Dose–Response Predictability of Urinary Bladder Hyperplasia by *N*-2-Fluorenylacetamide Feeding in Mice: Its Modification by Sex

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Abstract □ A serial sacrifice experiment involving urinary bladder hyperplasia, produced by feeding 8–86 ppm of *N*-2-fluorenylacetamide for 90 days to BALB/c mice, showed a dose–response relationship for males but not females. The information from this study was used in a statistical design to predict the percent of hyperplasia in relation to time and dose. A formula for establishing the confidence limits of the curve was derived. Sex-dependent spontaneous pericardial fibrosis and mineralization were also observed, with the male response being twice that of the female.

Keyphrases □ *N*-2-Fluorenylacetamide—production of urinary bladder hyperplasia in mice, dose–response predictability, modification by sex □ Urinary bladder hyperplasia—*N*-2-fluorenylacetamide feeding in mice, dose–response predictability, modification by sex □ Hyperplasia, urinary bladder—effects of feeding *N*-2-fluorenylacetamide to BALB/c mice, dose–response predictability, modification by sex

Haley *et al.* (1) reported a dose–response-related urinary bladder hyperplasia in both sexes of C57BL/6 and BALB/c strain mice fed *N*-2-fluorenylacetamide at dietary concentrations ranging from 0.001 to 0.05%. Clayson *et al.* (2) previously reported a similar observation after feeding the chemical at a concentration of 0.03% for 2 weeks. However, only two of 12 C57 × IF male mice responded, and the hyperplastic index was 68 for the treated and 58 for the controls by 11 weeks. The development of urinary bladder hyperplasia has been reinvestigated utilizing larger numbers of animals and five different concentrations of *N*-2-fluorenylacetamide.

EXPERIMENTAL

BALB/c strain mice, 1680, equally divided between the sexes were used. The animals were maintained in quarters thermostatically regulated to 24° (72 ± 5°F) with free access to food and water. The mice weighed from 20 to 30 g. The sexes were randomized as to cages and racks. The groups were composed of 480, 480, 240, 160, and 80 animals, equally divided by sex and fed the dietary concentrations of *N*-2-fluorenylacetamide in meal[1] shown in Table I. *N*-2-Fluorenylacetamide dietary content was determined by the methods of Bowman and King (3). There were 240 controls.

Sacrifice periods were 1, 3, 6, 9, and 13 weeks from the start of the experiment, and 336 animals were sacrificed at each time interval. All animals were necropsied and the following tissues were examined microscopically after staining with hematoxylin–eosin: lungs, heart, aorta, thymus, skeletal muscle, kidneys, adrenal, liver, spleen, gallbladder, pancreas, cerebellum, spinal cord, stomach, colon, ileum, duodenum, lymph node, salivary glands, lacrimal gland, eye, harderian gland, thyroid, trachea, esophagus, skin, tongue, sternum, testes, epididymus, preputial gland, seminal vesicle, coagulating gland, ovary, uterus, mammary gland, urinary bladder, prostate, pituitary, and any unidentified mass. The hyperplastic index at 13 weeks was calculated by the method of Clayson *et al.* (2) (Fig. 1). Hyperplastic bladder epithelium is scored as follows: 5 points for six to eight cell layers, 3 points for three to five cell layers, and 1 point for doubtful hyperplasia.

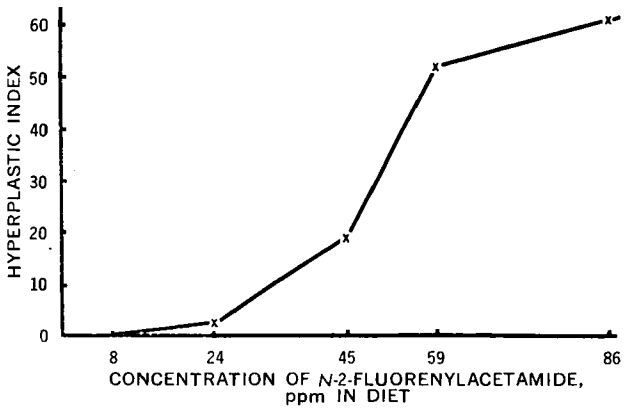

Figure 1—*Hyperplastic index for BALB/c male mice ingesting* N*-2-fluorenylacetamide in the diet for 13 weeks. Females gave indexes of 7.5 and 21.2 after ingesting 59 and 86 ppm, respectively, for the same time interval. Both sexes had a hyperplastic bladder epithelium of three to five cell layers, equivalent to a value of 3 points. The formula of Clayson* et al. *(2),* N/n, *where* N *is the total number points per group of animals and* n *is the number of mice per group, was used to calculate the plotted values.*

The urinary bladder hyperplasia response is of a quantal nature and was recorded for different dose levels and different sacrifice times. Therefore, it seemed appropriate to attempt to quantify this dose, response, and time relationship in a plane whose equation is:

$$Y = a + bX + cZ \qquad \text{(Eq. 1)}$$

when Y is the probit of the response, X is $\log_{10}$ (dose), and Z is $\log_{10}$ (time). The mathematics for such a model was discussed by Finney (4).

If one considers a fixed response (say 50% hyperplasia), then the resulting linear equation may be solved for dose for a given time or

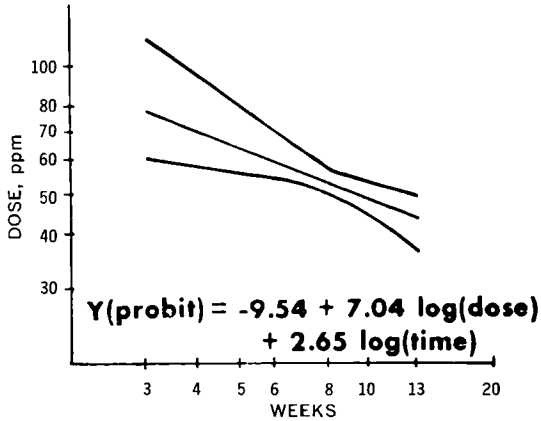

Figure 2—*A plot of* $5.00 = -9.54 + 7.04 \log (dose) + 2.65 \log (time)$ *with appropriate confidence limits on dose.*

[1] Purina.

