tration to help with regulation rather than indicating absolute certainty of no risk below the concentration.

The European Medicines Agency (EMEA) (61) has proposed that concentrations of less than 1.5 μg/day (corresponding to a 10^{-5} lifetime risk) would be acceptable for genotoxic impurities and contaminants in pharmaceuticals where there is a risk:benefit consideration. Humphrey has argued for a more flexible approach than a standard figure (62).

Conclusions

A wide range of curves and mathematical models can be fitted to experimental data but cannot prove the existence of an absolute threshold in a dose-response relationship. Equating a threshold with the identification of a NOEL/NOAEL based upon statistical significance using a hypothesis testing has serious limitations. Statistical methods based upon the estimation of the size of a response together with the associated confidence intervals can provide estimates of doses where there is high confidence of negligible risk. Approaches based upon the benchmark dose methodology and threshold of toxicological concern have the potential to be used in this way. Future advances in the field may include the development of more sophisticated mathematical models which include consideration of DNA repair and metabolic detoxification (63) and the use of multivariate methods in association with -omics technologies to investigate the pattern of responses in low dose studies.

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Regular article

Theoretical and Experimental Approaches to Address Possible Thresholds of Response in Carcinogenicity[1]

Kirk T. Kitchin[2]

Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, USA

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The determination and utilization of the actual low dose-response relationship for chemical carcinogens has long interested toxicologists, experimental pathologists, modelers and risk assessors. To date, no unequivocal examples of carcinogenic thresholds in humans are known. However, at least 5 examples of thresholds of preneoplastic foci or tumors have been observed in animals. The two largest dose-response studies utilized 20,880 mice (2-acetylaminofluorene) and 7,200 rainbow trout fry (aflatoxins). In both of these studies linear relationships were observed for DNA adducts and for liver tumors. A threshold relationship was observed for 2-acetylaminofluorene induced mouse urinary bladder cancer. Other comprehensive dose-response studies have examined the chemicals 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline, 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine and diethylnitrosamine. Taken collectively, the DNA adduct data for these 6 well studied chemicals are fairly linear. The foci and tumor data show either supralinear, linear or threshold curves, making it difficult to generalize. All the 6 studied chemicals cause multiple biological effects including genotoxicity, cytotoxicity and cell proliferation in complex dose and time dependent patterns that are not fully understood. We do know that there are multiple possible biological defenses (at least 7 pharmacokinetic and 7 pharmacodynamic) against the development of cancer. Currently, we have limited scientific and regulatory understanding of chemicals that act simultaneously or sequentially via both linear and nonlinear carcinogenic pathways (genotoxic and nongenotoxic). If an 100% experimental approach is used to elucidate the dose-response of chemicals of dual carcinogenic dose-response properties (linear and non linear), this would require studying 2 or more such chemicals in a large scale coordinated fashion employing at least 1,000 animals, 5 different treatment groups, 7 different study parameters and 8 different scientific disciplines.

Key words: cancer, threshold, dose-response, genotoxic, mutagenic

Introduction

Looking at the impact of cancer upon human health and society, one quickly sees the enormous incidence of disease, lost human potential and death. Indeed cardiovascular disease and cancer are the two major causes of human disease and death. Using data from the American Chemical Society's Cancer Facts & Figures 2008 report, relative to lifelong human probabilities, the birth to death percent chance of developing an invasive cancer is 44.9% in males and 37.5% in females. The birth to death rate for developing invasive cancer is 12.3 % for breast cancer in females and 16.7% for prostate cancer in males. The rate of invasive prostate cancer development in males aged 70 and higher is 13.4%. There is an approximate 100-fold or greater difference in the age specific rates of several invasive human tumors such as colon & rectum and lung & bronchus (of both sexes) and also urinary bladder and prostate cancer in males. Age related prostate changes and noninvasive prostate hyperplasia, foci and tumors are common in older men (1). Hence, there is a saying among urologists that their male patients will either acquire prostate cancer during their lifetime or die of some other cause prior to developing at least prostate cancer in situ.

Society in general and risk assessment organizations and officials have long struggled with several difficult issues in balancing the conflicting goals of protecting the public health from environmental carcinogens and minimizing the costs of environmental regulations. There are many difficulties that confront a risk assessor. First, there are often orders of magnitude between both (a) the concentrations of chemicals in human exposures and animal experiments and (b) the incidences of cancer in human populations (1–2% for the yearly invasive cancer rates in 60 to 69 year old Americans) and animal bioassay experiments (e.g. often 2 to 100%) (Fig. 1 and

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[2]Correspondence to: Kirk T. Kitchin, Environmental Carcinogenesis Division, Mail Drop B143-06, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA. Tel: +1-919-541-7502, Fax: +1-919-541-0694, E-mail: kitchin.kirk@epa.gov

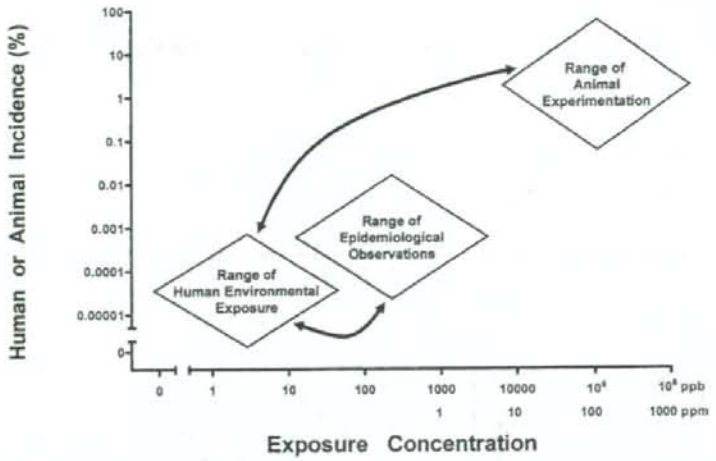

Fig. 1. In human risk assessment we wish to protect a large number of people who are exposed to low concentrations of chemicals. Epidemiological studies and experimental studies in animals can both provide useful and different types of information to risk assessment however the information often comes from quite different ranges of both exposure conditions and incidences.

Table 1. Depending on the chemical of interest and the source of the risk assessment information (epidemiological or animal study), there is often an enormous range of dose or effect extrapolation required to reach the common human exposure concentrations of environmental chemicals (e.g. 2 to $\sim 10^5$)

	SMALL	LARGE
DOSE	2–10	$\sim 10^5$
EFFECT	2	$\sim 10^7$

Table 1). For example, a 50 rodent experiment might at best be capable of telling the difference between a 0 and 2% incidence of cancer, but it cannot provide useful information about human cancer incidences in the range of one cancer case per thousand or one case per million people. At best the limit of sensitivity of common rodent bioassays is about 4–10%. Second, when human epidemiology information is available to assist in assessing risk, the human exposure assessment can never be as complete as in a typical animal bioassay experiment. In addition the human tissue concentration of the parent chemical or important metabolites are rarely known for organs in which cancers frequently develop (e.g. lungs and bronchus, breast, colon and rectum, and prostate). No matter how much information is available and how good it is, a risk assessor is always faced with major uncertainties. Among the largest and most important uncertainties are the true human dose-response relationships in the dose range in which no meaningful animal experiments can be performed.

