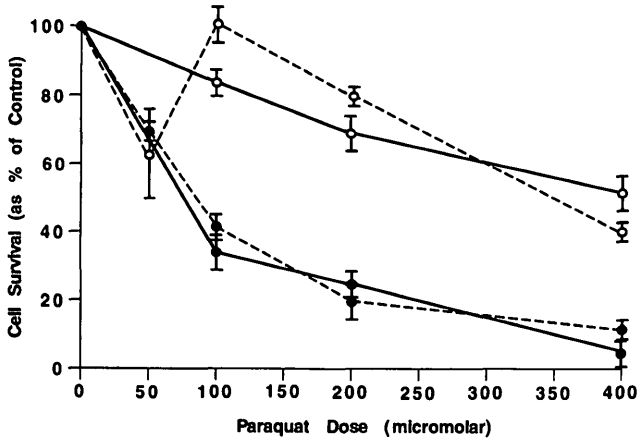

Figure 3. Two independent experiments measuring the survival (as a percent of control cell counts) of triplicate cultures of pooled early passage, replicating renal epithelial cells exposed to various concentrations of paraquat for 72 h. Experiment 1 (dashed lines) compares starling (open circles; cells pooled from three birds) and mouse (closed circles; cells pooled from three mice) renal epithelial cells. Experiment 2 (solid lines) compares budgerigar (open circles; cells pooled from three birds) and a different pool of mouse (closed; cells pooled from three mice) renal epithelial cells. Error bars are *SEM*s.

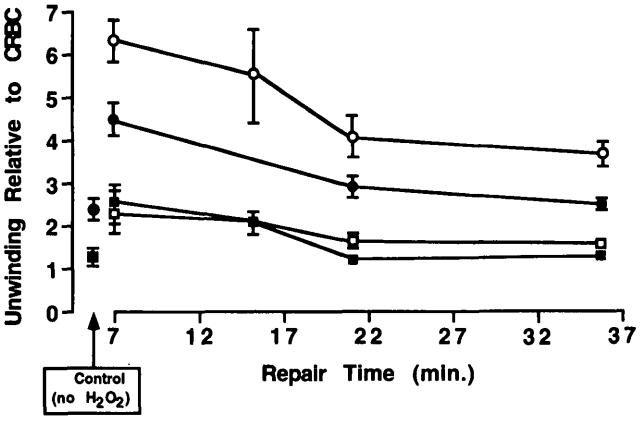

Figure 5. Flow cytometric measurement of alkaline-mediated unwinding of DNA (relative to control chicken red blood cell nuclei) in early passage, replicating renal epithelial cell pools of mouse (circles; cells pooled from three mice) and starling (squares; cells pooled from three birds). Shaded symbols represent control samples that received no H_2O_2 treatment. Closed symbols represent samples that were treated with 50 μM H_2O_2 for 5 min on ice followed by various repair times at 37°C. Open symbols represent samples which were treated with 100 μM H_2O_2 for 5 min on ice followed by various repair times at 37°C. Error bars are *SEM*s.

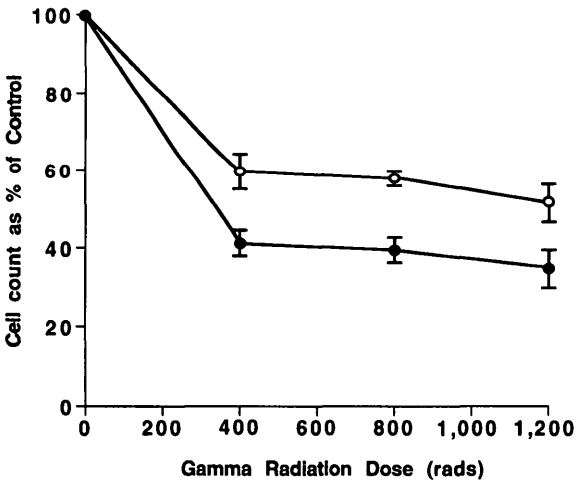

Figure 4. Survival (as a percent of control cell counts) of triplicate cultures of starling (open circles; cells pooled from three birds) and mouse (closed circles; cells pooled from three mice) early passage, replicating renal epithelial cell pools exposed to 0, 400, 800 and 1,200 rads of γ-radiation from a ^{137}Cs source, cultured for an additional 48 h, and then counted. Error bars are *SEM*s.

entirely consistent with our hypothesis. Moreover, the results were comparable for somatic cells isolated from two species of passerine birds (the canary and the starling) and from one species of psitticine birds (the budgerigar).

It would be of interest to extend these studies to longer-lived mammalian species. Preliminary results, comparing the survivals of quiescent cultures of human and avian renal tubular epithelial cells that are continuously exposed to 95% oxygen, do indeed indicate that the avian cells are more resistant, as might be predicted from a comparison of the lifetime energy consumption of humans (800 kcal/g/lifetime) relative to these three bird species (2000–3200 kcal/g/lifetime).

What might be the biochemical genetic mechanisms responsible for the superior resistance of avian somatic cells to oxidative stress? Based upon their comparative assays in pigeon and rat tissues, Ku and Sohal (18) have concluded that the pigeon may benefit from both lower rates of generation of superoxide and hydrogen peroxide as well as higher levels of various antioxidant defenses. Barja et al. (19) have proposed that the critical variable is the degree of free radical production near critical targets. Such critical targets would certainly include mitochondria, single copy nuclear DNA, and plasma cell membranes. The Barja group, like Ku and Sohal (18), found relatively low rates of generation of reactive oxygen species in birds, but in contrast to Ku and Sohal, found no evidence for enhanced levels of oxidative enzymes in birds; these levels were in fact found to be relatively reduced in birds as compared to shorter-lived species (20). One might therefore conclude that birds are resistant to oxidative damage mainly because fewer reactive oxygen species are generated per unit of oxygen consumption. Our work, however, indicates that when levels of oxidative stress are comparable (by the introduction of exogenous agents), bird cells are still more resistant than cells from a shorter-lived mammal (the laboratory mouse). It is therefore likely that a number of independent mechanisms have evolved in birds to result in their superior longevity in the face of their high metabolic rates. These mechanisms would certainly include alterations in the steady-state levels of certain nonenzymatic free radical scavengers. For example, remarkably high levels of uric acid have been noted in the tissues of the canary (21).

A genetic analysis has the potential to elucidate the relevant gene action. Given the relative vulnerability of cultured murine cells and the relative resistance of cultured avian cells to oxidative stress, it may be possible to design

a complementation assay suitable for the isolation and characterization of the avian genes conferring such resistance. These could potentially involve both nuclear and mitochondrial genetic information.

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