GENES CONTROLLING SUSCEPTIBILITY TO MAMMARY CANCER

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
This report documents results from DAMD17-97-1-7123, which is designed to identify nutritionally modulated genetic loci in the mouse that alter susceptibility to breast cancer. Calorie restriction (CR) is a potent nutritional intervention, well documented to reduce carcinogen induced mammary tumors in rodents. The study underway is currently documenting the relative mammary tumor prevalence in 8 inbred strains of mice after being dosed with carcinogen. The mice in each genotype have been divided into two dietary groups, control and CR. CR has been successfully implemented in all strains after the mice were treated with carcinogen, demonstrating the feasibility of this nutritional intervention after carcinogen treatment. Although CR has resulted in statistically significant reductions in body weight, only 7 out of 8 strains demonstrate significant increases in longevity. In terms of tumorigenesis, this study has demonstrated genotypic specificity in the response of mice to carcinogen exposure. Taken together, these observations will be valuable in the search for gene controlling the effects on breast cancer of nutrition intervention.
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PI - Signature    Date
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Introduction

The purpose of this project was to identify the gene or genes responsible for controlling the reduction in mammary cancer response of mice obtained with calorie restriction. Mice subject to calorie restriction generally exhibit a reduced incidence and/or delayed onset of cancer [1] as well as an increase in longevity [2]. The importance that identification of the gene or genes responsible for these effects of calorie restriction would have with regards to breast cancer is two fold. The first application would be to identify those individuals who might benefit from prophylactic dietary intervention to prevent and/delay breast cancer. The second benefit identifying genes controlling the response to calorie restriction would yield is in understanding the mechanism by which this response is elicited. The potential exists for the development of therapeutic interventions that could similarly decrease breast cancer incidence and/or increase its age of onset. The scope of this project is to identify strains of mice which demonstrate differential mammary tumorigenesis response to calorie restriction and then to identify the gene or genes responsible.
Body

Approved Statement of Work:

Phase I

A. Determine the range of mammary tumor susceptibility after exposure to the carcinogen 7,12-dimethylbenz[a]anthracene in a panel of 10 inbred strains of mice.

AND

B. Compare the differences in mammary tumor burden between ad libitum and calorie restricted animals for each genotype.

Ongoing and completed work

The initial experiment conducted as part of this project was to determine what modifications were necessary to adapt a published protocol for inducing mammary cancer with a single dose of 7,12-dimethylbenz[a]anthracene (DMBA) so as to maximize survival and permit analysis of the longitudinal response of the mice. We found a significant reduction in mortality in mice which received hydration support immediately following DMBA administration [3] (Appendix 3).

Having attained adequate survival, the next step was to proceed with the actual experiment i.e., comparing mammary cancer after carcinogen exposure in different strains of mice when animals were fed either ad libitum or were restricted to 60% of the caloric intake of ad libitum fed mice. Mammary cancer resulting from a single oral dose of 65 mg/kg DMBA had been reported at 18-20 weeks [4] and between 15 and 40 weeks [5] with incidences of affected animals ranging from 3-83% and 73% respectively [4, 5]. It was anticipated that the kinetics of mammary cancer induced by DMBA in mice would be similar to that reported in rats.

Cohorts of approximately 40 individuals from each of 8 distinct genotypes of mice were ordered for the purpose of comparing the effects of calorie restriction on the age of mammary cancer manifestation and overall incidence. The various genotypes of mice were dosed over a period of 22 weeks. The strains for study included one from Germany, the NMRI, in which calorie restriction was reportedly not protective against chronic exposure to DMBA [6]. If the conclusion drawn by the authors of this report was correct, the anticipated outcome was that these mice would fail to demonstrate a positive response to calorie restriction. A positive response in this experiment was defined as either an increase in average longevity and/or a decreased incident of tumor and/or increased age at which mammary tumors are observed.
The mice of each genotype were dosed with DMBA at 9 weeks of age and then were assigned to two diet groups such that the groups for each genotype were matched for weight at 12 weeks of age. The caloric intake of the calorie restricted cohorts were gradually reduced over a period of 3 weeks so that the body weight of the restricted mice were kept between 60 and 70% that of the ad libitum fed cohort for that genotype. The ratio of the body weights of the calorie restricted vs. ad libitum cohort for each genotype was used to titrate the food intake of each strain as long as the ad libitum cohort survived. Appendices 1a-i show the relative weight of each genotype for the duration of the experiment. A multivariate distribution-free significance analysis [7] which utilized a Wilcoxon rank test was used to compare the growth curves for the ad libitum and calorie restricted mice of each genotype. Significant (P < 0.001) differences in body weight were demonstrated for all 8 genotypes, indicating that a restriction in caloric intake sufficient to affect body weight was successfully attained for the strains in this study (Table 1).

An additional 9th genotype, the Werner homozygous knockout, was added to the genotypes under investigation and is currently being studied. Werner syndrome (WS) is a human genetic disease which resembles premature aging and is the apparent result of a mutation in a gene encoding for a DNA helicase. The clinical manifestations are many and include accelerated manifestations such as graying of the hair, cataractogenesis, atherosclerosis and cancer in humans [8]. It has been reported that a deletion within the murine WS helicase results in increased sensitivity to inhibitors of topoisomerase and loss of cellular proliferative capacity [9]. Further, it has been suggested that the WS helicase is involved in certain pathways of apoptosis, leading to the accumulation of cells that are highly susceptible to FAS induced apoptosis [10]. These observations suggested that the Werner (WRN) knockout mouse might be more susceptible to carcinogen induced mammary tumors. Thus, the response of mice, which were either homozygous (-/-) or heterozygous (+/-) for a deletion in the exon encoding region VI in the catalytic helicase domain, to exposure to the carcinogen DMBA are being compared in terms of cancer incidence and longevity. The WRN -/- mice were predicted and then subsequently demonstrated to lack detectable WN helicase protein (D. Lombard, personal communication). Using a similar analysis to compare the body weights of the -/- and +/- mice as was used for the different diet groups of the other genotypes, no significant difference between these two genotypes was observed (Appendix 1h)(Table 1).
Three additional strains of mice C57BL/6J-Lep$^{ob}$, C57BL/6J-AY and C57BL6J-tub/+ have been obtained for study. These mice have all been dosed with the carcinogen, but work with these mice lags far behind the other 9 genotypes used in this investigation and thus will not be further discussed in this report.