The term threshold has been used differently by different authors and for different disciplines. In respect to dose-response relationships, there seem to be three major uses of the term threshold. First, the term absolute threshold means that there is no biological effect caused by the exposure at all (2). Second, the term experimental (some people prefer the word practical) threshold means that no biological effect was detected in a particular experiment of a certain sample size and statistical analysis (2). This is somewhat similar to an experiment derived no observable effect level (NOEL). The largest treatment group sample sizes in cancer bioassay experiments is in the range of 2,109 mice (3) and 360 trout (4). Experimental designs for common cancer bioassays use only 50 animals per treatment group. Each individual experimental design will vary in its ability to detect small biological changes and also the degree to which its experimental parameters are correlated with adverse health outcomes such as cancer. The statistical concepts of sensitivity, positive predictivity and concordance are useful in describing the degree to which a particular biomarker is connected to an adverse health outcome (5). Third, the term pragmatic threshold (2) means that a biological effect has occurred but that it is biologically unimportant.

Theoretical Approaches

Moolgavkar originated a theoretical and useful view of carcinogenesis that divides carcinogenesis into the stochastic processes of sequential mutational events and of cell birth, apoptosis, necroses, differentiation and death (6). Figs. 2–4 show schematic versions of a type of multi-stage carcinogenesis model for N required mutations with intervening time for clonal expansion of the number of mutated cells possessing a certain number of mutations. In Fig. 2, the case of purely genotoxic car-

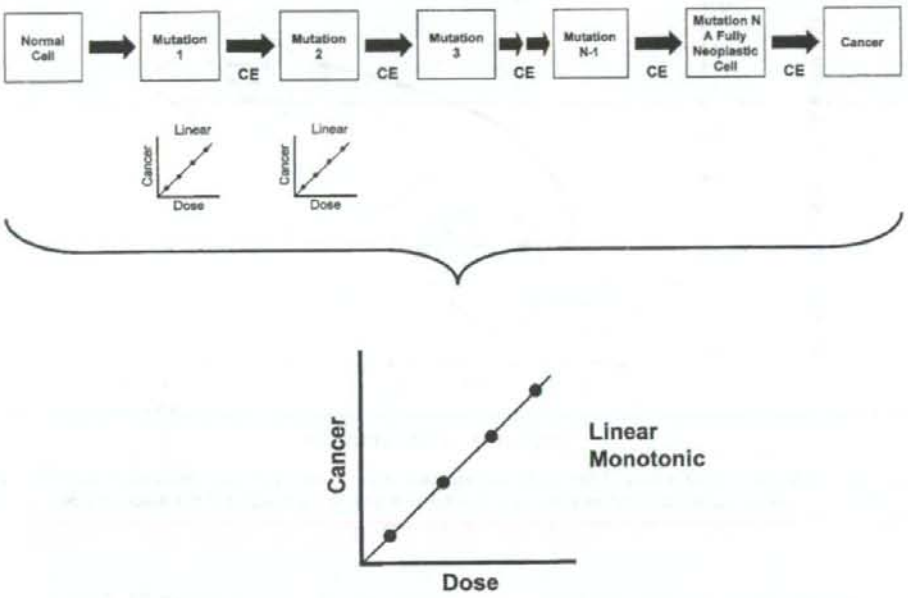

Fig. 2. Scheme of multistage carcinogenesis consisting of N mutational steps with clonal expansion (CE) of the number of mutated cells. In this example the genotoxic carcinogen increases the rates of mutational steps #1 and 2. For strongly mutational compounds the tumor dose-response relationship should be linear at low doses. At higher doses upper limits or asymptotes will be observed near 100% tumor bearing animals.

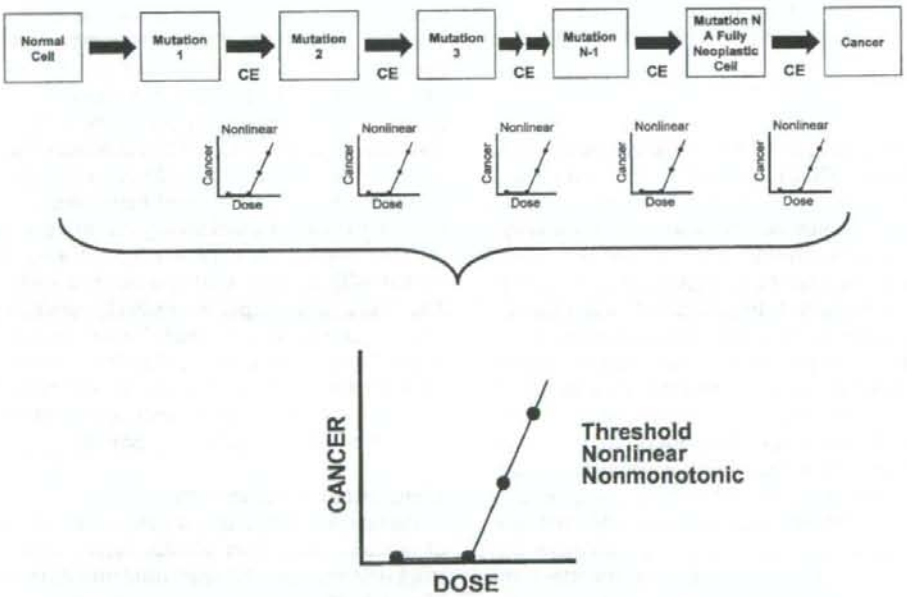

Fig. 3. Scheme of multistage carcinogenesis consisting of N mutational steps with clonal expansion (CE) of the number of mutated cells. In each of the individual clonal expansion steps as well as the overall carcinogenic process there is a nonlinear or threshold type of dose-response relationship.

cinogens which act in a linear dose-response fashion on two mutational steps in the low dose region is presented. Theoretically, if there is no important contribution from cellular kinetics, the best estimate of the dose-

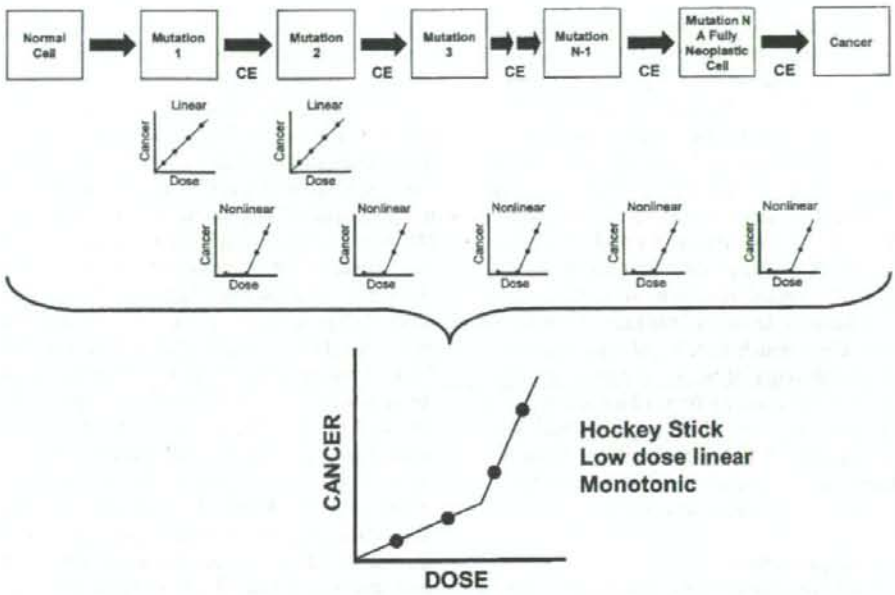

Fig. 4. Scheme of multistage carcinogenesis consisting of two mutational steps increased in rate by dose along with clonal expansion (CE) of the number of mutated cells. This scheme is the mechanistic and mathematical sum of the schemes of Figs. 2 and 3. The dose-response relationship of each of the clonal expansion steps is nonlinear or threshold. The two mutational steps should be linear, the same as in Fig. 2. Overall the relationship between cancer and dose is monotonic, low dose linear and/or hockey stick shaped. At higher doses several processes can contribute to carcinogenicity and upper limits or asymptotes found. At low doses only the mutational process is contributing and the dose-response relationship is linear.

response relationship between cancer incidence and exposure is both monotonic and linear at low concentrations and then at higher doses saturation and asymptote character as the function approaches 100% tumors.

