ELSEVIER

Experimental Gerontology, Vol. 32, Nos. 1/2, pp. 215–228, 1997

0531-5565/97 $17.00 + .00

PII S0531-5565(96)00037-X

PATHOBIOLOGY OF AGING RODENTS: INBRED AND HYBRID MODELS

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Abstract—The definition of inbred strains of animals is provided, underscoring the homogeneity of the individuals in a strain, as well as the lack of allelic variation within each individual. Inbred animals present long-term reproducibility and relative stability, which facilitates experimentation over a long period of time. The derivations of several specific groups of inbred animals including coisogenic, congenic, and recombinant inbred lines are detailed. Applications for inbred strains to the study of aging including analysis of longevity characteristics, genes involved in the control of age-related parameters and gene interactions with other genes or the environment are presented. The concept of aging as a consequence of genes and the ramifications of competitive pleiotropy are discussed. The distinction between aging and age-related diseases or lesions is explored. Cumulative lesion incidence is suggested as a biomarker of aging. *Copyright © 1997 Elsevier Science Inc.*

Key Words: mice, rats, pathology, gerontology, inbred, hybrid, nutrition, aging

INBRED STRAINS

A STRAIN of a species is inbred when virtually every genetic locus is homozygous. What this means is that all individuals within an inbred strain share the set of characteristics that uniquely define them compared to other strains. Typically, inbred strains are derived from 20 or more consecutive generations that have been brother × sister mated; the strain can then be maintained with this same pattern of propagation. Individual animals within an inbred strain are as identical as monozygotic twins. Inbred strains can also be made using other breeding programs, but the rate at which they become homogeneous is slower.

It is important to recognize that inbred animals are different from all others. They are not only genetically uniform, but they are homozygous at almost every locus. Noninbred strains of animals may be homozygous at some loci, but not homozygous at all loci.

There are several qualities of inbred strains that make them especially valuable for research. The first is their long-term relative genetic stability. This is important because it allows researchers to build on previous investigations. Genetic change can occur only as a result of mutation within an inbred strain. In outbred stocks, genetic changes come about through mutation and as well through changes resulting from selection and changes in gene frequency.

A second valuable quality of inbred animals is their isogenicity. Individuals are identical. Use of inbred mice controls for genetic variability. The homozygosity of inbred strains is a valuable quality because inbred strains will breed true. It is possible to produce individuals for replicate experimentation as well as for studies by other investigators. Additionally, because the genotype is uniform, it is possible to ascertain whether any particular individual is a member of a particular inbred strain.

When using an inbred strain to investigate any type phenomenon, it is important to be aware that the observations may be relevant to only that strain. Because an inbred strain differs from all others, there will be characteristics unique to it. It is, therefore, important to use more than one strain to confirm that any observation obtained pertains to the species and not just to the strain studied. This caveat is also applicable to the extrapolation of observations made in one species as a generalizable phenomena. It is wise to cross species lines as well as strain lines when attempting to label observations as having broad-based application.

Derivation of inbred strains

How are inbred strains derived? An extreme example in which two wild mice of the same species are utilized will be illustrative. This is an extreme example because many of the standard laboratory mice were derived from the collections of mouse "fanciers." Many of these animals had already been partially inbred when they were originally obtained for research purposes (Morse, 1981).

After breeding the pair of wild mice, mice in the first filial or F1 generation are produced. These will be heterozygous for any genes that were different but homozygous in each parent. Any gene that was homozygous and identical in the parents will be identical in all the F1 offspring. Any gene that was heterozygous in one or the other parent will segregate in the F1 generation, meaning that the F1 individuals will not be identical for that gene.

The next step is to select any brother and sister from the F1 generation and allow them to mate. The offspring produced are known as the F2 generation. The F2 are more variable with respect to genotype than were either the parental mice or the F1 generation.

Another brother and sister pair are selected from the F2 generation to produce an F3 generation. Assuming that the F1 mice were heterozygous for an allele, an F2 mouse will have a one in four chance of being homozygous for one isoform of the allele. The chance of selecting a pair of individuals homozygous for a given locus increases in succeeding generations (Green, 1981). Once an allele is the only one available within the population, the allele is now "fixed," unless a mutation occurs, recreating the lost allele. Experience and statistical analysis demonstrate that within 20 generations virtually all genetic variability will be lost from repeated brother × sister matings. Beyond this point, mutation introduces new variance at a rate at least as great as the rate at which inbreeding removes variation. An important point in this derivation of an inbred strain is that only one brother and one sister be mated in each generation. If the program is complicated by cousin × cousin, uncle × niece, or aunt × nephew pairings; although an inbred genotype will eventually be produced, it will take longer. This is because such matings may reintroduce alleles already removed from the line.