Within each genotype, the lifespan data, expressed in days, were analyzed by the Lifetest Procedure with the significance of difference between diet groups expressed as that of the Log-Rank chi-square (Table 1). Significant effects of calorie restriction on longevity in the inbred strains of mice following a single dose of DMBA, were observed in 7 out of the 8 strains in which it was examined (Appendices 2a-i). The longevity of the Werner knockout homo- and heterozygotes, similarly examined, have not demonstrated significant differences to date (Appendix 2h).

One major goal of this project was to identify a strain of mouse that did not respond to calorie restriction in terms of modulating mammary cancer incidence. A strain with this characteristic would provide the opportunity to identify the gene or genes controlling the response to this dietary intervention. It is significant, to have identified a strain of mouse, the FVB/J, which potentially does not possess the capability to respond to calorie restriction. Although calorie restricted (Appendix 1f), there appears to be no modulation in longevity to date (Appendix 2f). This strain may yield the identity of the gene or genes involved in controlling the responsiveness to calorie restriction observed so clearly in most rodents.

The single dose of DMBA which was utilized in these experiments does not, however, result in a carcinogenic response that was specific to mammary tissue in mice. The pathology observed in this work strongly suggests that the result of the DMBA dosing regime utilized increases the incidence and decreases the age of manifestation for the malignancy or malignancies to which given strains are predisposed. These observations are the basis of a recently submitted manuscript [11] (Appendix 4).

The ineffectiveness of the single oral dose of DMBA to elicit mammary cancer is a major problem affecting the capacity of this project to move forward with its stated goal of identifying genes controlling responsiveness of mammary cancer to dietary intervention. Toward solving this problem, a protocol has been submitted and approved by the institutional Animal Care and Use Committee in which mice will be anesthetized and have two compressed pellets each containing 20 mg medroxyprogesterone acetate implanted.
subcutaneously. Each mouse will be anesthetized with isoflurane and given a 1 mg dose of DMBA at 9, 10, 12 and 13 weeks of age by oral gavage as previously detailed [12,13]. The advantage of this combination of medroxyprogesterone coupled with 4 doses of DMBA is that it is reported in mice to result in shortened latency for mammary tumor development and higher mammary tumor incidence than DMBA alone [12, 13]. They report that 70% of mice study manifest mammary tumors with an average latency of 99 days [13]. This outcome is far superior to that observed in our most responsive strain, the C3H, where a single dose of DMBA resulted, in the mice examined to date, an incidence of mammary tumors that was only 33% with an average latency of 354 days [14]. Success using the reported protocol will greatly decrease the time necessary for the later components in this study.

It appears from our initial study that the C3H is the most responsive strain to DMBA induced carcinogenesis. Using the protocol for inducing mammary tumor detailed by Aldaz and colleagues [12, 13] the response of 4 strains of mice, C3H, C57BL/6, 129/J and FVB/J will be examined. The response of these 4 strains to calorie restriction has already been ascertained in the first experiments conducted. The first three strains manifest statistically significant increased lifespan when calorie restricted following DMBA administration. The FVB/J failed to show any effect on average longevity in response to calorie restriction. In addition, these strains have been selected for their propensity to manifest mammary tumor response to DMBA. The C3H mice were previously found to have the highest incidence of mammary tumors, 129/J mice had a relatively strong hyperplastic response of mammary tissue and C57BL/6 and FVB/J each had zero incidence of either mammary hyperplasia or mammary tumor in ad libitum fed mice after a single dose of DMBA. This next component in the experiment will establish the relative response of these four strains under conditions which have been optimized for mammary tumor formation.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Comparison</th>
<th>Body Weight</th>
<th>Longevity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>ad libitum vs. calorie restricted</td>
<td>≤ 0.0001</td>
<td>0.0005</td>
</tr>
<tr>
<td>BALB/c</td>
<td>ad libitum vs. calorie restricted</td>
<td>0.0001</td>
<td>0.0190</td>
</tr>
<tr>
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<td>≤ 0.0001</td>
<td>0.0010</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>ad libitum vs. calorie restricted</td>
<td>≤ 0.0001</td>
<td>0.0005</td>
</tr>
<tr>
<td>DBA/2</td>
<td>ad libitum vs. calorie restricted</td>
<td>≤ 0.0001</td>
<td>0.0001</td>
</tr>
<tr>
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<td>≤ 0.0001</td>
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<td>NMRI</td>
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<td>0.0010</td>
</tr>
<tr>
<td>129/J</td>
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<td>0.0022</td>
</tr>
<tr>
<td>Werner knockout</td>
<td>homozygote vs. heterozygote</td>
<td>0.1992</td>
<td>0.2855</td>
</tr>
</tbody>
</table>
Approved Statement of Work:

Phase II
(Choice dependent on the outcome of phase I)
A. Statistically significant difference in response to calorie restriction (CR) are found for two genotypes with comparable mammary tumor burden when ad libitum fed. Produce F2 generation in which to map the gene(s) controlling susceptibility CR effect on mammary tumor.

B. Statistically significant difference in response to calorie restriction are found for two genotypes with different mammary tumor burden observed when ad libitum fed. Use recombinant inbred strains to identify gene(s) controlling susceptibility CR effect on mammary tumor.

C. No significant difference in response to calorie restriction observed among the genotypes. Produce an F2 generation in which to map gene(s) for differences in mammary tumor susceptibility.

Planned experimental work

The results of the experiment in which C3H, C57BL/6, 129/J and FVB/J mice are implanted with medroxyprogesterone and given 4 doses of DMBA will determine which of the three proposed phase two lines of work are conducted. Obviously, if the FVB/J mice respond to this treatment with development of mammary tumors, we will proceed with choice A, producing an F2 generation with C3H x FVB/J mice in which to map the differential response to calorie restriction exhibited by these two strains. If neither of the three stains other than C3H respond to this treatment with the development of mammary tumors, then the next step will be choice C. F1 hybrids (C3H x C57BL/6) will be purchased from which an F2 generation will be produced in which to map differences in mammary tumor susceptibility following dosing with DMBA.

