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Mutations in liver DNA of *lacI* transgenic mice (Big Blue) following subchronic exposure to 2-acetylaminofluorene

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Summary

2-Acetylaminofluorene (2-AAF) was administered at levels of 0, 300 and 600 ppm in the diet for 28 days to female transgenic mice bearing the *lacI* gene in a lambda vector (Big Blue® mice). The lambda vector was excised from liver DNA and packaged in vitro into bacteriophage particles which were allowed to infect *E. coli* bacteria, forming plaques on agar plates. Approximately 10^5 plaques were screened per animal for the appearance of a blue colour, indicative of mutations in the *lacI* gene which had resulted in an inactive gene product. Background mutation rate was 2.7×10^{-5} (pooled results of two animals, 8 mutant plaques/289 530 plaques). At 300 ppm in the diet, the rate of 3.5×10^{-5}(8/236 300) was not significantly increased over background. At 600 ppm in the diet, the rate increased approximately 3 fold to 7.7×10^{-5} (17/221 240). In comparison to the usual single or 5-day carcinogen exposure regimes, the 4-week exposure protocol allowed the use of much lower dose levels (10–1000 fold lower). Overt toxicity could thus be avoided. The daily doses used were somewhat higher than those required in 2-year carcinogenicity studies with 2-AAF.

Transgenic animal technology is currently opening up new possibilities in biomedical research. One application of particular interest to toxicology is the development of in vivo mutation assays as quantitative tools to assess the mutagenic potency of chemicals. One such assay uses transgenic mice bearing the *lacI* gene (Big Blue® mice). The genome of these mice has been manipulated such that every cell contains, stably integrated into the DNA, multiple tandem copies of a lambda vector bearing the bacterial *lacI* gene. If the mice are exposed to mutagens, there is a small probability that a mutation will occur somewhere along the inserted sequence. Mutations that lead to an inactive gene product (the *lac* repressor protein) are readily detected in host bacteria using a colour test (Kohler et al., 1990).

Experiments with the *lacI* transgenic mice to date have concentrated on short-term exposures (1–5 days), under which conditions extremely

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large doses of known carcinogens, in the range of acute lethal doses, are necessary to produce a detectable increase in mutation frequency in target organs (Kohler et al., 1991b). However, to be useful for low-dose risk assessment, the detection limit of the assay would have to be improved. One possibility in this direction is a longer exposure period. We carried out a subchronic (28-day) exposure to the standard carcinogen 2-acetylaminofluorene (2-AAF). Results are compared to the quantitative dose–effect relationships in acute studies.

Materials and methods

Big Blue® mouse assay principle

The genome of the mice contains multiple copies of a lambda vector bearing the *lacI* gene, stably inserted into an autosomal chromosome. If the mice are exposed to genotoxins, there is a small probability that a mutation will occur somewhere along the inserted sequence. Following carcinogen exposure, the lambda vector bearing the target gene is excised from the genomic DNA and packaged in vitro into infective lambda bacteriophage particles. The particles are allowed to infect an excess of *E. coli* bacteria. On agar plates, plaques from infected bacteria show up as holes against a lawn of uninfected bacteria. Mutations in the marker *lacI* gene are detected using a simple colour test: the *lacI* gene encodes a protein that represses expression of *lacZ*, a bacterial gene which codes for the enzyme β-galactosidase (β-gal). If this *lacI* repressor is inactivated by a mutation, *lacZ* will be transcribed in the host bacteria and β-gal activity expressed. β-Gal activity is detected with the chromogenic substrate X-Gal, which makes a blue product upon cleavage. On the agar plates one thus finds isolated blue plaques containing the mutated target gene amongst a vast majority of colourless plaques bearing the intact repressor. (For more information on the *lacI* mutagenesis assay, see Kohler et al., 1990.)