Experiments that sequenced nearly all human genes in 22 cases of human glioblastoma multiforme and 24 cases pancreatic cancer have established that (a) hundreds of individual genes that are mutated in these two cancers and (b) on average 60 genes are altered in glioblastoma (7) and 63 genes in pancreatic cancer (8), respectively. The number of genetic alterations found in the 24 cases of different pancreatic tumors ranged from approximately 30 to 155 (8). These gene alterations included point mutations, amplifications and deletions (7,8), of which point mutations were the most commonly found.

In Fig. 3, a case of a chemical that lacks any linear contribution to carcinogenesis is presented. This is normally considered the nongenotoxic family of chemicals. In this causal scheme all of the driving factors are associated with nonlinear cellular kinetics (e.g. cytotoxicity and regenerative hyperplasia) and there is zero contribution from linear mutational processes. Overall the dose-response of such chemicals is described as threshold, nonlinear or nonmonotonic (Fig. 3). The word monotonic means always increasing or always decreasing.

In Fig. 4 a more interesting case of a chemical that effects both nonlinear and linear causes of carcinogenicity is presented. The contribution from nonlinear processes at high doses makes the slope large in this high dose range. However, at low doses, the dose-response relationship will still be linear even though the slope may be small.

Another theoretical approach to the problem of low dose carcinogenesis is the theory presented by Upton (9) that might be called the incremental exposure-incremental risk or additivity to background theory. This theory states that if chemical-induced biological effects add to already existing background carcinogenic processes, this will necessarily result in a monotonic or linear dose-response relationship (9).

In the history of toxicology, experimental pathology and carcinogenesis, we were first aware of the linear in dose-response, genotoxic and mutational type of carcinogens. Chemicals that are members of this group include ionizing radiation, cigarette smoking exposures, 2-acetylaminofluorene (CAS 53-96-3), aflatoxins, many polycyclic hydrocarbons and nitrosamine carcinogens.

Some of the first instances of nonlinearity in carcinogenesis were found with 2-acetylaminofluorene induced urinary bladder tumors (3), nitrosamine induced esophageal cancers (10), formaldehyde (CAS 50-00-0)

(11), carbon tetrachloride (CAS 56–23–5) (12), chloroform (CAS 76–66–3) (13) and non-DNA-reactive chlorinated hydrocarbons such as hexachlorobenzene (CAS 118–74–1) and *p,p'*-dichlorodiphenyltrichlorethane (CAS 50–29–3) (DDT). The funding for some of these studies came from large scale coordinated investigations into dose-response relationships (National Center for Toxicological Research (NCTR) (3), the British Ministry of Agriculture, Fisheries and Food (10), several Japanese studies (14,15)), general investigations into carcinogenesis and targeted research by organizations like the former Chemical Industry Institute of Toxicology (CIIT) in the USA which developed and advocated nonlinear risk assessments of some chemical carcinogens of low or zero genotoxicity (e.g. chloroform) (13). Unfortunately, there has been little experimental work on chemical carcinogens which possess both linear and nonlinear causes of carcinogenicity and have complex or hockey stick-shaped dose-response curves.

Experimental Approaches

Zeise *et al.* (16) have contributed a thorough review of carcinogenic dose-response relationships in both humans and experimental animals. This section of the present paper will present six studies of different chemicals in rats, mice and trout fry. Experimental design and results from studies of 2-acetylaminofluorene (2-AAF) (3), four aflatoxins (4) (aflatoxin B_1 (CAS 1162–65–8), aflatoxicol (CAS 29611–03–8), aflatoxin M_1 (6795–23–9) and aflatoxicol M_1 (CAS 64330–03–6)) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (CAS 77500–04–0) (MeIQx) (15) are presented in Table 2. The NCTR study of 20,880 female mice employed 7 dietary exposure concentrations and varied sacrifices from 9 to 33 months and thus offers a data set with both time as well as dose as the x axis (3). A linear relationship for liver tumors and a threshold relationship for urinary bladder tumors were observed in this much discussed and interpreted study. The 7,200 trout fry study of Bailey *et al.* (4) also stands out for the large number of experimental animals and the evenly spaced logarithmic dose selection in the experimental design. Up to 5 doses of 4 aflatoxins differing in their structure and potency were studied. Overall, the results showed linearity for DNA adducts with external dose and a fairly linear tumor response in trout liver (4). Some of the tumor data points were off a straight line. Three of the aflatoxin dose-response curves did not show any indication of thresholds (4). Tsuda *et al.* (15) used MeIQx as a genotoxic heterocyclic amine carcinogen in a 1,145 rat study of liver adducts and foci. Their logarithmic spacing of the five doses makes it difficult to demonstrate a linear tumor dose-response (if this is true for MeIQx), but the adducts were found to be linearly related to dose. In the experimental design of animal toxicity and carcinogenicity tests, experimentalists are often forced to design the study either arithmetically or logarithmically. Arithmetic designs are often appropriate for deciding between linear or threshold extrapolation models. Logarithmic designs are best for range finding and deciding what parameters are responsive in which dose regions.

Sample size and the sensitivity to detect biological effects are an important element of experimental design. Sensitivity is mainly determined by the biology of the experimental system, the experimental methods used, the background rate of the process(es) being studied and the sample size. To detect with 95% certainty a biological effect that occurs at a 10, 1, 0.1 and 0.01% incidence requires 29, 299, 2,995 and 29,956 animals, respectively (17). Thus, even the 2-AAF mouse cancer bioassay experiment cannot detect cancer incidences of 0.01% which is not an acceptable chemical-induced cancer rate in humans.