If, however, the new protocol fails to elicit a more robust mammary cancer response, the mapping of gene(s) responsible for differences in mammary cancer response following a single dose of DMBA between the C3H and FVB/J as well as those gene(s) responsible for the difference in responsiveness to calorie restriction between these two strains will be conducted. The time necessary to conduct this work will be significantly greater than originally anticipated.
Key Research Accomplishments

- Survival of mice following oral gavage with the carcinogen DMBA has been optimized. (Appendix 3).

- A strain of mouse, the FVB/J, which appears not to respond to the dietary intervention of calorie restriction by living longer or exhibiting decreased age-dependent incidence of cancer has been identified.

- The response of mice to exposure to the carcinogen DMBA has been demonstrated to exhibit genotypic specificity (Appendix 4).

- A strikingly high percentage of DBA/2 mice treated with DMBA exhibited cardiac calcinosis. While this strain has been demonstrated to have a genetic predisposition for this lesion [15], the average age of the affected animals was relatively young. Although the obvious response to the stress of carcinogen exposure is cancer, this response is limited to mitotically active tissue. It appears in a susceptible genotype, that an additional response to DMBA exposure is cardiac calcinosis (Appendix 5). This observation is especially relevant given that as the proportion of postmenopausal woman increases, along with breast cancer, they will be experience increased cardiac vulnerability. In addition, treatment related cardiac toxicity remains a problem in the treatment of breast cancer [16].

Conclusions

This work lays the foundation for identifying gene(s) which control responsiveness to the intervention of calorie restriction. The importance of this is that it provides researchers with a new mechanism by which calorie restriction elicits its effect AND provides clinicians with a means of discerning which patients will respond to dietary intervention as a means of delaying or decreasing the incidence of mammary and potentially other cancers.
References


List of Appendices and legends for figures

Appendix 1 Body Weight

Appendix 1a  Average body weight in grams for A/J mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P ≤ 0.0001).

Appendix 1b  Average body weight in grams for BALB/c mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P = 0.0001).

Appendix 1c  Average body weight in grams for C3H mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P ≤ 0.0001).

Appendix 1d  Average body weight in grams for C57Bl/6 mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P ≤ 0.0001).

Appendix 1e  Average body weight in grams for DBA/2 mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P ≤ 0.0001).

Appendix 1f  Average body weight in grams for FVB/J mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P ≤ 0.0001).

Appendix 1g  Average body weight in grams for NMRI mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P ≤ 0.0001).

Appendix 1h  Average body weight in grams for 129/J mice over time for ad libitum (AL) and calorie restricted (CR) animals, which were significantly different (P = 0.0012).

Appendix 1i  Average body weight in grams for Werner gene knockout mice over time for mice which were homozygous and heterozygous for the Werner gene deletion. The body weights of these two groups did not differ significantly (P = 0.12).
Appendix 2 Mortality kinetics

Appendix 2a Longevity curves for ad libitum (AL) and calorie restricted (CR) A/J mice, which differed significantly (P = 0.0005).

Appendix 2b Longevity curves for ad libitum (AL) and calorie restricted (CR) BALB/c mice, which differed significantly (P = 0.019).

Appendix 2c Longevity curves for ad libitum (AL) and calorie restricted (CR) C3H mice, which differed significantly (P = 0.0010).

Appendix 2d Longevity curves for ad libitum (AL) and calorie restricted (CR) C57BL/6 mice, which differed significantly (P = 0.0005).

Appendix 2e Longevity curves for ad libitum (AL) and calorie restricted (CR) DBA/2 mice, which differed significantly (P = 0.0001).

Appendix 2f Longevity curves for ad libitum (AL) and calorie restricted (CR) FVB/J mice, which did not demonstrate any significant difference (P = 0.38).

Appendix 2b Longevity curves for ad libitum (AL) and calorie restricted (CR) NMRI mice, which differed significantly (P = 0.001).

Appendix 2b Longevity curves for mice which were homozygous and heterozygous for the Werner gene knockout, which were not significantly different (P = 0.29).


Body weight for NMRI mice

- NMRI AL
- NMRI CR
Body weight for Werner knockout mice

- + heterozygous
- ■ homozygous
Survival Kinetics for A/J mice
Survival Kinetics for BALB/c mice

- BALB/c AL
- BALB/c CR

Graph showing the percentage of animals surviving over time (age in weeks).
Survival Kinetics for DBA/2 mice

- **DBA/2 AL**
- **DBA/2 CR**

**Axes:**
- **Y-axis:** animals surviving (%)
- **X-axis:** age (weeks)
Survival kinetics for FVB/J mice

- FVB/J AL
- FVB/J CR
Survival kinetics for NMRI mice

animals surviving (%) vs age (weeks)

- NMRI AL
- NMRI CR
Survival kinetics for 129/J mice

- 129/J AL
- 129/J CR

Animals surviving (%) vs. age (weeks)
Werner gene knockout mice

% surviving

age (weeks)

+ heterozygote
■ homozygote

DAMD17-97-1-7123
Improved Survival Rates in Mice that Received Prophylactic Fluids After Carcinogen Treatment

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Abstract | During the development of a model for 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary adenocarcinoma in mice, a high mortality rate was attributed to dehydration. Therefore, we compared the acute survival of mice given subcutaneous fluids prophylactically immediately following DMBA gavage to that of animals provided treatment only when clinical signs of dehydration were observed. Mortality in the prophylactically treated mice was 5% compared to 47% in animals treated only after the manifestation of dehydration. Prophylaxis with subcutaneous fluids significantly reduces mortality in DMBA-treated mice.

Because of species differences in physiology, experimental protocols based on one animal model may require modification when applied to a different animal. In light of previous work in rats, we developed a breast cancer model in mice by using the pro-carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), which forms depurinating DNA adducts in rat mammary epithelial cells (3). DMBA is widely used as a carcinogen in rodents, with a variety of doses, number, and routes of administrations reported (1, 2, 4-7). We selected the DMBA method described by Haag et al. (2) and Hsu et al. (1) because it appeared to allow for a smaller sample size and minimal stress to the animals (8). This method is reported to result in a 100% incidence of mammary adenocarcinoma in susceptible rat genotypes without early mortality (1, 2). The protocol involves only a single administration of carcinogen and thus minimizes both stress to the animals and the potential for environmental contamination. The modifications necessary to manage the hydration status of DMBA-treated mice and to minimize acute mortality are described.