Categories of inbred strains

There are several special categories of inbred strains that have an application for research.

Substrains. Substrains are defined as related inbred strains that were derived from a single inbred strain that have been separated for more than eight generations. This commonly occurs

when inbred animals are acquired by vendors, who then maintain inbred colonies of animals derived from the common source. Individual investigators, maintaining their own animal colonies will also eventually develop their own substrains.

Coisogenic strains. Coisogenic strains are defined as two strains of a species that differ at a single genetic locus. This difference is due to a single mutation in a genetic locus, which can occur spontaneously or can be induced through laboratory intervention. After identification of the mutation, the individual and its siblings are bred, followed by subsequent brother × sister matings, until the new strain is inbred. This new inbred strain will differ from the parental line from which it was derived by only one gene.

Congenic strains. The laboratory approximation of coisogenic strains are congenic strains. Two congenic strains are two inbred strains that differ by a single gene, the difference being that the mutation did not originally occur within the strain used to derive the congenic pair. When a mutation of interest occurs in mice of ill-defined genetic background or on a background other than the one of interest, an individual with the mutation can be bred with an inbred mouse with the desired background. Individuals in succeeding generations expressing the trait of interest are then bred with individuals of the desired inbred strain. This process of backcrossing continues until the inbred strain and the newly derived inbred strain differ by only the locus of interest.

Recombinant inbred strains. Recombinant inbred (R.I.) strains effectively scramble two different genomes and result in a series of inbred lines, each of which has a unique assortment of genetic material from the two initial parental genomes. The starting points are two inbred genotypes that are used to produce a group of F1 hybrids. Brother × sister pairs of F1 hybrids are mated to create an F2 generation, in which all genes now segregate independently. Next, a series of brother and sister pairs are established. In each subsequent generation from each pair only a single male and female are mated. After 20 generations, one has many inbred lines that differ from each other due to random differences in gene segregation, a process begun with the F2. All the R.I. lines contain only those genes that were present in one or another of the parental strains. R.I. lines have been very useful in genetic mapping of traits that differ between inbred strains. An example of the application of R.I. strains to biological questions was a study demonstrating that there are definite chromosomal regions correlated with the differences in longevity between the C57BL/6 and DBA/2 strains of mice (Gelman *et al.*, 1988). The longevity of 20 recombinant inbred lines were examined: two strains had significantly shorter survival than average and five strains demonstrated increased longevity. The conclusion was that for these R.I. strains there was no single genetic marker that accounted for the variance in survival times, although groups of six or more markers appeared adequate to account for the differences. The investigators concluded that several genes act in groups to confer longer life span. This work illustrates the complexity of the genetic contribution to aging and/or longevity and serves as a first approximation for mapping chromosomal regions coding for genes controlling longevity.

Mapping genes

Work with R.I. lines for identifying areas in the genome that modulate longevity demonstrates the utility of R.I. lines in identifying genes. These sets of inbred strains provide one means for determining the area in which genetic information of interest can be located. Another technique for identifying location of genes involved in inheritance of a trait is the back cross. Consider a simple case involving a mutant phenotype found in mice of defined coat color. To determine

whether the gene mutation is linked with the coat color, one mates mice with the mutation with mice of a second coat color without the mutation. If the gene for the mutation and coat color are closely linked on the same chromosome, then every mouse exhibiting the mutant phenotype will have the first coat color. If the two genes are distant on the same chromosome, or on different chromosomes, then only 50% of the mice with the mutant phenotype will have the first coat color. The use of the back cross for gene linkage analysis is a stochastic process requiring classical statistical-genetic analysis.

Genetic mapping initially relied on physical characteristics such as coat color, neurological markers, or body size as the polymorphisms or genetic variations with which to establish linkage. This is because these phenotypes were the only genes with multiple alleles expressed in a manner that could be monitored. As the technologies developed, isozymes or allelic variants of enzymes could be added to the list of genes expressed in several forms that could be identified with relative ease. This increased the number of markers that could be monitored and, thus, used for mapping (Ruddle and Roderick, 1968). As the construction of genetic linkage maps progressed, it was recognized that important data was often missing. One solution developed was the use of variation in DNA sequences conveniently observed in restriction fragment length polymorphisms (RFLP) (Lander and Botstein, 1986). Further investigation demonstrated that simple sequence repeats (SSRs), utilizing tandem repetitions of di-, tri-, or tetranucleotides, provide an abundant source of genetic markers that can be typed using polymerase chain reaction assays (Love *et al.*, 1990). There are commercially available markers for the SSRs, permitting establishment of genetic linkage of a mutation in several months instead of years. Computer programs such as Mapmaker readily translate the information generated and calculate probable gene order and interlocus genetic distances (Dietrich *et al.*, 1992).

