Environmental and Molecular Mutagenesis 21:209–211 (1993)

Can Nongenotoxic Carcinogens Be Detected With the *lacI* Transgenic Mouse Mutation Assay?

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Key words: Di(2-ethylhexyl)phthalate, DEHP, heptachlor, phenobarbital, 2-acetylaminofluorene, Big Blue Transgenic Systems, liver, cell division

INTRODUCTION

In a commentary in Vol. 20, No. 3, of this journal, Ashby and Leigibel [1992] warned of possible confusion in the understanding of genotoxic and nongenotoxic carcinogens because the new transgenic mutation assays might open the possibility of detecting mutagenic activities of chemicals unrelated to their presence in the cell at the moment of mutation initiation. They reasoned that carcinogens able to stimulate the rate of cell division [Butterworth, 1990; Cohen and Ellwein, 1991] could accelerate the accumulation of "spontaneous" mutations in marker genes arising from endogenous DNA damage [Ames, 1989; Loeb, 1989; Lutz, 1990].

We have just completed a study to investigate whether the measurement of *lacI* mutations in *lacI* transgenic mice [Kohler et al., 1990] could indeed be used to detect nongenotoxic carcinogens such as heptachlor and phenobarbital as "indirect mutagens," following a 4-month feeding period at dose levels positive in the 2-year bioassays. Di(2-ethylhexyl)phthalate (DEHP) was included in the study as a carcinogen that could, in addition to having mitogenic activity, be indirectly genotoxic via oxygen radicals from peroxisomal hydrogen peroxide. 2-Acetylaminofluorene (2-AAF) served as positive control for the induction of *lacI* mutations. The stimulation of liver cell division was investigated in the same animals, using immunohistochemistry for bromodeoxyuridine incorporated into DNA.

The negative results, both for *lacI* mutations in liver DNA and for the rate of hepatocyte division, show that the nongenotoxic carcinogens investigated do not give rise to a generally increased level of mutations or a sustained general increase in the rate of cell division.

MATERIALS AND METHODS

Female 6-week old *lacI* transgenic C57BL/6 mice (Big Blue® from Stratagene, Taconic Farms, Germantown, NY) were randomized by weight into groups of three. They were treated for 120 days with di(2-ethylhexyl)phthalate (DEHP), heptachlor, or sodium phenobarbital mixed into the diet at levels of 3,000 and 6,000, 10 and 20, and 250 and 500 mg/kg feed, respectively. 2-Acetylaminofluorene (2-AAF) at 75 and 150 mg/kg feed was used as genotoxic positive control. Extracts of food pellets were checked with capillary gas chromatography for the carcinogen concentrations. Levels were found to be within −20/+15% of the nominal values.

On day 119, a 24-hr osmotic minipump (Alzet 2001D from Alza Corp., Palo Alto, CA) containing 220 μl bromodeoxyuridine solution (BrdU; 20 mg/ml) was subcutaneously implanted into each treated mouse and three of the controls. Twenty-four hours later, all animals were killed. The large liver lobe was put into 4% formalin for the immunohistochemical determination of BrdU incorporated into DNA (Dietrich and Swenberg, 1991), the remaining liver was shock frozen in liquid nitrogen and stored at −70°C. Isolation of DNA, packaging of the lambda vector (bearing the *lacI* gene) into viable bacteriophage lambda particles, and analysis of plaques for *lacI* mutants was performed according to the Instruction Manual distributed by Stratagene (La Jolla, CA 92037). On average, 106,000 plaques were counted per treated animal, and 180,000 for the controls. Dose responses were evaluated by linear regression of the data. Individual dose groups were compared to the controls for an effect using the Dunnett t-test.

RESULTS

The average weight gain of the animals was not affected by the treatments. Implantation of the BrdU-minipump had no effect on the spontaneous mutation frequency in the liver, which was 10×10^{-5} ($\pm 4 \times 10^{-5}$; average ± 1 S.D. from three mice; a pooled total of 55 mutants were detected in

Received November 24, 1992; revised and accepted January 7, 1993.

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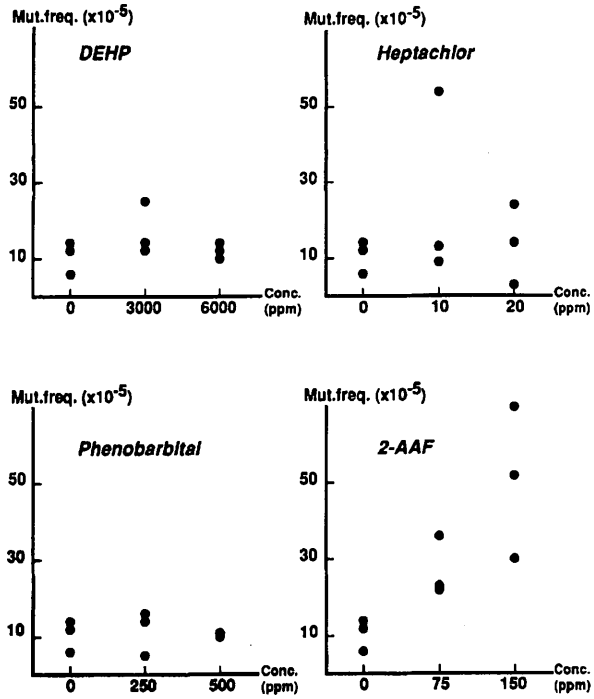

Fig. 1. Mutation frequencies in the *lacI* gene of liver DNA isolated from female *lacI* transgenic mice 4 months after feeding with three nongenotoxic and one genotoxic hepatocarcinogen at two dose levels. Each spot represents one animal. On average, 180,000 and 106,000 plaques were analyzed for each control and treated mouse, respectively. Confidence intervals for the single values can be estimated from the Poisson distribution. Mean mutation frequencies are given in the text.