Material and Methods

This study was approved by the USDA Human Nutrition Research Center on Aging Animal Care and Use Committee. Two cohorts of 20 6-wk-old female C3H/HeNsd mice (Harlan Sprague Dawley, Indianapolis, IN) were individually housed in 8" x 8" x 8" suspended, polycarbonate cages and provided ad libitum access to NIH-31 diet (Harlan Teklad, Madison, WI) and purified water sterilized by UV irradiation. The mice were acclimated to appropriate environmental conditions for 3 wk prior to carcinogen exposure (9, 10). At this time, all animals were observed daily for clinical signs of disease and weighed each week.

Working within a fume hood, we dissolved DMBA (Sigma Chemical, St. Louis, MO) in sesame seed oil (Sigma Chemical) to a concentration of 5.2 mg/mL. The first cohort of 20 mice were anesthetized with Aerrane (isoflurane; Fort Dodge Animal Health, Fort Dodge, IA) in a negative-pressure hood and orally gavaged with 0.13 mL DMBA to provide 65 mg DMBA/kg body weight. The second cohort were similarly dosed, but prior to recovery from anesthesia, each mouse was injected subcutaneously (SQ) with 1.0 mL 0.9% NaCl (Abbott Laboratories, North Chicago, IL). In addition to the water bottle with sipper tube present in each metabolic cage, this second cohort of mice was also given a jar of drinking water. The difference between the two cohorts was the timing of the treatment for dehydration rather than the treatment itself.

Three days after dosing, 80% of a DMBA dose is reportedly present in the excreta (11), and no biologically active carcinogen remains in vivo 5 d after an oral gavage (12). Accordingly, mice were housed in metabolic cages (Lab Products, Maywood, NJ) for 1 wk to facilitate collection of all feces and urine potentially contaminated with DMBA. A plastic bag was used to enclose the entire urine/feces separation unit to minimize potential carcinogen contamination of the area. All excreta were disposed of as chemical waste. Personnel safety procedures including protective face shield and disposable garb were used as previously described (13). Access to the animal room (maintained at negative pressure) was restricted.

Comparison of mortality incidence between groups was carried out with a 2 x 2 $\chi^2$ analysis. Average body weights of the mice were compared by using a two-tailed t-test. Statistical analyses were conducted with STATOOLS (14).

Results

No difficulties were experienced while gavaging the mice, and all animals were ambulatory and active upon recovery from anesthesia, which occurred within 1-2 min after dosing. All mice appeared to be in a similar condition 24 h after receiving DMBA. Between 48 h and 72 h after DMBA dosing, three animals in the first cohort (no prophylactic fluids) were lethargic, with clinical signs of dehydration, including anorexia, anuria, loss of skin elasticity, and skin turgor. Mice observed with any of these clinical signs were given 1.0 mL saline SQ and a water jar was placed in their cage. Despite this supportive fluid therapy, the condition of these mice did not improve, and they died within 48 h. Another eight mice in this cohort had similar clinical signs up to 4 wk after DMBA treatment and were provided with supportive hydration; these mice subsequently died within 48 h. The two cohorts did not differ with respect to the hydration measures taken, but rather the time point after dosing at which they were initiated.

The difference in post-dosing mortality was significantly different ($p \leq 0.05$) between groups (Figure 1). The cumulative 4 wk post-procedure mortality for the first cohort of mice was 47%. This value compared with a loss of only 5% (one mouse) during the same 4-wk period for mice receiving the prophylactic injection of saline immediately after DMBA dosing. At 2 weeks after dosing, the average weight of the mice in the first cohort that
FIG. 1. The survival of mice given prophylactic vs therapeutic hydration support as a function of time after dosing with DMBA.

died during weeks 3 and 4 (18.6 ± 1.51 g) was significantly less than that of the prophylactically treated mice (22.3 ± 2.3 g; P ≤ 0.005). The average weight of the surviving mice 4 weeks after DMBA administration did not differ (21.3 ± 1.8 g for control animals vs. 22.3 ± 2.3 g for those prophylactically treated).

Discussion

The induction of mammary tumors in the rat with the use of chemical carcinogens is a commonly utilized model for the study of breast cancer (15). Although the rat model is ideal for some experiments, there are valid reasons for examining phenomena in other species, including the facilitation of specific analyses or the comparison of effects between species. We highlight here the great importance of prophylactic hydration to survival of mice treated with DMBA.

Dehydration in mice leads to diminished food intake, generalized weakness, decreased ability to regulate body temperature, hypovolemia, and electrolyte imbalances with renal and cardiovascular failure. Basic veterinary care, i.e., provision of supportive fluid therapy upon presentation of clinical signs, was insufficient to prevent the high mortality associated with the effective DMBA dose. The timing of fluid administration as a supportive measure is an important factor and may be narrowly defined in rodents (16). Prophylactic interventions to facilitate hydration may be prudent for the adaptation of other rat protocols to mice, as a variety of important physiologic functions including immune responses, renal cortical blood flow, and drug distribution are altered by hydration status in mice (17–19). Subcutaneous fluid administration as a means of rehydration is effective in other species, including humans (20). As also suggested by Dieterich et al. (17), this study reinforces the necessity of performing small-scale pilot studies to adapt published protocols from one animal model to another prior to initiating large experiments.

Acknowledgments

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References


The Response of Different Mouse Genotypes to Treatment
with 7,12-dimethylbenz[a]anthracene

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Summary

The aim of this experiment was to determine strain dependent differences in response to exposure to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). Cohorts of 16-19 female mice from each of seven strains (A/J, BALB/c, C3H, C57BL/6J, DBA/2J, NMRI and 129/J) were studied. Mice were given a single oral gavage of DMBA (65 mg/kg) at 90 days of age followed by prophylactic administration of fluids as previously described [4]. DMBA is a polycyclic aromatic hydrocarbon which forms depurinating DNA adducts and is considered a potent procarcinogen. This experiment shows that exposure to a carcinogen results in different tumors manifest in different strains. The occurrence of several distinct neoplastic lesions in individual animals was not an uncommon observation. Administration of DMBA appeared to accelerate the expression of the neoplastic lesions to which these strains were predisposed.