Genetic consequences and inbred strains

As inbred strains are being developed, several lines are propagated simultaneously because the original wild heterozygotes from which the process was begun likely carried one or more alleles conveying undesirable traits or even lethality. Additionally, each founder animal may have carried alleles that interacted lethally with a portion of the genome from the other founder. This problem of lethality with inbreeding also occurs as recombinant inbred lines are developed. Many lines of brother × sister matings are set up at each generation, but, inevitably, some lines die out because of deleterious recessive genes in either of the founder stocks, which by chance become homozygous and fixed in a line, assuring extinction of that line.

There are several ways in which extinction is observed to occur. These include embryonic lethality, death in the perinatal period, runting with death at the time of weaning or sterility in either or both genders; in addition, recessive lethal characteristics can be manifested later in the onset of degenerative, inflammatory, or neoplastic disease processes. Most of these later-acting processes have been discovered in relatively young animals, because few strains of rodents are routinely maintained through old age. A few examples of such diseases occurring in young adulthood are: NZB mice, which develop autoimmune hemolytic anemia within the first few months of life (Auer *et al.*, 1974); LPR mice, which develop fatal lymphoproliferative disease that begins by eight weeks of age (Green, 1990); and NOD mice, which develop early diabetes in nonobese animals (Makino *et al.*, 1980). Some examples of lethal processes that occur in slightly older adult mice are the Purkinje cell degeneration by eight months of age in the Harlequin hair coat (Hq) mutation (Bronson *et al.*, 1990), and motor neuron degeneration mouse (mnd), which develops neuronal ceroid lipofuscinosis with severe neurodegeneration starting

between eight and nine months of age (Bronson *et al.*, 1993). It is essential that the gene or genes responsible for adult death in inbred strains that die in early or late adulthood be genetically mapped and eventually cloned.

However, one outcome of inbreeding is that inbred mice may be quite normal. They may have somewhat smaller litters, but can have life spans nearly as long as outbred stocks of mice. C57BL/6 mice are inbred but have a fairly long life, for example. Other laboratory rodents were originally derived from a few breeding pairs and are, by default, inbred stocks. These include the Syrian hamsters, which originated from three individuals obtained from a single litter captured in Syria in 1930 (Van Hoosier and Ladiges, 1984). These inbred animals we study in science and keep as pets are very successful, in spite of their inbred status.

Some highly inbred stocks are known to continue to be quite successful, even in the wild. Cheetahs are almost completely inbred (O'Brien *et al.*, 1983), but are not yet extinct. Inbred status is especially undesirable, however, in a variable environment. In the wild, it is these inbred stocks that are doomed to extinction if environmental conditions change, because there is no allelic diversity from which to select those traits that may be useful for survival in the new environment. Even in the relatively controlled conditions of the laboratory, inbred animals are at an environmental disadvantage compared to outbred or hybrid lines. Inbred strains are likely to be less resistant to minor perturbations in the environment. Paradoxically, though they are genetically uniform, they are likely to be more diverse phenotypically than partially outbred mice or even F1 hybrids. This results from the increased sensitivity of inbred strains to environmental alteration. Simply stated, because inbred mice have the same alleles at every locus, their response to even minor physiological insults is controlled by only a single genetic response. This contrasts with F1 hybrid mice, which have two different alleles for many genes and, therefore, increased diversity of gene products that may serve to dampen the magnitude of the observed response to minor environmental fluctuations (Phelan and Austad, 1994).

Genetic contamination and mutation

It is important to maintain proper animal husbandry to insure production of the anticipated genotype. The risk of production of offspring arising from an accidental outcross is increased in a facility in which several strains with similar phenotypes are maintained in a single room. Under these conditions, it is possible for an animal to be placed in a cage that is either incorrectly labeled or that houses individuals of the opposite gender with a different genotype. Either may eventually result in the production of offspring without the intended genotype. Additionally, when several lines are housed in a single room, it is easier for a stray animal of one genotype to encounter animals with other genotypes.

The idealized situation would be for inbred populations to be perfectly stable. Mutation rates are low and the manifestation of observable phenotypes often permits detection of changes to the genotype. However, not all mutations are rapidly noted and it can be, that over a period of time, there is notable change to an inbred population. New mutations can occur and become quite widely dispersed within a stock before being discovered. The most common means for this to occur is for an individual to be bred with other members of the strain, thereby dispersing the recessive gene among their progeny. Eventually, the gene will appear in homozygous form in some descendant of the progenitor animal. If the trait results in embryonic lethality, when two heterozygotes are bred a 25% reduction in litter size will be observed.