In addition to sample size the length of time an ex-

Table 2. Summary of three major studies of the dose-response relationships for adducts, foci and tumors

NCTR (FARMER ET AL., 1979)	BAILEY ET AL., 1998	TSUDA ET AL., 2003
2-ACETYLAMINOFLUORENE (2-AAF)	4 AFLATOXINS	2-AMINO-3,8-DIMETHYL-IMIDAZO[4,5-f]QUINOXALINE (MeIQx)
20,880 FEMALE MICE (BALB/cStCrlfC3HNctr)	7,200 TROUT FRY	1,145 MALE F344 RATS
0, 30, 35, 45, 60, 75, 100 AND 150 PPM (DIET)	4 TO 64 PPB OR 80 TO 1280 PPB (DIET)	0, 0.001, 0.01, 0.1, 1, 10, AND 100 PPM (DIET)
7 DOSES FROM 30 TO 150 PPM, 9–33 MONTHS; LIVER & URINARY BLADDER	UP TO 5 DOSES FROM 4 TO 1280 PPB FOR 2 WEEKS, THEN NORMAL DIET FOR 1 YEAR; LIVER	6 DOSES FROM 0.001 TO 100 PPM FOR 4, 16 OR 32 WEEKS; LIVER
TUMORS: HEPATIC–LINEAR BLADDER–THRESHOLD	ADDUCTS–LINEAR TUMORS–FAIRLY LINEAR	ADDUCTS–LINEAR FOCI–THRESHOLD

Table 3. Summary of another three major rat studies of the dose-response relationships for adducts, foci and tumors

FUKUSHIMA ET AL., 2004	WILLIAMS ET AL., 1999	PETO ET AL., 1991
2-AMINO-1-METHYL-6-PHENOLIMIDAZO-[4,5-b]-PYRIDINE (PhIP)	DIETHYLNITROSAMINE (DEN)	DIETHYLNITROSAMINE (DEN) DIMETHYLNITROSAMINE (DMN)
1,759 MALE F344 RATS	390 MALE F344 RATS	4,080 MALE AND FEMALE RATS
0, 0.001, 0.01, 0.1, 1, 10, 50, 100, 400 PPM (DIET)	0, 25, 50, 100 AND 200 μMOL/KG/WEEK	0.03, 0.07, 0.13, 0.26, 0.53 1.0, 1.6, 2.1, 2.6, 3.2, 4.2, 5.3, 6.3, 8.5, 16.9 PPM
8 DOSES FROM 0.001 TO 400 PPM FOR 16 WEEKS; COLON	4 DOSES FROM 25 TO 200 μMOL/KG/WEEK FOR 5 OR 10 WEEKS, BY MOUTH, THEN 24 WEEKS OF PHENOBARBITAL PROMOTION; LIVER	15 DOSES FROM 0.033 TO 16.9 PPM IN DRINKING WATER FOR LIFESPAN; LIVER, ESOPHAGUS
ADDUCTS–QUITE LINEAR COLON FOCI–THRESHOLD	ADDUCTS–SUPRALINEAR FOCI–SUPRALINEAR LIVER TUMORS–THRESHOLD	TUMORS: LIVER–LINEAR ESOPHAGUS–THRESHOLD

periment is run is of great importance in determining the outcome of a cancer biomarker or bioassay experiment. As an example in the NCTR 2-AAF mouse bioassay study, there is clearly significant carcinogenicity happening at 12 months and longer in urinary bladder and at 18 months and longer in the liver (3). However, it is also clear that there is no significant 2-AAF induced carcinogenicity present at 9 months in the urinary bladder and at 14 months and earlier in liver (3). Thus, even this extremely large animal experiment would not have detected any 2-AAF-induced carcinogenicity if the study had been terminated too early. DNA adducts will typically be present during a chemical exposure. However, unless there is sufficient time the animals may not be found experimentally positive for cancer biomarkers, foci or tumors.

Table 3 presents three other informative studies of the dose-response relationship for the genotoxic carcinogens 2-amino-1-methyl-6-phenolimidazo-[4,5-*b*]pyridine (CAS 105650–23–5) (PhIP) (14), diethynitrosamine (CAS 55–18–5) (DEN) (18) and dimethylnitrosamine (CAS 62–75–9) (DMN) (18). In the study by Fukushima *et al.* (14), 1,759 rats were used in an experimental design of both logarithmic (0.001, 0.01, 0.1, 1, 10, 100 ppm) and additive (50, 400 ppm) nature. In this 16 week exposure, DNA adducts were linear and colon foci were found to have thresholds of response. The Gary Williams group has published several elaborate studies of the dose-response relationship for DEN-induced liver foci and cancer. In one of their experimental designs, 390 male rats and 4 doses arranged arithmetically (25, 50, 100 and 200 μmol/kg/week for 5 or 10 weeks) were followed by phenobarbital promotion (18). The conclusions of this interesting study were that supralinearity was observed for adducts and foci. However the liver tumors showed a threshold of response. Peto *et al.* (10) used a much larger number of animals (4,080 rats), 15 doses of DEN and DMN and longer lifespan exposures in their much discussed experiment. Their overall findings were that the dose-response for liver tumors was linear but that there was a threshold for esophageal tumors.

When the experimental designs and results of the selected 6 studies are displayed in a grid (Table 4) one immediately notices large differences between the studies. Three different species were used (rat, mouse and trout). Some studies used only female animals, some used only male animals and other studies used rodents of both genders. Doses were selected with different experimental purposes in mind and varied from logarithmic to arithmetic experimental designs. The lengths of time varied from weeks (4, 5, 10, and 16 weeks of exposure to the genotoxic agent) to as long as 33 months (3) or the entire animal lifespan (10).

Only three of these studies proceeded to very long times–lifespan for the nitrosamine rat study (10), up to 33 months for the 2-AAF mouse study (3) and one year for the aflatoxins and trout study (4). The shorter times used in other studies may have minimized the biological effects observed and influenced the shape of the dose-response relationship.

Generally, the results support an interpretation that adducts are linear with external dose. With foci as the endpoint, both threshold (14,15) and supralinear (18) dose-response curves were observed. Both linear (3,4,10) and threshold (18) dose-response curves were observed for tumors. Both experimental scientists and risk assessors would have difficulty in interpreting such a different set of experimental designs and this incomplete and conflicting set of observations. It is certainly true to say that linear relationships for animal liver tumors have been observed in well conducted experiments for 2-AAF in mice (3) and for nitrosamines in rats (10). Thresholds of carcinogenic response have also been ob-

Table 4. A simplified summary of 6 major studies of the dose-response relationships for adducts, foci and tumors

AUTHORS	FARMER ET AL., 1979	BAILEY ET AL., 1998	TSUDA ET AL., 2003	FUKUSHIMA ET AL., 2004	WILLIAMS ET AL., 1999	PETO ET AL., 1991
CHEMICAL	2-AAF	AFLATOXINS	MeIQx	PhIP	DEN	DEN, DMN
LENGTH OF EXPERIMENT (WEEKS)	143	54	32	16	38	LIFESPAN
ADDUCTS	LINEAR & LINEAR	LINEAR	LINEAR	QUITE LINEAR	SUPRA-LINEAR	
FOCI			THRESHOLD	THRESHOLD	SUPRA-LINEAR	
TUMORS	LINEAR & THRESHOLD	FAIRLY LINEAR			THRESHOLD	LINEAR & THRESHOLD

served for 2-AAF in mouse urinary bladder (3) and for nitrosamines in rat esophagus (10). However, linear, threshold and even supralinear dose-response relationships have been observed in some experiments for the endpoints of foci or tumors (Table 4). Thus, it is difficult to determine what is generally or often the case based on such a set of animal data.

In cellular studies of dose response relationships, similar conflicting dose-response data are available. For example the mutational dose-response relationship of methylmethane sulfonate (CAS 66-27-3) (MMS), methynitrosurea (CAS 684-93-5) (MNU), ethylmethane sulfonate (CAS 62-52-0) (EMS) and ethylnitrosurea (CAS 759-73-9) (ENU) were studied in human lymphoblastoid cells (19). In respect to inducing mutations in the hypoxanthine phosphoribosyltransferase (HPRT) mutation assay, the overall results were that MNU and ENU were linear and MMS and EMS showed threshold type curves. The two mutagens with a linear dose-response, MNU and ENU, had lower values for their Swain Scott constants and higher degree of DNA adduct formation to the oxygen atoms of dG, dT and dC.