Animals and treatment

Six female 12-week-old C57BL/6 mice bearing the *lacI* gene (Big Blue® mice) were obtained from Stratagene (Taconic Farms, Germantown, NY, USA). Animals were housed in macrolone cages on sawdust bedding. After 1 week's acclimatisation, the mice were randomly assigned to groups of two receiving pelleted lab chow (Nafag Feed 890, Gossau, Switzerland) containing 300 or 600 mg/kg 2-AAF (Fluka, Buchs, Switzerland; > 99% pure). Two control animals received pelleted chow lacking 2-AAF. Feed consumption and weight gain were monitored twice weekly. After 28 days, the mice were killed by heart puncture under ether anaesthesia, the liver was removed, immediately frozen in liquid nitrogen, and stored at −20°C (for 2 weeks) until workup.

DNA isolation

A detailed protocol for the isolation of DNA and the mutation assay, based on established procedures as described in Sambrook et al. (Sambrook et al., 1989), is available from Stratagene, La Jolla, CA, USA. The methods (all carried out under aseptic conditions) are summarised briefly below. A small slice of liver from the large lobe (approx. 150 mg) was homogenised briefly with 3 ml ice-cold douncing buffer (14 mM sodium phosphate, 20 mM EDTA, 140 mM NaCl, 5 mM KCl, pH 8.0) and incubated for 3 h at 50°C with an equal volume of proteinase K solution (2 mg/ml proteinase K from Boehringer-Mannheim, Mannheim, Germany, 2% SDS, 100 mM EDTA, pH 7.5). Protein was removed by two extractions with 6 ml phenol:chloroform (1:1). The upper aqueous layer was removed with a wide-bore pipette to prevent shearing the DNA. A final extraction with chloroform removed most traces of phenol. The DNA was precipitated by slow addition of 2 volumes of room-temperature ethanol and then resuspended in 3 ml TE buffer (10 mM Tris · HCl, 1 mM EDTA, pH 7.5). DNA concentrations were measured photometrically (A_{260} = 20 at 1 mg/ml) and adjusted to approx. 1 mg/ml with TE buffer. The more concentrated samples required over 1 week to go fully into solution.

DNA packaging

Aliquots of 20 μl DNA solution were transferred with wide-bore pipette tips to Eppendorf tubes. Transpack® in vitro packaging extract components (Stratagene) were thawed, mixed as

described in the kit and added to each liver DNA sample. Transpack® is a patented mixture of lambda phage coat proteins and enzymes, containing all components necessary to excise lambda vectors from genomic DNA and construct viable phages. Samples were incubated for 3 h at room temperature or 37°C. The packaging reactions were stopped by diluting the samples to 1 ml with SM buffer (Sambrook et al., 1989) and storing on ice. The lambda vectors containing the bacterial *lacI* marker gene were now packaged into infectious bacteriophage particles.

Mutation assay

E. coli bacteria lacking the ability to cleave methylated DNA (strain SCS-8, Stratagene) were freshly inoculated from an overnight culture and grown to mid log phase in liquid NZ-Y medium (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% NZ amine, pH 7.0) supplemented with 0.25% maltose and 12.5 mM $MgSO_4$. The bacterial pellet was spun down and suspended in 10 mM $MgSO_4$ to a concentration corresponding to an absorbance of 0.7 at 600 nm. Bacteria (2-ml aliquots) were mixed in 60-ml polyethylene tubes with 333 μl of bacteriophage suspension from the packaging reaction above (i.e., each packaging reaction was divided into three aliquots) and incubated at 37°C for 15 min. In the meantime, 1 g X-Gal (Staehelin, Basle) was dissolved in 3.5 ml dimethylformamide and added to 500 ml molten (52°C) top agar (NZ-Y medium with 0.7% agarose). Top agar (25 ml) was added to each tube and the contents poured onto a 23.5 cm-sided square plate containing NZ-Y bottom agar. Plates were incubated at 37°C overnight.

The next day, plates were scored for mutant plaques (blue); the number of plaque-forming units (pfus) on the plate was estimated by manually counting plaques in representative sectors. After overnight incubation at 4°C, which intensifies the blue colour of X-Gal-positive plaques, plates were reexamined for faint blue plaques. Plaques were confirmed by coring from the plates, suspending in SM buffer containing chloroform and replating at low density with bacteria and X-Gal on small NZ-Y petri dishes. About 10% of the putative mutants were screened out by this process. The resulting mutation frequencies were expressed as number of blue plaques divided by the total number of plaques. Approximately 100000 plaques from the liver were screened per animal for the appearance of blue mutants.