An important concern is that the discovery of new mutations will be aided if inbred strains of mice are maintained by strict brother × sister mating. In this situation, any new recessive

mutation can be observed in the F2 or later generations, because mice heterozygous for the gene will have a maximal chance of producing homozygous offspring. On the other hand, if any mouse of an inbred line is mated to any other within the line, individuals homozygous for the mutation may not be produced for many generations. This is one explanation for the assumed lack of spontaneous mutations in some large commercial breeding laboratories, where inbred animals are not propagated by brother × sister mating. It may be a perceived advantage by such firms who prefer to market their inbred animals as being free of mutations. This may placate naive customers but it is not an honest assessment of their product.

The genetics of limited colonies is not only different from the colony from which it was derived, it is different from all other colonies of animals. For inbred strains, these populations would be defined as substrains. However, for outbred populations, these colonies are genetically isolated groups of animals that are heterozygous by definition. Through chance alone, some alleles will be more prevalent in one group than in another. Unique random mutations will inevitably occur in all the different isolated populations. Furthermore, over time there will be differences in the environmental conditions for the different isolated groups that may hasten genetic divergence between the various groups. One means by which this could occur is through natural selection of mutations that confer enhanced fecundity under the conditions in which the animals are housed. Genetic changes, such as these, within isolated populations, are known as "genetic drift" and occurs in colonies of outbred as well as inbred populations.

Genetic drift is difficult to assess in outbred populations. There are, however, means to monitor the genotype of the animals within inbred colonies, especially for mice. Each inbred strain has a unique pattern of genomic markers. Any genomic deviation indicates that either a mutational event has occurred, or an error involving animal husbandry has introduced genetic variation into the colony.

An important example, germane to the biology of aging, of a phenotypic change occurring in an inbred population has been observed in the F344/N, an inbred strain of rat. Over a period of 11 years there has been a trend towards increased average body weight among the F344/N (Rao *et al.*, 1990). During this same period, there has been a concomitant decrease in longevity, due in large part to an increased incidence of leukemia.

It is not known at this time what genetic or environmental change accounts for the change in longevity and age-related pathology in these rats. It will, therefore, be difficult to determine the cause of the observed changes. There is no living cohort of individuals that demonstrate the previous phenotype, so direct comparison of individuals manifesting the two phenotypes is not possible. There have been several proposals to remedy this situation. One entails selecting for lighter weight animals in an attempt to rederive the strain for its previous weight. This is not a genetically supported approach because the F344/N had been maintained for many generations through brother × sister matings and so all genes would be fixed. It is not possible to select traits in inbred populations. Another suggested solution has been to modulate the weight of the strain by controlling the caloric consumption of the group. The physiology of such an animal population would be characteristically different from the previously *ad libitum*-fed population and possibly inappropriate (Hart *et al.*, 1995).

Do outbred lines exist? And are they a reproducible resource?

Brother × sister matings will result in the rapid generation of an inbred line. However, any population of animals will inevitably become inbred if it is reduced to some critically small size, perhaps as high as a few thousand animals. This has implications in the management of

endangered species; it is also relevant to the management of animal colonies. Colonies of so-called outbred animals are complicated by two genetic issues. The animal colonies of commercial vendors are typically established with a small number of males and females. Because the number of founder animals is limited, the genetic diversity present in the colony at its onset is a small portion of that within the original outbred population. The diversity is then further limited through interbreeding within the colony. Although brother × sister matings result in the generation of an inbred strain in the least number of generations, other familial breeding pairs will also generate inbred strains. The end result is that the colony will become inbred.

There are serious issues regarding the genetic heterogeneity of commercially available outbred strains. Several outbred strains were derived from inbred strains. This is illogical because there was miniscule heterogeneity at the onset. The vendor may be describing these animals as outbred simply because the stock are not maintained by brother × sister mating. This, however, does not generate broad based genetic variability. A second compromise to the genetic heterogeneity of outbred strains arises when commercial vendors improve their physical facility, and select a single pair or small number of animals from which to rederive their colonies of outbred animals. This acts as a bottleneck, further reducing the potential genetic variability of the population. It is not possible to breed heterogeneity into a homogenous population without the use of genetically diverse individuals.