54,100 plaques analyzed). As expected, treatment with the genotoxic carcinogen 2-AAF resulted in a dose-dependent and highly significant increase of the mutation frequency to 27×10^{-5} ($\pm 8 \times 10^{-5}$) and 51×10^{-5} ($\pm 20 \times 10^{-5}$) at 75 and 150 ppm in the diet, respectively.

With the nongenotoxic hepatocarcinogens, no significant increases in mutation frequency could be observed. The average mutation frequencies ($n = 3$; $\times 10^{-5}$) were 17 ± 7 and 12 ± 2 for DEHP, 25 ± 25 and 14 ± 10 for heptachlor, and 12 ± 6 and 10 ± 1 for phenobarbital (low and high doses, respectively). The animal-to-animal variation in response was quite marked with heptachlor. Figure 1 shows the mutation frequencies for all animals. It clearly illustrates the difference between the genotoxic carcinogen 2-AAF and the three nongenotoxic carcinogens, produced at dose levels that would have resulted in a similar liver tumor incidence with all four carcinogens. At the high-dose levels, for instance, a net increase in liver tumors of 34, 67, and 52 percent was reported for the nongenotoxic carcinogens DEHP [Ashby and Tennant, 1988], heptachlor [Ashby and Tennant, 1988], and phenobarbital [Thorpe and Walker, 1973], whereas 2-AAF induced a 41% increase after 2 years [Littlefield et al., 1980].

At the time point examined, the rate of cell division in the liver was also unaffected by exposure to the nongenotoxic carcinogens. The labelling index for the incorporation of BrdU into DNA (number of BrdU-positive hepatocytes divided by the total number of hepatocytes), i.e., the fraction of hepatocytes in S-phase within a 24-hr period, was 0.3%, with no differences between the groups (data not shown).

DISCUSSION

Our data indicate that the two classic nongenotoxic carcinogens heptachlor and phenobarbital did not give rise to an increase in *lacI* mutations in the transgenic mice treated for 4 months. DEHP also produced no increase, supporting the idea that oxygen radical-induced DNA damage and mutagenicity is unlikely to be involved in its carcinogenicity [Butterworth, 1987]. The idea expressed by Ashby and Leigibel [1992], based upon a plausible mechanistic hypothesis, could not be supported with our experiments. How can the negative data be explained?

Strain Differences?

Carcinogenicity of phenobarbital in the liver of the male mouse has been shown to be strongly strain dependent. Males of the C57BL/6 strain were not responsive [Becker, 1982]. Only two strains of female mice have been investigated (CF-1 positive [Ponomarkov and Tomatis, 1976]; BALB/c negative [Cavaliere et al., 1986]). No sex or strain difference has been found in the carcinogenicity of heptachlor. DEHP was carcinogenic for both rats and mice. It is unlikely that the mice used in this study would not have shown any increased liver tumor formation within 2 years, particularly in the heptachlor and DEHP groups. Nevertheless, for transgenic animal strains to become valid short-term test systems for chemical carcinogenesis, a few long-term studies will have to be performed. Future experiments should be based on the recently available *lacI* transgenic B6C3F1 hybrid mouse, for which a large carcinogenicity data base exists (NTP studies [Ashby and Tennant, 1988]).

Nonsustained Mitogenic Effect

The mitogenic effect of nongenotoxic hepatocarcinogens can be very prominent at the beginning of a treatment but fades away upon adaptation [Melnick, 1992]. Our negative data support the understanding that mitogenesis by nongenotoxic carcinogens is not a sustained effect upon the entire organ. This also means that measuring the average stimulation of cell division might not be a reliable short-term test for nongenotoxic carcinogens.

Delayed Cell Death

The nongenotoxic carcinogens tested have tumor-promoting activity in an initiation-promotion protocol, probably due to providing a growth advantage to initiated cells.

Clonal expansion of these cells is not necessarily based on an increased rate of cell division but could also be the result of a decreased rate of death of initiated cells, which would also give the cells more time to take the next step in malignant transformation. This process is not accompanied by a measurable increase in mutations in the whole organ.

lacI as Marker Gene

The hypothesis tested was that nongenotoxic carcinogens could provide a general mitogenic stimulus to the target tissue, thereby randomly accelerating the fixation of spontaneous DNA damage as mutations. The negative results indicate that the mechanisms of nongenotoxic carcinogens are more subtle. The *lacI* gene is unrelated to the process of carcinogenesis. If the nongenotoxic carcinogens are selectively expanding clones of cells that have undergone a mutation in a gene involved in growth control (i.e., "initiated" cells), then the *lacI* gene would not be a sensitive marker for their detection. It might be better to measure mutation frequency directly in a proto-oncogene or tumor suppressor gene. Nonetheless, if the process is specific to isolated preneoplastic clones of cells, looking at the entire liver will dilute the investigated effect.

In conclusion, our data indicate that the measurement of rates of cell division or mutations in the liver as a whole is unlikely to generate data that accurately describe the process of hepatocarcinogenesis. The clonal nature of the process of carcinogenesis has to be taken into account. Rather than analyzing the tissue as a whole, we need to search for ways to selectively examine cells that have already moved along the path toward tumor formation.

ACKNOWLEDGMENTS

We thank Dr. Peter Schmid for the analysis of the animal feed for the levels of the carcinogens, Ms. Regula Candrian and Dr. Daniel Dietrich for their help with the minipump implantation and the immunohistochemistry, and Dr. Christian Sengstag for numerous tips with the molecular biology techniques.

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Accepted by —
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