Key words:
mice, genotype, DMBA, cancer, pathology
1. Introduction

An assumption regarding carcinogen exposure is the specificity of the tumor resulting from a defined dosage of the carcinogen studied. Commonplace among experimental study design is a morphologic description of the specific tumor, its latency, incidence and/or total tumor burden in a particular strain of mouse or rat. Often results regarding expression of a particular gene or genes will be measured and/or the efficacy of putative interventions designed to modulate tumor expression are reported. However, it is critical that an individual inbred or hybrid strain be recognized as a group of individuals which are genetic replicates of one another. An experiment performed in one inbred strain is a repetition of the same interaction between the defined perturbation and one set of alleles. One positive aspect resulting from this constraint is the validity of the comparison between treatment groups, each consisting of multiple individuals receiving their respective treatments. Another ramification of such experimental design is, however, that the observations in the strain studied may or may not be applicable beyond that strain to the entire species.

The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) is metabolism-dependent procarcinogen described as a mammary carcinogen in rats [1]. DMBA is a polycyclic aromatic hydrocarbon capable of forming DNA adducts [2]. As polycyclic aromatic hydrocarbons have been demonstrated to be mutagenic and are found in our environment [3] it is of interest to document the range of effects which result from exposure.

In the present study, cohorts of seven strains of mice were dosed with the DMBA and followed to establish genotypic patterns of tumorigenesis. The results of this study have importance in terms of assessing carcinogenic potential. In conjunction with the ever more detailed map available for the mouse genome, these finding are a step in determining genetic differences associated with specific tumor predisposition.
2. Materials and methods

2.1 Animals

Six week old, virgin female mice were obtained as follows: A/J, BALB/c, C3H, C57BL/6, DBA/2 (Harlan Sprague, Indianapolis, IN), 129/J (The Jackson Laboratory, Bar Harbor ME) and NMRI (B&K Universal, Fremont, CA). The mice were maintained under 12 h light/12 h dark conditions at 23° and 45% humidity. The mice had ad libitum access to NIH-31 diet (Harlan Teklad, Madison WI) and sterilized water. This study was approved by the USDA Human Nutrition Research Center on Aging Animal Care and Use Committee.

2.2 Animal husbandry

Each individually caged mouse was weighed every two weeks and monitored daily for health throughout the course of the experiment. Mice which demonstrated weight loss of >20% in 2 weeks, exhibited pain or distress or failed to consume food in a two day period were terminated with the use of CO₂ inhalation.

2.3 Carcinogen administration

The mice were acclimated to the laboratory environment for 3 weeks before dosing with DMBA. Each mouse was anesthetized with isoflurane (Fort Dodge Animal Health, Fort Dodge, IA), orally gavaged with 65 mg DMBA/ kg body and provided with 1.0 cc 0.9% NaCl for the prevention of dehydration. The mice were placed in metabolic cages after dosing to facilitate safe collection of carcinogen contaminated excreta as previously
described [4]. The animals were then transferred to conventional suspended polycarbonate cages and maintained for up to 18 months of age.

2.4 Histologic Preparation

The inguinal mammary fat pads were recovered from all mice, unless autolized. After dissection, the dissected mammary tissues were placed on paper prior to being placed in fixative. By allowing the tissue to adhere to the paper, it insured suitable flatness for sectioning of the mammary tissue. The paper was removed after 5 minutes. Other tissues including lung, liver, pancreas, spleen, ovary and uterus were routinely collected from each mouse. In addition, samples of any grossly abnormal appearing tissues were sampled. All tissues were fixed in Telyesniczky’s fixative (20:2:1 of 70% ethanol, 37% formalin and glacial acetic acid). Tissues were dehydrated and then embedded in paraffin. Five μm sections were cut, stained with hematoxylin and eosin and coverslips were applied with Permount.

2.5 Statistics

The data for lesion incidence and longevity were compiled using the relational database, FoxPro (Microsoft, Redmond WA). The average longevity for each genotype after a single oral gavage with DMBA is presented in Table I. A lesion was defined as common if it was observed in at least 10% of at least one genotype. The data on the incidence of the common lesions is expressed as the percentage of affected animals along with the mean age of affected animals ± standard error (Table II). Comparison of lesion incidence among genotypes utilized \( \chi^2 \) analysis. The level of significance, \( P \), for the difference in incidence among the genotypes was corrected for the multiple comparisons run (i.e., the number of
common lesions) using the Bonferroni adjustment. The adjusted significance value was accepted as demonstrating significant difference at $P \leq 0.05$. Analysis of the mean age of mice observed with each common lesion were compared using a two tailed t-test [5].

3. Results

The mice in each of the genotypes studied were active within a few minutes after dosing as previously reported [4]. Mice which succumbed acutely, which was defined as within 10 days after dosing, were as follows: 2 A/J, 3 BALB/c, 2 C3H, 1 C57BL/6 and 2 DBA/2. The number of acute deaths did not differ significantly among genotypes. These animals were not included when calculating the percentage of mice in each genotype which did or did not have specific lesions.

The proportion of mice found dead during the course of study or were sacrificed did not differ significantly among the 6 genotypes studied. The average age of the mice in months for each genotype is presented in Table I. Post-hoc comparison demonstrated that only the longevity of the 129/J, the shortest lived and the C57BL/6, the longest lived strain differed significantly. Except for the possibility of these two strains, differences among genotypes can therefore not be the result of differences in lesions observed among the genotypes.

The list of lesions commonly observed differed among the strains studied as can be seen from Table II. The most stringent of data corrections for multiple tests, the Bonferroni correction, has been applied to data and yet the data still demonstrate highly significant differences among the genotypes studied as to the incidence of common lesions observed.

The list of lesions commonly observed in this study after a single oral gavage of DMBA were among those lesions observed to occur in these strains of mice in association with
normal aging [6, 7]. As these historical data were obtained on animals with an older average age, it suggests that the DMBA served to accelerate processes to which the mice were already predisposed.