Heterologous stock

If the premise for the use of outbred strains is a model that more closely approximates the genetic variability present within human populations, a more appropriate solution may be the use of heterologous stock. Well-known heterologous stock have been generated starting with eight inbred strains of mice that produced 28 distinct F1 hybrids. The next generation was derived by crossing individual F1 hybrids such that there were no common ancestors between them at the parental or grandparental level. This results in 420 possible combinations, although in practice, it is common to use a subset of 10% or less (Strauss *et al.*, 1992). If generations beyond the F2 are produced, there is still the tendency to drift towards homogeneity. The F2 stock are heterogeneous populations that can be replicated. Although this is a painstaking approach, it does result in the desired genetic heterogeneity in a population that can be repeatedly produced.

F1 hybrids and hybrid vigor

Inbreeding results in the creation of a population with no genetic variance, i.e., all individuals are identical. A second characteristic of inbred populations is that each individual is homozygous at virtually all loci. Having a population of identical individuals is highly desirable; homozygosity may not be. The use of F1 hybrids separates these two phenomena. The first filial generation derived from two inbred strains are as identical to one another as individuals in each of the parental strains. The major difference is that they are heterozygous at many loci as opposed to the homozygosity of the parents. Examination of coefficients of variation (the standard deviation divided by the mean), demonstrates that F1 hybrids are phenotypically less variable than the inbred strains from which they were derived (Phelan and Austad, 1994). It has been observed that the F1 hybrids derived from two inbred strains most often have greater longevity than either parental strains (Russell, 1941). One consideration for the use of F1 hybrids may be the somewhat increased maintenance costs because colonies of two inbred lines must be maintained for the production of the F1 hybrid.

GENETICS OF AGING

Numerous studies demonstrate a range of median longevity for inbred strains of mice and rats. This suggests that the time frame in which animals of a given species die is not fixed within the species, but rather there are genetic factors involved in its control. Furthermore, there are differences in the mortality rates for different inbred strains, as well as differences in mortality kinetics as indicated by the shapes of the mortality curves for different inbred strains. This suggests that there are differences in rates as well as processes involved with aging. Several strategies have been proposed for identifying genes controlling life span (Vijg and Papaconstantinou, 1990).

The use of genetically tightly defined populations for research on aging allows the study of age-related processes in an informed fashion as a result of the wealth of available background information. It is difficult to observe significant effects if the variability between individuals for the parameter being followed is large. Inbred, and especially F1 hybrid animals, are models in which this variability is minimized. Specific interventions to alter the time table of aging can be assessed only in groups of animals where the patterns of aging have been previously defined. In the study of aging, it is important to be aware of changes that occur with age as well as the incidence of disease processes that may affect the parameter(s) of interest.

A means for genes to influence aging is in interaction with the environment. The phenotypic manifestations of any characteristic are derived from environmental factors as well as the genotype of the individual. The use of animals with definable and reproducible genetic backgrounds permits the distinction to be drawn between innate and environmental influences on physiological characteristics. It is an especially critical consideration in life span studies, where environmental factors impact individuals over a protracted period. Laboratory rodents present an advantage for the study of aging, in addition to the availability of multiple inbred strains, because conditions for maintaining them are well regulated. Barrier-housed animals do not have unintentional exposure to microbes or known mutagens or carcinogens. The light/dark cycle, water mineral content, room humidity, and temperature, as well as diet composition, are tightly controlled.

An example of the interaction between environment and genotype in aging is observed with age-related amyloidosis. The deposition of amyloid in the kidney, intestines, liver and other internal organs in mice had, for many years, been understood as a disease process that occurred in older animals and was assumed to be solely a disease of aging. It was clear that there was a genetic component as some inbred strains were susceptible, while others were not. Upon closer examination, however, even for susceptible strains, there appeared to be environmental factors necessary for development of the disease. It was demonstrated that when housed individually rather than in cages with other animals, C57BL/6 and DBA/2, two inbred lines that are susceptible to amyloidosis, do not manifest the disease (Lipman *et al.*, 1993).

The use of genetics in the study of aging raises questions related to how genes affecting the processes of aging or longevity might be selected. Most age-related changes are not beneficial. Such genes might be responsible for the downregulation of physiological functions that demonstrate age-related deterioration. By definition, the action of such genes directly acting to cause these changes would be considered deleterious. Genes that confer beneficial effects in young animals, and that have deleterious effects beyond reproductive age, would still be positively selected in a population. Additionally, while there is selective pressure for genes conferring advantage to young animals, there is no similar selection for beneficial characteristics expressed in the older individual. The selection of such genes is known as antagonistic pleiotropy, where

selection is based on early beneficial effect, without regard to the consequences manifest in the older individual. As any population evolves, more and more of these genes may accumulate. Antagonistic pleiotropy provides one mechanism by which deleterious traits of aging could be postulated to accumulate (Rose, 1991).