In humans, no convincing thresholds of cancer have been observed. Human exposure assessment, biomarkers, selection of the best dose metric and the concentration of carcinogenic chemicals and/or their active metabolites in cancer susceptible interior human organs could all be improved (20,21).

In the experimental studies presented in this paper, DNA adducts, DNA oxidation, cell proliferation and foci are all examples of biomarkers of exposure or effects. While biomarkers of carcinogenesis have appeal and some utility in determining dose-response relationships, there are limitations to the use of cancer biomarkers as well. Biomarkers of carcinogenesis never have 100% positive predictivity and 100% concordance with tumors (5). Regardless of what the cancer biomarker is, false negatives and false positives are normally observed. Thus, at present it is appropriate for risk assessors to give more weight to tumor data than to biomarkers of tumors data.

Table 5. Possible pharmacokinetic causes of thresholds in dose-response relationships of genotoxic carcinogens

PHARMACOKINETIC:
Exclusion from the animal, cell or nucleus
Requirement for enzymatic activation
Detoxification/conjugation enzymes
High capacity for excretion
Transport systems reduce exposure
Sequestration reduces exposure
Adduct formation not with DNA

Theoretical and Experimental Approaches

Although completely experimental approaches attempting to answer the question: "What is the dose-response relationship between tumors and external chemical dose?" lead to conflicting data sets, there have accumulated a number of theories and reasons to explain the nonlinearities that are sometimes seen with chemical carcinogens of zero, low or medium degrees of genotoxicity. Table 5 presents some of the pharmacokinetic reasons why thresholds could exist for chemical carcinogens. Exclusion from important compartments such as the nucleus, cell or even the entire organism would be expected to introduce nonlinearities to a dose-response curve. A high capacity for detoxification, conjugation or excretion can also cause nonlinearities. A requirement for particular enzymes for activation of a procarcinogen to an active carcinogen could also introduce nonlinearities. Sequestration or adduct formation with macromolecules other than DNA could cause nonlinearities. In all these cases it is nonlinearities between the external chemical concentration and the interior concentration of the chemical needed at the correct location for carcinogenesis that is causing the nonlinearity.

Table 6. Possible pharmacodynamic causes of thresholds in dose-response relationships of genotoxic carcinogens

PHARACODYNAMIC:
Adduct formation not with DNA
High capacity of DNA repair
High fidelity of DNA repair
Inducible defense systems
Requirement for DNA replication (fixing)
Apoptosis of initiated cells
Necrosis of initiated cells
Immune surveillance

Pharmacodynamic reasons why a threshold might occur are presented in Table 6. From a Moolgavkar type of two stage clonal growth or a multi-stage model as presented in Fig. 2, it is easy to see that if substantial apoptosis, or necrosis or immune system based death of cells with important mutations occurs, this could drive a pharmacodynamic nonlinearity. DNA repair is known to be both of high capacity and high fidelity (22,23). However, tumors do spontaneously appear in humans of many different ages leading us to the inevitable conclusion that the biological processes that defend us against carcinogenesis (i.e. DNA repair, cell cycle check point controls, apoptosis, immune system etc) are not 100% effective. The mathematical march toward acquiring more and more mutations in one's cells as time progresses is best seen in two well known facts. The first is that there is a strong age dependency for most human tumor types. The second is that humans have a high birth to death chance of developing invasive cancers (about 37% for women and 45% for men).

Conclusion

We have good scientific and regulatory understanding about dose-response curve shape for linear (and often genotoxic) chemicals and also for nonlinear (usually nongenotoxic) carcinogenic chemicals. Examples of this are 2-AAF and nitrosamines for genotoxic chemicals and chloroform for chemicals with a lower degree of genotoxicity. Formaldehyde causes DNA protein cross-links as well as cell death and regenerative cell proliferation (11). However, we do not have as good scientific and regulatory understanding of chemicals that can simultaneously or sequentially act via both linear and nonlinear pathways of carcinogenesis (e.g. Fig. 4). These types of chemicals are sometimes called mixed or dual mode of carcinogenesis chemicals.

Looking at the current state of understanding of chemical carcinogenesis, we can see what type of information has been most useful to risk assessors. To make future major experimental progress with chemicals of dual carcinogenic dose-response properties (linear and nonlinear), we would need to study 2 or more such representative chemicals (e.g. chemicals with modes of action of cytotoxicity, receptor occupancy, hormonal activity, oxidative stress etc.) in a large scale coordinated fashion involving:

(a) $\geqq$1,000 animals per experiment
(b) $\geqq$5 different dose groups
(c) $\geqq$7 different study parameters
(adducts, 8OHdG, apoptosis, necrosis, cell proliferation, foci, tumors and many other desirable parameters)
(d) $\geqq$8 different scientific disciplines
(physiological based pharmacokinetics (PBPK), toxicology, experimental pathology, molecular biology, genotoxicity, statistics, modeling and risk assessment)

Some of the reasoning used to develop this particular views on the design of large scale multi-disciplinary cancer experiments is given below. At least 5 experimental points are needed to fairly consider equations such as the four parameter asymmetric sigmoid equation in the data analysis along with other common equations such as a line, linear-quadratic, power equation and other transition equations. The inclusion of an intercept term in the data analysis (to account for background carcinogenesis) requires an additional data point for any equation. While sensitivity calculations can be used to calculate the sample size needed to detect a response of a particular incidence, the expense and difficulty of running large animal studies is often of greater practical importance. To increase the sample size per treatment group from 50 to 200 animals will not even increase the sensitivity by a factor of ten. Few animal cancer bioassay studies have used more than 2,000 animals. Some of the more useful to environmental dose-response issues large experimental studies are tabulated in Tables 2 and 3. The three largest studies using 4,080 rats [5], 7,200 trout [2] and 20,880 mice [1] have been much discussed, cited and presently form part of the foundation of contemporary human cancer risk assessment.

For comparative purposes, any future large scale bioassay should include at least the study parameters used in the prior meritorious studies presented in Tables 2–4 (i.e. tumors, foci, DNA adducts, oxidized DNA bases and cellular kinetic parameters (apoptosis, necrosis and cell proliferation). It is quite desirable to include other biomarkers of carcinogenesis as well. To exclude a needed scientific discipline from the experimental design process and the data interpretation of a large scale coordinated bioassay experiment would be a costly mistake. For example individuals very familiar with risk assessment approaches need to be included in the experimental design to insure that the questions being asked are actually going to provide data that will be useful in answering major risk assessment questions. Similarly genotoxicity experts need to be included because they may be able to include in the experimental design *in vivo* parameters of mutagenesis that would be valuable in the data interpretation.

Naturally the brainstorming, developing, planning and agreeing on a specific experimental protocol, as well as the funding and executing of such a multi-disciplinary experiment is an extremely difficult task. However, substantial progress is unlikely to be made without such future large scale multi-disciplinary experimental studies.