Statistics

The confidence intervals for the mutation frequencies were calculated on the basis of a Poisson distribution (Werner, 1984):

$$k/(n \cdot F_{[\infty,2k]}) \leq p \leq \left[(k+1) \cdot F_{[2(k+1),\infty]}\right]/n$$

where p = real mutation frequency, k = number of mutants, n = total pfus counted, $F_{f1,f2}$ = uncertainty factors from 2-sided F-distribution table for $\alpha = 0.05$.

TABLE 1

MUTATION FREQUENCY IN THE LIVER OF FEMALE TRANSGENIC MICE BEARING THE *lacI* GENE (Big Blue® MICE) FOLLOWING 28-DAY EXPOSURE TO 2-ACETYLAMINOFLUORENE IN THE DIET

2-AAF dose	Plaques counted	Mutant plaques	Pooled mutation frequency	95% confidence interval of mutation frequency (MF; $\times 10^{-5}$)
0 (Control)	116760	3	2.7×10^{-5}	$1.2 \leq MF \leq 5$
	172770	5		
300 ppm	140160	4	3.5×10^{-5}	$1.5 \leq MF \leq 7$
	96140	4		
600 ppm	127220	10	7.7×10^{-5} *	$4 \leq MF \leq 12$
	94020	7		

Each line represents one animal.
* $P = 0.02$.

In our situation, with low mutation frequencies and total pfu counts below 500000, the animal-to-animal variation in mutation frequency was smaller than the mathematical uncertainty inherent in each frequency itself. For this reason, the data from the two animals in each dose group were pooled.

To compare the mutation rates of treated vs. control groups for significant differences, the statistical method 'comparison of Poisson rates' was used from the software package 'Statgraphics' (procedure as in Mansfield (1980).

Results and discussion

The mutation frequencies observed in control and 2-AAF-treated animals are summarised in Table 1. An approximately 3-fold increase in mutation frequency was observed in the liver of mice given 600 ppm 2-AAF in the diet for 1 month, whereas the increase over background seen in the mice receiving 300 ppm was not significant. In principle, confidence limits for the mutation frequencies – and with this, the statistical significance of a difference – is dependent on the number of plaques counted. In our case, for instance, it would require the examination of a total of more than 10 million plaques (> 2.5 million per animal) for the 30% increase in mutation frequency seen with the 300 ppm animals to become significant.

In comparison to the literature reports (Myhr, 1991; Kohler et al., 1991a,b; Hoorn et al., 1993), our subchronic exposure regimen considerably increased the sensitivity of the assay. The former data are based on 1-5 doses of the carcinogens. The doses of carcinogen used and the mutation frequencies induced in the liver with acute and subchronic regimens, in comparison to the carcinogenic potencies of the test compounds, are summarised in Table 2. As the data base is relatively small at this time, liver data from *lacZ* mice from Hazelton, Kensington, MD, USA (Myhr, 1991; Hoorn et al., 1993) were included in the comparison. Carcinogenic potencies are expressed as the 'TD_{50}', defined as the daily dose of a chemical (per kg body weight per day) that results in a 50% tumour incidence in laboratory

TABLE 2

MUTAGENICITY DATA FROM THE LIVER OF *lacI* AND *lacZ* MICE, COMPARING DOSES AND POTENCY OF CARCINOGENS USED TO THE MUTAGENIC EFFECTS SEEN WITH ACUTE OR SUBCHRONIC EXPOSURE PROTOCOLS

Assay and carcinogen	Daily dose [mg/kg bw] (No. of days)	Estimate of carcinogenic potency [TD_{50}; mg/kg bw/day] [a]	Daily dose/TD_{50}	Mutation rate ($\times 10^{-5}$)		Reference
				Control	Treated [b]	
lacI mice, subchronic						
2-AAF	90 (28)	42	2	2.7	7.7	this study
lacI mice, acute						
Methylnitrosourea	100 (5)	2.6	40	1.9	19	Kohler et al., 1991a
lacZ mice, acute						
Ethylnitrosourea	100 (1)	0.07	1500	1.7	13	Myhr, 1991
	20 (5)		300	1.7	14	
Chlorambucil	10 (1)	0.13	80	2.2	12	Hoorn et al., 1993

The lowest dose giving rise to a significant effect is shown.