There are also interactions among different genes that may play a role in aging. The one gene-one phenotype model is too simple to fit the biological reality. There are mutations, for example, whose expression is controlled in part by the genetic background in which the mutation resides. The mutant gene may be completely or partially compensated for by a modifying gene. This situation is exemplified by the identification of *Mom-1*, a gene that reportedly controls 50% of the genetic variation in tumor number in the Min (multiple intestinal neoplasia) mouse, which carries the mutant *Apc* gene (Dietrich *et al.*, 1993). Although complicated, complex multigene interactions can be analyzed.

AGE-RELATED DISEASE PROCESSES IN AGING

Large studies in which significant numbers of animals are examined at a variety of ages can be used to explore several interesting points including the relationship between age and disease processes, the interaction of genetics, age, and disease incidence, as well as the extent to which environmental factors have a role in determining disease incidence.

An interesting and significant aside that can be gleaned from the comparison of experiments with either longitudinal and cross-sectional design is the rate at which disease processes affect mortality data. The question is whether lesions observed in a cross-sectional analysis are similar to those observed in a longitudinal study. If disease processes that result in the death of the animals progress rapidly, the incidence of such processes might not be accurately represented in a cross-sectional study. Discrepancies of this sort are dependent on time periods between examination as well as the rate at which lesions result in the animal's death. When representative animals were examined every six months, while there were strong similarities between the most commonly observed lesions and those found in longitudinal studies (Bronson and Lipman, 1991) the prevalence and incidence rates for all lesions were not identical. This is seen with the relatively frequent observation of atrial thrombosis in the C57BL/6J mouse from unpublished longitudinal studies conducted by Harrison and Archer at the Jackson Laboratories compared with at least two cross-sectional studies (Bronson, 1990; Bronson and Lipman, 1991). However, many of the mouse malignancies such as alveolar histiocytosis, FCC lymphoma, harderian gland adenoma, hemangiosarcoma, hepatocarcinoma, hepatoma histiocytic sarcoma, and lung adenoma are commonly observed in experiments of either design. This somewhat surprising observation suggests that progression of these lesions in mice occurs over a period of time greater than six months in length. There are important practical implications for this observation. It suggests that experiments that are designed to be cross-sectional will be nearly as informative regarding the disease processes present as a function of age as would be perfectly executed experiments which are longitudinal in design. However, it is practical to have animals specifically designated for examination at convenient times, rather than to process them individually as they die. Rodents undergo rapid autolysis and have the unfortunate predisposition to succumb during the night. This set of circumstances results in a number of animals being rendered useless for study of their tissues. This demonstrates a benefit of cross-sectional experiments, where the tissues from animals can be processed so as to provide the most informative sections possible.

A fortuitous observation regarding lesions is that for a given strain, or even a given species, there is a finite number of lesions that will be observed to occur. In three studies involving large

numbers of rodents, the number of distinct lesions observed ranged from 178 for rats and 186 for mice (Bronson, 1990), 135 for rats (Bronson and Lipman, 1991) to 450 for rats (Lipman *et al.*, 1996). Part of the discrepancy for the variety of lesions observed is due to different numbers of strains studied. Each strain may have lesions which are unique to their genotype. Another source of variation is the level of detail considered. Although 450 lesions were observed to occur in three genotypes of rat, only 54 actually occurred in more than 6% of the animals in at least one gender-genotype cohort. This is comparable to the 51 common lesions out of 395 observed in another large study of rats, in this case the F344 (Maeda *et al.*, 1985).

It would be a mistake to conclude that it is appropriate to ignore rare lesions. Rare lesions contribute significantly to the average lesion burden of the group, comprising, for example, 88% of the observed lesions in the F344 × BN F1 hybrid. Infrequently observed lesions are also of significance to the individual animal. One method for dealing with such data is to examine the total lesion burden observed per animal as a function of age.

The age-related variability in prevalence of lesions is demonstrated by calculating the average number of lesions that animals develop over their life span. This number increases as age increases, suggesting the measure is a biomarker of aging. The utility of lesions in this capacity can be illustrated using data generated during an ongoing project characterizing the pathology present as a function of age in different inbred and hybrid strains of mice and rats. Examination of the average number of lesions observed at necropsy in female B6C3 F1 hybrid mice, as seen in Table 1, demonstrates a progressive increase with age. Analysis of the effects of calorie restriction, an experimental intervention repeatedly demonstrated to increase longevity, shows the average number of lesions per mouse is decreased at each age examined as compared with *ad libitum* fed animals. While it is true that the lesions noted in the youngest ages are of dubious biologic significance, it is of interest that even at six months of age, the difference in average number of lesions per animal for the calorie restricted mice (0.667 ± 0.6) is significantly less than that of the age-matched, *ad libitum* fed mice (2.1 ± 1.0), $p = 0.0001$. This indicates that there are effects of calorie restriction that are measureable early, and that the processes modulated by calorie restriction are not limited to those that are terminal.