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Commentary

Extrapolation of the Animal Carcinogenesis Threshold to Humans[1]

Minako Nagao[2,3,5], Satoko Ishikawa[2], Hitoshi Nakagama[3] and Masahiko Watanabe[4]

[2]Keio University Faculty of Pharmacy, Tokyo Japan
[3]National Cancer Center Research Institute, Tokyo Japan
[4]School of Pharmacy, Shujitsu University, Okayama, Japan

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The presence or absence of a threshold in carcinogenesis for genotoxic carcinogens was reevaluated. The ED01 study of 2-acetylaminofluorene, performed in the U.S. using more than 24,000 mice, provides us with information about the practical limits of an attainable experimental approach for determining carcinogenesis thresholds. The data indicated that the dose response was highly non-linear and an apparent threshold existed for bladder carcinogenesis, but that it was linear-no-threshold for liver carcinogenesis in the same animals. Despite smaller study sizes, we attempted to evaluate the carcinogenesis dose response to 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in rats, using published data. Mammary tumors were induced in female F344 rats by PhIP in a linear-no-threshold dose response, as was lymphocytic leukemia in male and female rats. However, colon tumors were induced in a non-linear dose response, possibly with a threshold, in the same male animals. Liver tumors were induced in male F344 rats by MeIQx, and preneoplastic changes in the liver were induced in non-linear dose response, possibly with a threshold. From these findings, it can be deduced that linear or non-linear dose response with or without thresholds changes depending upon the exposing chemical, species and target organs. Considering heterogeneity of humans there would be no appropriate animal models to evaluate threshold in humans for carcinogenicity of chemicals.

Some types of genotoxic carcinogens, such as methyl methanesulfonate, ethyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, show highly non-linear dose response with an apparent threshold in mammalian cells or bacteria *in vitro*. Involvement of repair mechanisms strongly supports the presence of a threshold. Although it is necessary to confirm non-linear dose response for animal carcinogenesis of a compound showing a threshold for *in vivo* genotoxicity, it is expected that such compounds exhibit thresholds for human carcinogenesis. Dose response studies of genotoxic carcinogens will provide information on using valuable chemicals safely.

Key words: genotoxic carcinogen, threshold, dose response, linear-no-threshold, non-linear, target organ

Introduction

There is a movement to review the current risk evaluations of genotoxic carcinogens that there is no threshold for hazard effect. Since biological systems have DNA repair mechanisms, it can be speculated that there should be thresholds for mutagenesis and carcinogenesis induced by genotoxic carcinogens.

Extensive studies were performed approximately 30 years ago in the U.S. to clarify the threshold for carcinogenesis by a genotoxic carcinogen, 2-acetylaminofluorene (AAF) evaluating more than 24,000 mice (1,2). Highly non-linear dose response to AAF with an apparent threshold was demonstrated for bladder tumors in female BALB/c mice, but linear dose response without threshold for liver tumors in the same animals. On the other hand, linear dose responses were found in DNA-AAF adduct formations in the bladder and liver of BALB/c mice (3). Linear-no-threshold dose response for mutagenesis is generally accepted.

Recently, it was claimed that there are thresholds for carcinogenesis, preneoplastic changes, and mutagenicity when using heterocyclic amines (HCAs) (4–6). These investigators attempted to demonstrate the presence of thresholds using very low doses, including that comparable to human HCA exposure.

In the present article, we introduce the AAF-ED01 study. In this study, we compared the mode of carcinogenesis dose response of two mutagen-carcinogen HCAs, 2-amino-1-methy-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). Existing HCA experimental data were not sufficient to evaluate presence or absence of thresholds, so we compared the mode of dose response in inducing neoplastic change in different organs of the same animals. Even using small numbers of

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[5]Correspondence to: Minako Nagao, Keio University Faculty of Pharmacy, 1-5-30 Shibakoen Minato-ku, Tokyo 105-8512, Japan. Tel/Fax: +81-45-822-1081, E-mail: mnagao@m8.dion.ne.jp

animals, we found that PhIP induced mammary tumors and leukemia in a linear-no-threshold dose response, and it induced colon tumors in a non-linear dose response with a possible threshold (7). As seen in the AAF carcinogenesis study, the PhIP carcinogenesis study indicated that the mode of dose response differed depending upon the target organs. In this article, we present published scientific data that is necessary to understand how to regulate genotoxic carcinogens correctly.

Genotoxic carcinogens are generally considered to have no threshold for genotoxicity. In this study, we really confirmed the absence of thresholds for *in vivo* genotoxicity for several compounds including HCAs by use of published data (8,9). However, some DNA-reacting genotoxic agents were demonstrated to show highly non-linear dose responses *in vitro*, with an apparent threshold (10,11). Involvement of repair mechanisms was indicated for this non-linearity (11), and it was calculated that the repair was about 99.99% below the threshold (19). Therefore, it is reasonable to expect that there are thresholds for carcinogenesis of these compounds. By clarifying genotoxicity dose response of chemicals concerned, some genotoxic chemicals can be utilized below the threshold, safely.

Information Obtained from Dose-response Study in AAF Tumorigenesis

We plotted the reported data of the ED01 study of AAF (CAS 53-96-3) (24,192 female Balb/c mice with lifetime dosing) (12), on an arithmetic scale (Fig. 1). It indicates the presence of an apparent threshold for bladder tumors with a highly non-linear dose response and an absence of threshold for liver tumors when the incidence was extrapolated from doses where a significant increase was observed. After 33 months of treatment, the dose response curve for bladder tumors showed a semi-logarithmic fit with an apparent threshold (Fig. 1a), as reported by Waddell (13). The equation and correlation coefficient we obtained are $y = 112.37\mathrm{Ln}(x) - 460.77$, $R = 0.98$. However, at 18 and 24 months, linear fit was seen with apparent thresholds. The logarithmic fit for bladder carcinogenesis may be due to saturation in incidence with 150 ppm dosing at 33 months, and linear dose response with an apparent threshold was estimated below 100 ppm. The apparent threshold (the point where the line crossed zero) decreased with increased treatment duration, as pointed out previously (13).

For liver carcinogenesis, the dose response curve over 33 months showed a linear fit (Fig. 1b). The apparent threshold was calculated to be 3 ppm ($y = 0.47x - 1.42$, $R = 0.97$). The apparent threshold values decreased with increased treatment duration, and we calculated it to be 0 ppm by 34 months, by use of an equation deduced by Waddell (13). Thus, there would be no threshold, as was also previously reported (12,14). In organs where tumors develop spontaneously, carcinogens may show linear dose responses without thresholds. Background tumor incidences in the liver were 1.1%, 2.6%, and 17 %, at 18, 24, and 33 months, respectively, compared with 0.4%, 0.3%, and 1% in the bladder at 18, 24, and 33 months (12).

In this article, the dose response relationship for bladder carcinogenesis is designated as nonlinear and that for liver carcinogenesis as linear-no-threshold.

Information Obtained from Dose Response Studies of HCA Induced Neoplastic Changes

Dose response studies of PhIP (CAS 105650-23-5) and MeIQx (CAS 77500-04-0) carcinogenesis have been performed with limited numbers of animals and selected dose groups. However, since these compounds induce malignant changes in multiple organs, analysis of experimental data would be useful to help define whether the dose response curves are dependent on target or-

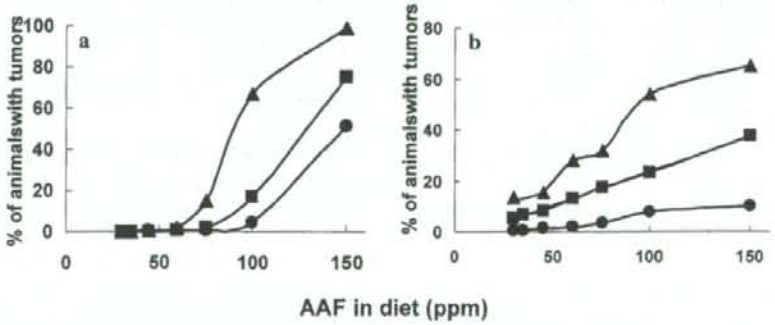

Fig. 1. Summary of ED01 study. Percent of Balb/c mice with tumors, after administration of diet containing AAF for 18 (●), 24 (■), or 33 months (▲). The values of animals with tumors (%) were subtracted values with that of control. Experimental data were from (12). **a**: Bladder tumor; **b**: Liver tumor.