Table I—Incidence of Urinary Bladder Hyperplasia in BALB/c Strain Mice Fed *N*-2-Fluorenylacetamide

Sacrifice Interval, weeks	Sex	Dose of *N*-2-Fluorenylacetamide, ppm					
		0	8	24	45	59	86
1	♂	0/24[a]	0/48	0/48	0/24	0/16	0/8
	♀	0/24	0/48	0/48	0/24	0/16	0/8
3	♂	0/23	0/47	0/48	1/24	2/16	6/8
	♀	0/24	0/48	0/48	0/24	0/16	0/8
6	♂	0/24	0/47	0/48	2/24	11/16	8/8
	♀	0/24	1/47[b]	0/47	0/24	0/16	0/8
9	♂	0/23	0/47	2/48	5/22	11/15	8/8
	♀	0/24	0/47	0/46	0/24	0/16	2/8
13	♂	0/24	0/46	3/44	7/23	13/15	8/8
	♀	0/24	0/47	0/47	0/24	2/16	3/8

[a] Number responding out of total number in group. [b] Animal had cystitis.

time for a given dose and confidence limits placed on either. This may be accomplished by considering the region of $(Y - Y_0) \geq t^2$ var (Y), where Y_0 is a fixed response and:

$$\text{var}\,(Y) = 1/Snw + X^2/Snwx^2 + 2XZ/SnwXZ + Z^2/SnwZ^2 \quad \text{(Eq. 2)}$$

$$X = X - \bar{X} \quad \text{(Eq. 3)}$$

$$Z = Z - \bar{Z} \quad \text{(Eq. 4)}$$

By replacing $Y = Y + bX + cZ$, expanding this, and taking the equality, one has a quadratic in X or Z. When fixing Z and solving the quadratic in X, the solution is:

$$X = \bar{X} + \frac{-B \pm \text{root}\,(B^2 - 4AC)}{2A} \quad \text{(Eq. 5)}$$

where:

$A = b^2 - t^2/SnwX^2$
$B = 2(bw - t^2Z/SnwXZ)$
$w = \bar{Y} + cZ - Y_0$
$C = (w^2 - t^2/Snw - t^2Z^2/SnwZ^2)$

and the terms are from the usual maximum likelihood probit solution.

If one then increments the time variable (Z), upper and lower confidence limits are produced for the dose variable (X). This may be repeated for any fixed response or contour (Y_0).

RESULTS

Table I shows the time sequence for the development of urinary bladder hyperplasia in BALB/c mice fed dietary concentrations of *N*-2-fluorenylacetamide ranging from 8 to 86 ppm. The first response was observed at 3 weeks and was seen only in the males. Moreover, the response became progressively greater with each sacrifice interval as this sex responded with increased frequency at lower dosage levels. Only at the two higher dosage levels and at the 13th-week interval was hyperplasia observed in the females. The one female that responded at 8 ppm at the 6th-week interval was suffering from acute cystitis, a condition that produces hyperplasia. The difference in response between the sexes might involve metabolic conversion of *N*-2-fluorenylacetamide to its *N*-hydroxy derivative, the proximate carcinogen (4), with the females showing a lower conversion rate.

There is apparently a critical level of *N*-2-fluorenylacetamide ingestion involving dosage rate, time, and total dose for the development of urinary bladder hyperplasia. Figure 2 indicates the interplay of these factors in the calculation of hyperplasia responses. The model from the *Experimental* section was fit to male hyperplasia data for 3–9 weeks and doses ranging from 24 to 86 ppm. The estimated model was:

$$Y\,(\text{probit}) = -9.54 + 7.04\,\log\,(\text{dose}) + 2.65\,\log\,(\text{time}) \quad \text{(Eq. 6)}$$

The model was an adequate representation of the data as expressed with the χ^2 goodness of fit with 13 *df*, $X_{13}^2 = 20.27$. The 95% confidence intervals on dose for the 50% response contour are shown in Fig. 2.

Other than urinary bladder hyperplasia, the only consistent histopathological findings were spontaneous pericardial fibrosis and mineralization, apparently unrelated to chemical ingestion because they occurred with equal frequency in the controls. The overall incidence was males 218/824 (26.4%) and females 115/829 (13.8%). All other tissues examined did not differ from the control tissues.

DISCUSSION

Reports of *N*-2-fluorenylacetamide inducing urinary bladder hyperplasia in mice have been confirmed (1, 2). In addition, it has been shown that the level below 300 ppm is equally effective in inducing this pathological change in the urinary bladder epithelium. The sex difference in response was similar to the observation of liver tumors in male but not in female mice (5).

Calcareous pericarditis was previously reported for several inbred strains of mice: DBA, C, CBA, C3H, A, and BALB/c (6–9). The calcified lesions varied from 77 to 94% for the first four strains, but figures were not given for the BALB/c strain. In contrast to the observation of a greater incidence of the lesion in male BALB/c mice, the other strains showed no sex difference.

The successful fitting of a model to the data for male hyperplasia gives hope that such a model may also be fit to tumor data and that the time factor can be given appropriate consideration in extrapolating to safe doses.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 10, 1974, from the *Department of Health, Education, and Welfare, Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079*

Accepted for publication July 3, 1974.

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