A utility for large data sets is for analysis of variability based on gender, strain, species, and environmental conditions. Comparison of average number of lesions observed in male BN and F1 hybrids rats derived from BN and F344 demonstrated that the hybrid animals had significantly lower lesion totals ($p \leq 0.001$) (Lipman *et al.*, 1996). This is the outcome predicted on the basis of hybrid vigor. These hybrid rats had decreased incidence of a variety of lesions

TABLE 1. COMPARISON OF LESION BURDEN FOR *AD LIBITUM* FED AND CALORIE RESTRICTED B6C3 F1 HYBRID FEMALE MICE

Age (months)	Ad libitum *fed: Average number of lesions/mouse*	*Calorie restricted: Average number of lesions/mouse*
6	2.1	0.7
12	4.5	2.1
18	8.3	4.3
24	13.0	6.9
30	15.6	8.3
36		12.0

including heart enlargement, hydronephrosis, pancreatic acinus hyperplasia, and extracortial nodules in the adrenal glands. An interesting dichotomy in prevalence was observed with a set of three lesions: interstitial cell hyperplasia, interstitial cell adenoma, and testicular atrophy. The hybrid rats had a statistically significant increased incidence of both interstitial cell hyperplasia and adenoma, while the BN rats had an increased incidence of testicular atrophy. It could be concluded that the increased incidences of the interstitial cell lesions contradicts the concept of hybrid advantage; however, it may just be that the increased incidence of a separate lesion, testicular atrophy, simply precludes the occurrence of the lesions involving the interstitial cells. This is a relatively simple example of the potential for one lesion to impact on the incidence and perhaps progression of another.

The demarcation between aging and age-related diseases is clearly not distinct. The incidence of a variety of disease processes increases with chronological age. It is possible to conclude that the process of aging is simply a compilation of deleterious physiological changes including accumulation of lesions. This would define aging merely as the accumulation of structural and functional abnormalities. Such a definition blurs the distinction between normal aging and disease process. The lesions observed in an individual are dependent on a number of factors including genetic inheritance, environmental factors, and stochastic events, as well as age. While patterns of lesions will demonstrate individual variability, there are also generalized age-related patterns of lesions. Aging includes phenotypic changes, the number and often severity of which increase over time.There are various aspects of the aging process that are modulated by lesion status. Cataloging the lesions present in cohorts of inbred strains will permit comparison of the different ways of aging in relation to patterns of lesions. It is necessary to know the generalized lesion accumulation for a strain of animals to distinguish the disease-related from the age-related components of processes that change with time. This is a difficult task, the importance of which is that not every difference between young and older animals is a consequence of aging per se. It is difficult to distinguish between modification of aging, remediation of disease, or combinations of these. For example, in the F344, a rat strain with progressive kidney degeneration differences between young and old animals in metabolites found in urine may be more a function of disease progression than age-associated deterioration. The glomerulonephritis observed in the F344 is observed to occur in other strains of rats; however, it occurs relatively late in life and is quite mild in the Brown Norway, for example (Bronson, 1990), suggesting genetic control. This exemplifies the intertwined relation among pathology, aging, and genetics.

The general trend is for laboratory rodents to develop lesions with increasing frequency as a function of age. This is in spite of their tightly controlled environments. It is possible to conclude from this that development of lesions is endogenous to the process of aging in these populations and to extrapolate that the same is true for other populations of mammals. Lesion incidence functions as a biomarker of aging in mice by definition because the number of lesions per individual increases as a function of age. The utility of such lesion information is in comparison between groups for rates of aging. For example, comparison of lesion incidence of similarly aged animals demonstrates a marked effect of caloric restriction on lesion incidence, presumably indicating a difference in aging between the different diet cohorts (Bronson and Lipman, 1991).

While cumulative number of lesions at a given age functions as a biomarker for the population, genetics also has an important effect on the number and variety of lesions present within populations. There are clear examples of lesions that occur significantly more frequently in one genotype than another; in one gender than the other; as well as in one species than in another. Two notable applications for such information are the selection of animal models that are not

prone to specific lesions that may perturb specific experimental results, and, secondly, as a guard against the assumption that the observations obtained from the study of one genotype are characteristic of the entire species. Lesion incidence and longevity are intimately linked and it is often informative to consider these parameters together. Comparison of the age at which 50% mortality is observed for several strains of rats demonstrates phenotypic differences between genotypes (Lipman *et al.*, 1996). Examination of the confidence intervals demonstrates that the age at which 50% mortality is observed differs significantly between strains. The F344 have reduced longevity as compared with either the Brown Norway (BN) or the F1 hybrid derived from the cross between them (F344BNF1). Examination of the lesion pattern and incidence with longevity demonstrates the interrelation between these parameters. There is decreased longevity observed in the F344 compared with the BN (Table 2).