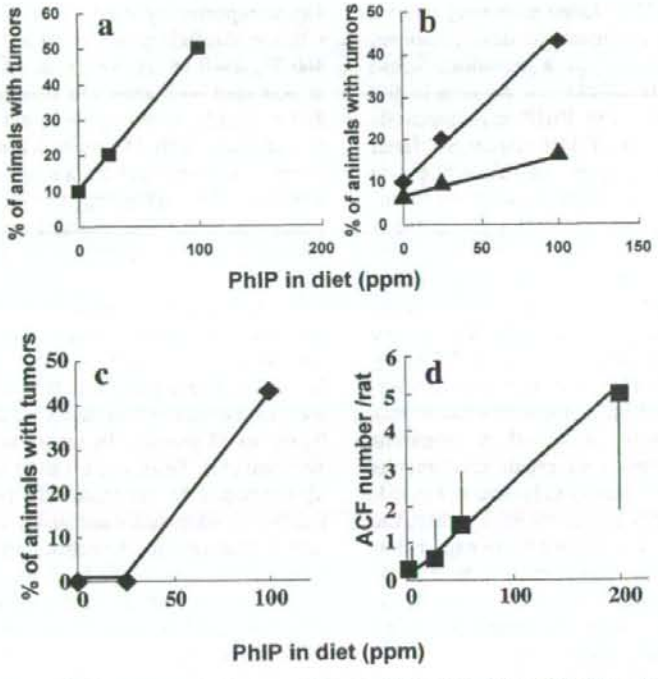

Fig. 2. Dose effect of PhIP on neoplastic or preneoplastic changes in rats. **a**: Mammary tumors after 104-week administration to female F344 rats. Data were from (7). **b**: Lymphatic leukemia after 104-week administration to F344 rats. Data were from (7). ■: Male, ▲: Female. **c**: Colon tumors after 104-week administration to male F344 rats. Data were from (7). **d**: ACF after 16-week administration to male F344 rats. Vertical bar indicates standard deviation. Data were from (4).

gans. Published data show that PhIP induces tumors in mammary glands, the hematopoietic system, colon, and prostate in rats (7,15). MeIQx induces tumors in the liver, skin, Zymbal glands, and clitoral glands of rats (16,17). To estimate the shape of the dose response, all experimental data including those which did not show significant difference from the control group were included, except where indicated.

PhIP–mammary tumorigenesis: Hasegawa *et al.* (7) reported a linear dose response to PhIP dosing using female F344 rats. Each group of 30 rats was fed a diet containing 0, 25, or 100 ppm of PhIP for 104 weeks. Mammary tumor incidences (adenoma or carcinoma) were 10%, 20%, and 50% in the 0, 25, and 100 ppm groups, respectively; the dose response was linear-no-threshold (Fig. 2a), fitting to $y=4x+10$, with correlation coefficient, $R=1$. In their experiment, a statistically significant increase was observed only for the 100 ppm group.

PhIP–leukemogenesis: Lymphocytic leukemia was induced in male and female F344 rats by feeding PhIP for 104 weeks (7). The incidences of leukemia were 10 %, 20%, and 43% in male rats, and 7%, 10%, and 17% in female rats, with diets containing 0, 25, and 100 ppm of PhIP, respectively (Fig. 2b). The dose response was liner-no-threshold, fitting to $y=0.33x+10.8$, with $R=0.998$ for the male rats, and $y=0.097x+7.1$, with $R=0.996$ for female rats. The control group incidences were 10% and 7% in male and female rats, respectively. A statistically significant increase was observed only with 100 ppm group of male rats.

PhIP–colon tumorigenesis: Colon tumors (all carcinomas, no adenomas were observed) were induced in male F344 rats, by feeding PhIP in diet at 0, 25, and 100 ppm for 104 weeks (7). The dose response was non-linear, with tumor incidences of 43% at 100 ppm and 0% at 0 ppm or 25 ppm (Fig. 2c). A possible threshold was indicated.

PhIP–colon preneoplastic change: The dose effect of PhIP in preneoplastic changes was examined by Fukushima *et al.* (4). They analyzed aberrant crypt focus (ACF) formation after feeding PhIP in the diet at 0, 0.001, 0.01, 0.1, 1 10, 50, 100, or 400 ppm for 16 weeks, and claimed that there was a threshold (4). They used 61- 244 rats for each group, but the standard deviation for the ACF number/rat was very large [2 or more coefficients of variation (standard deviation/average) in the groups receiving 0 to 10 ppm] where no significant

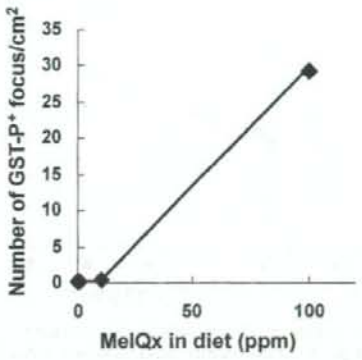

Fig. 3. Dose effect of MeIQx on liver preneoplastic changes. GST$^+$ focus numbers per cm^2 was measured after 16-week administration. Vertical bar indicates standard deviation. Data were from (6).

increase from the control group was observed. We reevaluated threshold possibilities using their data which did not show large coefficients of variation (less than 2), then plotted the findings on an arithmetic scale. The ACF number/rat used were 0.3 ± 0.7, 0.6 ± 1.0, 1.5 ± 1.4, and 5.0 ± 2.8 at 0, 50, 100, and 400 ppm, respectively. The data for these experimental groups were significantly different from that seen for the control animals, and a linear-no-threshold dose response, fitting to $y = 0.0123x + 0.120$, $R = 0.998$ was observed (Fig. 2d). Supportive evidence for the presence of threshold for colon carcinogenesis was not obtained by the study of preneoplastic changes.

MeIQx–liver tumorigenesis: Kushida *et al.* reported that liver tumors were induced in F344 male rats by feeding diets containing MeIQx for 52 weeks (17). Tumor (adenoma or carcinoma) incidences were 0%, 17%, 90% and 100% at 0, 100, 200, and 400 ppm, respectively. Since the experimental period was short compared to the life span, these data are not appropriate for analysis of mode of dose response. Murai *et al.* fed MeIQx for 104 weeks ($n = 51$) (5). Tumor incidences were 0, 0, 0, and 39%, with 0, 0.001, 1 and 100 ppm, respectively. Because the dosing spaces were so large, no information about mode of dose response was obtained.

MeIQx–liver preneoplastic changes: As for preneoplastic changes, the dose response for glutathione *S*-transferase placental form (GST-P)-positive foci was analyzed using 150 rats (dosed with 0 or 1 ppm) or 50 rats (dosed with 10 or 100 ppm) for each group (6). We plotted the results on an arithmetic scale and confirmed that the dose response was non-linear (Fig. 3). The response rate changed greatly at around 10 ppm and a threshold possibly exists. The study of preneoplastic changes indicates the possibility of presence of a threshold for liver tumorigenesis.