4. Discussion

The results presented in this paper demonstrate that the effects of exposure to the carcinogen DMBA are pleiotropic and at least in part, dependent on genotype. This suggests that attention to genotype as an experimental variable needs to reassessed in terms of its contribution to the disease processes being studied. The systemic administration of DMBA in Sprague-Dawley rats has been reported to result in a similar dose dependent patterns of DNA adduct formation in both mammary gland and lymphocytes, which respectively are a tissue that gives rise to neoplastic lesions and one that does not [8]. One interpretation is that the formation of DNA adducts alone is not sufficient for tumorigenesis. Taken with the results of this study, it suggests that the underlying genotype plays a role in defining the tissues which are predisposed to tumor development.

It has recently been demonstrated that angiogenic factors produced by tumors restrict the proliferation of metastasis and/or growth of additional neoplasias [9, 10]. Although the data substantiating this type of competitive inhibition is exciting, it may not have application to all instances of neoplastic disease. In this study, the proportion of mice with multiple neoplasias ranged from 6% in the BALB/c up to 28% in the A/J mice. These are conservative estimates as it included animals with no neoplastic lesions. If only those animals with at least 1 neoplastic lesion are considered, the average incidence of multiple neoplasias present in the genotypes studied is 33%. It could be argued that this situation of multiple neoplasias results from response to carcinogen exposure. The observation, however, of multiple malignancies of distinct origin within an individual is commonplace in
mice as they age [7, 11]. While it could be posited that these observations of multiple distinct malignancies are germane only to rodents, autopsy data indicate that this phenomena also occurs in humans resulting from chemical poisoning [12] or simply as a matter of course [13, 14]. In mice, the number of neoplastic lesion per animal has been demonstrated to increase as a function of age [15]. The incidence of multiple neoplasias in this study did not, however, correlate with the average age attained by the strain (data not shown). One explanation may be that the animals in this study were relatively young. It would also seem that carcinogen exposure does not modulate expression of all age-related lesions. Both of these factors could contribute to the lack of correlation between the age and the lesion burden of the mice studied.

The use of multiple genotypes in which to study the impact of specific carcinogens has become uncommon experimental practice. This is undoubtedly due, in part, to the increased costs in study design its inclusion brings. However, the importance of gene-environment interactions can be of large proportion. For example, although cigarette smoking is a well accepted cause of lung cancer, the majority of cigarette smokers never develop lung cancer [16]. While the supposition can be made that the smokers that don’t, succumb to other disease process prior to the development of lung cancer, the observation can still be interpreted as being highly suggestive of profound interactions between genes and the environment. Earlier work has demonstrated pleiotropic effects of the carcinogen DMBA in neonates [17]. That study demonstrated that in newborn mice given a single dose of DMBA, genotype played an important role in determining the principle sites of tumor formation. The current study extends this observation to older mice. In addition, comparison of the tumors which were observed commonly in the two studies suggest that either differences in age at which DMBA exposure occurred and/or manner in which it was delivered affected what tumors develop. There were certain commonalities in the tumors observed, such as the high incidence of lymphoma in C57BL/6 and lung adenoma in the
BALB/c. But, for example, while lung adenomas and liver hepatoma were the two most common lesions among the C3H mice injected as neonates [17], the most common lesion observed among the adult C3H mice orally dosed with DMBA in this study was mammary adenocarcinoma. Notwithstanding these differences, the incidence of lymphoma in C3H dosed with DMBA was consistent at about 20% in both studies. Differences in tumor type with age at which carcinogen exposure occurred is consistent with the observation that the biology of young, old and older individuals differ in profound ways. That age is an independent risk factor for specific neoplastic lesions is supported both from clinical [18] and laboratory [19] based studies. Age has been reported to be the greatest single risk factor for the development of cancer [20].

Attention to basic, fundamental experimental details such as genotype [17], caloric intake [21] and ages at which intervention occurs [22] are critical variables to explore in order to understand the biology controlling neoplastic disease. These experimental details are essential for appropriate interpretation of results. Strain dependent differences in response to carcinogen treatment afford the opportunity not only to identify genes modulating susceptibility to carcinogenesis and tissue specific differences in susceptibility, but the use of several genotypes will aid in determining the specific oncogenic pathways resulting in the cascade of changes which yield tumors.

Acknowledgments

This work could not have been done without the meticulous attention to the animals health and care provided by Maureen Kelliher. I would like to thank Dr. R. T. Bronson for the many valuable discussions we have had regarding this project. The work was supported by U.S. Army DAMD17-97-1-7123 and the U.S. Department of Agriculture, under agreement No. 58-1950-9-001. Any opinions, findings, conclusions, or recommendations
expressed in this publication are those of the author and do not necessarily reflect the view of the U.S. Dept. of Agriculture.

References


Table Legends

Table I  The number of mice in each genotype following oral gavage with DMBA at 90 days of age presented along with their average age in months ± standard error.

Table II  The percentage of mice in each genotype observed with any of the lesions commonly observed in these cohorts of animals. The Bonferroni corrected significance value, P, is presented for only those lesions where significant differences among the genotypes were observed.
<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>n</th>
<th>average age ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>18</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>BALB/c</td>
<td>16</td>
<td>10.3 ± 1.1</td>
</tr>
<tr>
<td>C3H</td>
<td>19</td>
<td>12.4 ± 0.7</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>17</td>
<td>12.8 ± 1.2</td>
</tr>
<tr>
<td>129/J</td>
<td>19</td>
<td>9.3 ± 0.4</td>
</tr>
<tr>
<td>DBA/2</td>
<td>18</td>
<td>9.9 ± 0.8</td>
</tr>
<tr>
<td>Organ or System</td>
<td>Specific Lesion</td>
<td>Mouse Genotype</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------------------</td>
<td>----------------------</td>
</tr>
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<td>extramedullary hematopoiesis</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>lymphoma</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>lung</td>
<td>adenoma</td>
<td></td>
</tr>
<tr>
<td>mammary gland</td>
<td>adenocarcinoma</td>
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<td></td>
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<tr>
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<td>galactorrhea</td>
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</tr>
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<td>papilloma</td>
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<tr>
<td>stomach</td>
<td>papilloma</td>
<td></td>
</tr>
<tr>
<td>uterus</td>
<td>hemangiosarcoma</td>
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</tr>
</tbody>
</table>
The response of the DBA/2 heart to aging, carcinogen exposure and calorie restriction