In every study of aging involving inbred, hybrid, or outbred animals, some animals die early and others later. This phenotypic variability within inbred strains is theoretically troublesome. There are also some differences between animals in pathology profiles. These variabilities can be explained in outbred stocks, because they are genetically different. Within inbred and hybrid strains, however, the individuals are genetically identical. It is difficult to postulate differences to account for one mouse of an inbred strain dying at two years of age with lymphoma and another at three years of age from pituitary adenoma. Factors such as "residual heterozygosity," environmental differences such as cage position or dominance order for group-caged animals have all been posited as accounting for such differences. There is a general reluctance to consider that aging involves processes that are stochastic in nature. However, if aging is viewed as increased loss of homeostasis, then as animals age they would be predicted to become more and more different from each other, by chance alone. This adds a random component to outcomes such as time and cause of death.

Experiments that extend over the lifetimes of the subjects are expensive. It is less costly to study a group of animals whose maximum longevity is two years rather than three years. However, it may be problematic to study aging in a model that dies at an early age. It is unclear whether animals whose longevity is truncated due to a specific disease can be said to undergo the process of aging. While some diseases themselves are considered as models for accelerated aging, there may be fundamental differences between the processes involved in accelerated and "normal" aging. Additionally, if death occurs prior to the physiological changes that normally accompany advanced chronological age for the species, it may be that the animals are reaching the end of their life spans before significant aging has occurred.

The designs of experiments to study aging are often fraught with compromise. Experiments with a longer lived strain of animal are inherently more expensive due to increased cost of

TABLE 2. COMPARISON OF MORTALITY KINETICS FOR BN, F344, AND F344BNF1 RATS*

Genotype	*Age in weeks for 50% mortality (male)*	*Upper 95% confidence interval*	*Age in weeks for 50% mortality (female)*	*Upper 95% confidence interval*
BN	129	142	133	141
F344	103	108	116	123
F344BNF1	145	151	137	143

*Reprinted from Lipman *et al.* (1996).

housing animals for longer times. The use of F1 hybrids, with decreased variability, may help offset this by reducing the number of animals required per group for statistical significance. It is advantageous to use cohorts of animals that are relatively lesion free for a longer period of time because they provide a larger window in which to compare changes associated with age without the complication of disease. It can be argued that it may be advantageous to use strains with fewer causes of death because group homogeneity is preserved even at the end of their life span. For whatever animals chosen, it is of critical importance to know the pathology and understand how common lesions will effect measured parameters. Use of inbred strains or F1 hybrids is important in gerontologic investigations due to the element of time. Comparison of animals of different ages requires use of cohorts born at different times or measurement of parameters on different dates. Either time factor potentially contributes to variability. Maximizing genetic homogeneity among the individuals studied will help minimize such complications.

The study of lesions is a necessary component for the study of aging as well as an independent parameter for the study of aging. Inbred strains are of value for the study of age-related disease processes because there are strain specific patterns of lesions. Aging in mammals is accompanied by age-related changes ranging from cosmetic to the malignant. It is of theoretical interest to determine the extent to which these changes are linked to aging. An alternate perspective is that these lesions constitute aging. The set of changes an individual develops with age is partly dependent on environment including exposure to infectious agents and various toxins, diet, and genetics. The use of inbred and F1 hybrid animals as model systems in which to study aging helps to focus the study on the processes of aging. The use of older animals to evaluate the applicability of specific drug therapies for geriatric medicine depends critically on the ability to distinguish spontaneous events from toxicological ones. Background information on the animals that includes incidence of lesions under the defined experimental conditions is necessary to make this distinction. The evaluation of drug toxicity in older animals in the absence of spontaneous disease is the unattainable gold standard. However, informed choice of animals that includes the specific disease processes to which they are predisposed will facilitate the generation of useful information.

Acknowledgments—This research was supported by National Institute on Aging grant AG07747 and in part from Federal funds from the U.S. Department of Agriculture, Agriculture Research Service under contract number 53-1950-5-003. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture nor that of the National Institutes of Health, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. I would like to thank Drs. Roderick Bronson and Jeffrey Blumberg for their generous support.

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