[a] TD_{50} is defined as the daily dose of a chemical (per kg body weight per day) that results in a 50% tumour incidence in laboratory animals after lifetime exposure (2 years in rats or mice). TD_{50} values were from the database of Gold et al. (1991). Where more than one carcinogenicity study with significant results was found in the database, the TD_{50} value most closely matching the conditions of the mutation assay was selected (i.e., data with mice, data from the same sex and organ as examined in the mutation assay).

[b] If more than one expression time was used following dosing, the most sensitive result is given here.

animals after lifetime exposure. The TD_{50} values were taken from the carcinogenic potency database of Gold and coworkers (1991). The 4-week treatment with 2-AAF showed a significant effect at a daily dose level which was about 10–1000 times lower than in the acute studies. If the dose of carcinogen is expressed as a multiple of the TD_{50} value (column 3), it becomes apparent that a daily dose of $2 \times TD_{50}$ sufficed to induce a significant increase in mutation rate when given for 28 days.

No signs of 2-AAF-induced toxicity were observed at the dose levels used in this study: both food consumption and weight gain were normal in the treated groups. However, in the acute studies with chlorambucil (Hoorn et al., 1993), ethyl- and methylnitrosourea (Myhr, 1991; Kohler et al., 1991a), doses lay in the acutely lethal range (e.g., LD_{50} of chlorambucil ≈ 20 mg/kg). Toxicity can result in regenerative hyperplasia, which in turn can accelerate the accumulation of mutations. Such results would be more difficult to use for extrapolation down to low-dose human exposure levels.

The increase in sensitivity observed with the 4-week exposure is the result of a steady increase in the number of 'stem cells' (defined in this context as cells capable of dividing) that carry a *lacI* mutation. Stem cell division in a fully grown tissue is considered to result in one cell committed to differentiation and death, and another stem cell capable of further divisions. DNA damage can give rise to mutations in both types of progeny cells. If the mutation is generated in the differentiated cell, its contribution to the mutation frequency determined in the DNA isolated from the whole tissue will be limited by the lifespan of this cell. If, on the other hand, the mutation is in the secondary stem cell, it will be passed on to all future progeny, and all subsequent divisions will result in an increase in mutation frequency until a steady state is reached between birth and death of the mutated differentiated cells of this lineage.

Specific mutations are rare events. Therefore, the number of *lacI*-mutated stem cells will always be much smaller than the number of stem cells not mutated at this locus. The number of stem cells that can be recruited to undergo a particular mutation remains essentially constant over the life-time of the animal; thus, the mutation frequency can increase linearly over time. This also means that additionally lengthening the treatment period would further increase the sensitivity of the assay.

Despite the reduction of the daily dose achieved by the longer exposure period, it is still necessary to give high doses of chemicals in order to induce measurable increases in mutation frequency in these transgenic mouse mutation assays: 150 ppm 2-AAF in the diet for 2 years led to a 40% increase in liver tumour incidence in BALB/c mice (Littlefield et al., 1980). Thus, with the 1-month exposure protocol, the assay with the transgenic mice was actually less sensitive than the long-term carcinogenicity bioassay. It is clear that further refinements of the assay are necessary. Refinements to the presently existing system could occur on the following levels: (1) reducing the mathematical variability of the data by counting more plaques per animal, (2) reducing the biological uncertainty of the data by increasing the number of animals per group, (3) lowering the detection limit by further lengthening the exposure period. In addition, the creation of new transgenic lineages and comparison of their inherent sensitivities, currently progressing in several labs, could lead to better systems.

Mutagenicity assays using transgenic animals offer new promise in the testing of chemicals for mutagenic activity in vivo in an organ-specific manner. It is to be hoped that the sensitivity of the assays can be sufficiently increased in the future that they will be useful not only for mechanistic studies at clearly carcinogenic doses, but also allow investigations at low dose levels.

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