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keywords: DBA/2 mice, cardiac, DMBA, pathology, calorie restriction

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Introduction

Cardiovascular disease is a common cause of death in humans; notwithstanding its association with mortality, there are relatively few age-related structural changes in the heart. While it is critical not to confuse occult disease with normal aging in the development of a model for human aging, it is pertinent to account for disease related changes and their overall contribution to mortality. The DBA/2 mouse is a strain in which the incidence of heart disease is greater than other strains [Bronson, 1990 #1] and this incidence increases with age [Bronson, 1991 #2]. Although not all humans develop cardiopathology with age, the DBA/2 mouse is an excellent system in which to study cardiac aging compromised by disease.

It is of interest that both normal aging and various progeroid syndromes include cardiac manifestations and increased cancer incidence [Lee, 1997 #10; Yancik, 1997 #8; Pagano, 1998 #7]. Although the response to various insults elicited in mitotically active tissue is likely to include a replicative component, the response of quiescent cells will be something other than replication. For example, as heart muscle cells do not have the capacity to divide, their response to carcinogen exposure must, by definition, be something other than neoplastic in nature.

In this report, the various age-related lesions affecting the heart in the DBA/2 mouse are presented. The effects of calorie restriction (CR), a nutritional intervention long documented to increase longevity [Weindruch, 1989 #9] on the overall incidence and the average age of affected individuals with heart lesions are compared. Finally, the capability of CR to modulate the effects on the heart of carcinogen administration are examined.

Materials and Methods

The cross-sectional experimental design, in which approximately 30 mice of each sex-diet cohort were examined at 6 months intervals utilized DBA/2NNia mice bred and maintained at the National Center for Toxicologic Research as detailed[Turturro, 1999 #3]. The actual number of mice examined and their average ages are shown in Table 1. Briefly, the mice were individually housed and were fed either NIH-31 ad libitum (AL) or NIH-31 supplemented with 1.67X the vitamin mix such that their calorie intake was equal to 60% of the AL intake (CR). The mice were shipped to the USDA Human Nutrition Research Center on Aging by air cargo and were processed for histological examination within 48
hours of their receipt. Prior to sacrifice, each mouse was deeply anesthetized with Avertin, their vasculature was flushed with physiologic saline and then their tissues fixed by intracardiac perfusion with Bouin’s solution, as previously described [Bronson, 1991 #2]. The animals were fixed for several weeks prior to tissue dissection which included the longitudinally bisected hearts and samples of thigh muscle. Tissues were dehydrated in alcohol, embedded in paraffin and 5 µm sections were cut and stained with hematoxylin and eosin. Tissues were graded on the presence or absence of lesions.

The cardiac effects of carcinogen exposure were examined using 6 week old, virgin female DBA/2 mice obtained from Harlan Sprague (Indianapolis, IN) and were maintained in our facility, fed NIH-31 ad libitum as described [Lipman, 2000 #5]. At 9 weeks of age, each mouse was anesthetized with isoflurane and given 65 mg/kg 7,12-dimethylbenz[a]anthracene (DMBA) in sesame oil via oral gavage followed by 1.0 ml of physiologic saline administered subcutaneously to prevent dehydration [Smith, 1999 #6]. Three weeks after DMBA administration, the mice were divided into two groups matched for weight. One cohort was fed ad libitum (AL) and the intake of the other was gradually restricted (CR) until the average body weight of the mice approximately 30% less than the AL cohort. The body weights and mortality kinetics for the AL and CR cohorts are shown in Figures 1 and 2. The mice were sacrificed using CO₂ inhalation at 18 months of age or when they demonstrated 20% loss in body weight, anorexia or appeared moribund. Tissue from animals which were sacrificed or found dead were fixed in Tellyesniczky’s fixative and were processed as above.

The data on age, diet and presence of specific lesions for each mouse was compiled using the relational database FoxPro (Microsoft, Redmond WA). Comparison of the mean ages for mice in the sex-diet cohorts of the cross-sectional experiment were analyzed using a two tailed t-test [Dallal, 1986 #4] and are shown in Table 1. The incidence of myocardial degeneration, cardiac calcinosis and skeletal muscle mineral deposition in these mice are shown in Tables 2-4 and the incidence of each lesion was compared among the different sex-diet cohort using χ² analysis [Dallal, 1986 #4]. Comparison of lifespan between the AL and CR mice which had been DMBA treated used SAS for Windows version 6.12 (SAS Institute, Cary, NC) and involved the log-rank estimate to correct for right censoring of the data. The incidence of cardiac calcinosis were compared between the diet groups for these DMBA treated mice also used χ² analysis [Dallal, 1986 #4].
Results

Determining whether the ages of the mice in each cohort are comparable is a necessary consideration (Table 1) prior to comparing the incidence of various lesions in the various cohorts of mice. A two-tailed student t-test showed that for both the males and females, the CR animals were significantly older than the AL, $P \leq 0.00001$ and $P = 0.02$ and males and females respectively. In addition, the AL males were significantly older than the AL females ($P = 0.04$) and the CR males were older than the CR females ($P = 0.00000$).

The two main effects of CR on myocardial degeneration examined were the age at which individual mice were found to manifest this lesion and its incidence within the cross-sectional populations. The data demonstrates that the incidence of myocardial degeneration was significantly greater among male and female mice that were ad libitum fed as compared with CR cohorts, $P \leq 0.0001$ and $P = 0.003$, respectively (Table 2). Comparison of the average age of affected individuals with myocardial degeneration demonstrated that the age at which CR mice were observed to be affected was significantly greater than the AL males ($P \leq 0.0001$). The difference in average age of affected individuals between the AL and CR cohorts did not reach significance for the females. Additionally, it was observed that in both dietary groups, the incidence of myocardial degeneration was significantly greater among the males than the females ($P = 0.00006$).