Table 1. Summary of treshold

Chemical	Species	Organ	Threshold
AAF	Mouse	Bladder	+
AAF	Mouse	Liver	−
PhIP	Rat	Mammary gland	−
PhIP	Rat	Systemic (lymphocytic leukemia)	−
PhIP	Rat	Colon	+?
MeIQx	Rat	Liver*	+?

Presence or absence of threshold was estimated from line shape of dose response curve.
+: Apparent threshold.
−: No threshold.
+?: Possible threshold.
*: Preneoplastic change

Dose Response Mode Changes by Target Organs

Table 1 shows a summary of the thresholds for AAF, PhIP, and MeIQx carcinogenesis. In the ED01 mouse study of AAF, non-linear dose response with an apparent threshold for bladder carcinogenesis was demonstrated, and linear-no-threshold dose response for liver carcinogenesis was noted under the same experimental conditions. In a PhIP study, liner-no-threshold dose response was suggested for mammary carcinogenesis and lymphocytic leukemogenesis. However, a non-linear dose response was observed for colon carcinogenesis under the same experimental conditions. In a MeIQx carcinogenesis study, a non-linear dose response was observed for liver preneoplastic changes. The possibility of presence of a threshold remained when a non-linear dose response was observed (Table 1). It is suggested that a particular carcinogen's dose response changes by the target organs.

Dose Response in Genotoxicity

LacZ transgene mutations in the large intestine of mice after administration of 0, 2, or 20 mg/kg/day of PhIP by gavage for 4 days (sacrificed 7 days after the last treatment) showed a linear-no-threshold dose response (18). Hoshi *et al.* (8) reported that MeIQx (after feeding to rats for 16 weeks) induced *LacI* mutation in the liver with nonlinear dose response and the presence of a threshold was shown by plotting on log-log scale without subtracting the background value. The doses tested were 0.001 to 100 ppm in diet over 10-fold spaces, with 10 control rats and 5 rats per experimental group. The average mutant frequencies increased dose-dependently, but had large standard deviations. By plotting on an arithmetic scale, the shape of the dose response curves, either with full scales of 100 ppm or 1 ppm (inset) did not show a threshold (Fig. 4a).

The dose response of two genotoxic agents, mitomycin C (MMC) and 1-β-D-arabinofuranosylcytosine (Ara-C), were reported following analysis of 10^6

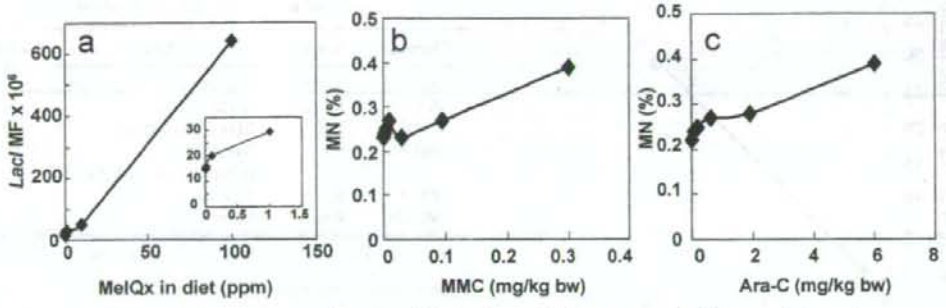

Fig. 4. Dose response of MeIQx, MMC, and Ara-C in genotoxicity *in vivo*. **a**: Big blue rats were fed diet containing various amounts of MeIQx for 16 weeks. *LacI* mutant frequency (MF) in the liver was determined. Data were from (8). **b**: Micronucleus (MN) induction in the peripheral blood reticulocytes of CD-1 mice by MMC administered by a single intraperitoneal injection. Data were from (9). **c**: MN induction in peripheral blood reticulocytes of CD-1 mice by various amounts of Ara-C administered by a single intraperitoneal injection. Data were from (9).

peripheral blood reticulocytes for micronucleus formation using flow cytometry (9). Although the presence of a practical threshold was reported by plotting on a log-log scale without subtracting background values (or on a semi-log scale), no threshold was indicated on an arithmetic scale (Figs. 4b and 4c). Thus, it may be considered that the genotoxic compound dose-response *in vivo* is generally linear-no-threshold.

However, two DNA-reacting genotoxic agents, methyl methanesulfonate and ethyl methanesulfonate were reported to show pragmatic thresholds for *HPRT* mutagenesis and micronucleus induction in human cells *in vitro*, although methylnitrosourea and ethylnitrosourea showed a linear-no-threshold dose response (10). The presence of a threshold for *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis has also been reported (11). In the case of MNNG, the threshold was demonstrated to be due to DNA repair action, using repair-proficient and repair-deficient strains of bacteria (11). Repair efficiency and the capacity of repair enzymes in this assay system were also estimated from the shapes of the dose response curves, and indicated that the repair was about 99.99% below the threshold (19).

Although MNNG genotoxicity dose response *in vivo* has not been demonstrated, it is thought that a threshold might be present for mutagenicity *in vivo*. It is worthwhile to confirm whether a threshold is present in MNNG carcinogenesis.

Conclusion

It has been demonstrated that the mode of carcinogen dose response changes depending upon the species, exposure chemical and target organ. The same chemical may show a linear-no-threshold dose response for one target organ even as it showed nonlinear dose response with an apparent or possible threshold in a different organ. It can be thought that for organs where background levels of tumor incidence are fairly high, carcinogens show linear dose responses, as observed in the mouse liver with AAF or in the rat mammary gland and hematopoietic system with PhIP. However, aflatoxin B1 showed a linear dose response in rat liver carcinogenesis (20), although the background level is extremely low. Considering the heterogeneity of the human species, it is wondered whether appropriate animal model systems are present for evaluation of the presence or absence of thresholds for carcinogenesis in humans. Further, the genotoxicity of DNA-reacting carcinogens generally shows a linear dose response *in vivo*. Therefore, it is reasonable to regulate genotoxic carcinogens based on the characteristics of linear-no-threshold. The U.S. Environmental Protection Agency recommends linear extrapolation as a default in carcinogen risk assessment (21). Application of the concepts of 'virtually safe dose' (VSD) or 'threshold of toxicological concern' (TTC) (22) would be feasible for regulation of very low doses of genotoxic carcinogens.

However, some types of genotoxic carcinogens show thresholds in genotoxicity *in vitro*, due to DNA repair. It could be that such types of carcinogens will show thresholds for carcinogenesis if the DNA repair enzymes similarly function in human cells. Such types of genotoxic carcinogens could be used safely below their threshold levels.

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Erratum

The authors would like to draw the reader's attention to an error in the title of the Volume 30 Number 3, August 2008 by K. Inami and M. Mochizuki (Genes Environ. 2008; 30: 71–6).

Contents: Activation Mechanism of 2-Acetylamino-9-**fluorenone** as a Mutagen in *Salmonella typhimurium*. Keiko Inami and Masataka Mochizuki.
Page 71, Title: Activation Mechanism of 2-Acetylamino-9-**fluorenone** as a Mutagen in *Salmonella typhimurium*. Keiko Inami and Masataka Mochizuki.

The authors apologize for this error.