The impact of diet on the average age of affected individuals and the incidence of cardiac calcinosis was more complicated (Table 3). The average age of the animals with heart calcinosis did not differ significantly among diet or sex groups. The incidence of heart calcinosis was significantly greater among the AL males than the CR males ($P = 0.00000$) and in addition, amongst the AL cohorts, the incidence was significantly greater in the males than the females ($P = 0.00001$).

With the observation of cardiac calcinosis, we sought to determine whether mineral deposition was a generalized age-related response of this strain. Examination of skeletal muscle (Table 4) and the tongue (Table 5) demonstrated that this was the case. The average age of individuals with mineral deposition in either skeletal muscle or tongue did not show significant differences among the diet groups. However, for both these lesions, the incidence was significantly greater among the CR females and males than among their AL
counterparts (P = 0.02). In addition, for both skeletal muscle and tongue mineralization, among the CR mice, the incidences were significantly greater in the males than in the females ( = 0.01) and the average age of affected individuals was significantly greater for the males than the females (P = 0.02). This pattern of increased incidence as well as increased age of affected individuals in the males is similar to that observed for the cardiac calcinosis in these mice.

Among the female DBA/2 mice dosed with DMBA, the average body of the CR mice was maintained at about 75% that of the AL fed cohort (Figure 1). The average body weight of the AL mice dosed with DMBA was comparable that reported for other DBA/2 mice of the same age which were not given DMBA [Turturro, 1999 #3]. As seen in Fig 2, the longevity of the CR mice was significantly greater than that of AL fed animals (P = 0.00001). While the incidence of cardiac calcinosis did not differ between these two diet cohorts of DMBA dosed individuals (Table 5), the average age of CR mice with this lesion was significantly greater than AL cohort (P = 0.04). The cardiac calcinosis was observed at a significantly younger age in mice that were treated with DMBA than those animals which were part of the cross-sectional study (P = 0.004). In addition, the incidence of cardiac calcinosis was significantly greater in this carcinogen exposed group for both AL and CR cohorts (P ≤ 0.00001).

Discussion

A significant difference in the age of the mice studied in the cross-sectional study which must be taken into consideration when examining lesion incidence among the four sex-diet cohorts. Thus, the increased incidence of cardiac calcinosis and the mineral deposition in skeletal muscle in the CR males as compared with the AL males may in part, simply reflect the increased average age of the CR cohort. It cannot be concluded from these data that CR increases the risk of manifesting these lesions as age is an associated factor implicated in lesion development. Additionally, the cardiac calcinosis and mineral deposition in skeletal muscle, although observed in all sex-diet groups were not commonly found in either diet cohort of females, nor among the AL males. The significantly increased incidence observed in the CR males coincident with the significantly greater age of this cohort suggests an association may exist between the two.

The myocardial degeneration, however, was observed to have a significantly increased incidence in the AL cohorts, even though they contained a smaller proportion of older
individuals. The decreased incidence of this lesions in concert with the increased age of the population strongly suggests that CR is associated with a decreased risk of developing myocardial degeneration. As the average age of the CR males manifesting this lesion was greater than the AL males, it suggests that the development of the disease is delay as well.

The incidence of the mineral deposition lesions examined appear to be more common among the CR cohorts than the AL mice examined. This may, in part, reflect the older average age of the individuals in these cohorts and perhaps a dependency on age for lesion development.

The high proportion of DMBA treated mice exhibiting cardiac calcinosis is striking, especially in light of the relatively young average age of the individuals examined. Although one response to the stress of carcinogen exposure is cancer, this would be limited to mitotically active tissue. Another response of tissue to injury, however, is mineral deposition at sites of inflammation (ref). Consistent with the observation of this type of response in the DBA/2 mice, it has previous been established that a locus on chromosome 7 determines susceptibility to dystrophic cardiac calcinosis in mice [Ivandic, 1996 #11]. Although this disease process is not well understood, it has been hypothesized to be the generalized manifestation of cellular injury from any number of possibilities including age, infectious agents and chemical agents. The data presented are consistent with the view that the dramatic cardiac calcinosis in relatively young DBA/2 mice is merely reflective of the permissive genetics that potentiates this response. It is arguable that exposure to carcinogen potentiated the development of disease to which these mice were predisposed.
Table 1: Age of DBA/2 mice in the cross-sectional study

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>Average age ± S.D.</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>17.09 ± 6.55</td>
<td>114</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>19.00 ± 6.81</td>
<td>149</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>18.57 ± 5.12</td>
<td>98</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>22.37 ± 6.83</td>
<td>147</td>
</tr>
</tbody>
</table>

Table 2: Incidence of myocardial degeneration in the cross-sectional cohorts of DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>Average age ± S.D.</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>21.87 ± 3.69</td>
<td>40%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>23.20 ± 2.58</td>
<td>23%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>21.39 ± 3.67</td>
<td>78%</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>26.60 ± 3.94</td>
<td>46%</td>
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Table 3: Incidence of cardiac calcinosis in the cross-sectional cohorts of DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>Average age ± S.D.</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>24.00 ± 0</td>
<td>2%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>16.00 ± 7.48</td>
<td>5%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>24</td>
<td>1%</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>27.71 ± 3.31</td>
<td>23%</td>
</tr>
</tbody>
</table>

Table 4: Incidence of mineral deposition in skeletal muscle in the cross-sectional cohorts of DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>Average age ± S.D.</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>20.00 ± 3.64</td>
<td>3%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>21.47 ± 5.73</td>
<td>10%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>24 ± 0</td>
<td>3%</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>26.0 ± 3.64</td>
<td>20%</td>
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Table 5: Incidence of tongue mineralization in the cross-section component of this study

<table>
<thead>
<tr>
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<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>15.00 ± 9.86</td>
<td>5%</td>
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<tr>
<td>female</td>
<td>CR</td>
<td>16.29 ± 7.14</td>
<td>14%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>no affected individuals</td>
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</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>23.14 ± 2.27</td>
<td>5%</td>
</tr>
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Table 6: Incidence of cardiac calcinosis in DMBA treated DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
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<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>12.3 ± 3.1</td>
<td>26%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>16.4 ± 3.0</td>
<td>40%</td>
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