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Needs for Biological Risk Assessment in Interspecies Extrapolation

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This paper suggests that not all chemicals shown to be carcinogenic in animals may exert this effect in humans exposed to much lower amounts of the chemical. It is possible that agents which differ in their effects in humans and animals may be identified through the application of Biological Risk Assessment to the experimental results. Chemicals tested in systems in which untreated animals develop high background yields of tumors or in which high-dose toxicity may be a critical factor in the induction of carcinogenesis are suggested as candidates requiring very careful consideration before their carcinogenicity in humans is assumed.

Introduction

The regulatory process for chemical carcinogens embraces three separate but interrelated components: (a) hazard identification, (b) risk assessment, and (c) risk management. Carcinogenic hazard identification is generally based on rodent bioassays employing a range of doses including the highest tolerable level of the test substance. This procedure compensates for the use of relatively small groups of rodents (50 animals of each sex/dose level), which is based on economic feasibility (*1*).

Risk assessment thus involves the determination of the probable effects on the human population of exposure to lower levels of substances shown to be animal carcinogens at higher doses. There are usually two stages in this process: high- to low-dose extrapolation and interspecies extrapolation. Dose extrapolation is generally based on one of a number of mathematical models that fit the data at the relatively high animal experimental exposures and assume the nature of the shape of the dose-response curves at lower levels. The weakness inherent in these models is clearly demonstrated by the variability of their predictions at low doses, which may differ by as much as 1,000- to 10,000-fold, scarcely an acceptable degree of variability (*2,3*). Interspecies extrapolation is generally based on two more assumptions, or "articles of faith": (a) Any animal carcinogen will be, or is highly likely to be, carcinogenic in man; (b) The human is as sensitive or more sensitive to the effects of a carcinogen as is the most sensitive experimental animal species. In this paper, these assumptions will be questioned in two ways:

- Are there situations in which it is reasonable to believe there is a probability that a chemical carcinogen determined at high exposure levels in rodents will not exert an effect in humans exposed to lower levels of the agent?
- Is there a better way to demonstrate the quantitative nature of the interspecies differences between humans and experimental animals than the guess embodied in article of faith (b)?

If the approaches that are raised to answer these questions are to be acceptable, there is a further need to consider how other toxicological evidence may be used to help establish or deny their acceptability. The evidence that a rodent bioassay conducted at high dose levels is inapplicable to humans must be very convincing indeed. The derivation and validation of answers to these and similar questions is conveniently called "biological risk assessment."

Nonrelevant Animal Carcinogens

Attempts to use differences in metabolic activation to explain absolute differences in species response to carcinogens have not been overly successful. Miller et al. (*4*) suggested that the reason why the guinea pig did not respond to the carcinogenic effects of *N*-2-acetamidofluorene lay in the failure of this species to metabolically activate the precarcinogen. However, Takeishi et al. (*5*) showed, using *in vitro* techniques, that guinea pig liver homogenates were able to conduct such activation and suggested the resistance of the guinea pig *in vivo* was due to its superior ability to detoxify the activated metabolite. Similarly, the rat has for many years been considered resistant to the bladder carcinogen 2-naphthylamine (*6*). But Hicks and her colleagues (*7*) showed that using test conditions that favored the production of the active metabolite (i.e., high doses given at less frequent intervals rather than low doses given continuously), 2-naphthylamine is an effective rat

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bladder carcinogen. Overall, metabolism *per se* has not been shown to be a key factor in identifying agents active in animals but inactive in humans. Other biological factors also need to be considered.

High Tumor Incidences in Untreated Animals

A number of bioassay systems for the identification of carcinogens are no longer considered reliable indicators of possible carcinogenicity for humans. These include the lung adenoma test in strain A mice (*8*), the induction of bladder tumors in the presence of urinary calculus (*9*), and bladder implantation (*10,11*). A high rate of occurrence of tumors in control animals is apparent in the lung adenoma test, whereas urinary calculus by itself leads to a high yield of bladder carcinomas (*11*). No major attempt appears to have been made to determine if these exceptions to the general assumption that animal bioassay results apply to humans represent just isolated examples or reflect a more general situation.

A possible approach to the difficulty in judging whether a chemical is or is not carcinogenic in such systems is implied by the two-stage theory of carcinogenesis (*12–15*). If naturally occurring tumors, like those that are induced, arise from initiated cells through a form of survival competition, it is possible that carcinogens identified in the presence of high tumor rates in untreated animals will include both those that have the ability to initiate and promote tumor development and those that are only able to promote or enhance development of initiated cells to tumors. If the human tissue (in contrast to the animal tissue) is deficient in initiated cells, carcinogenicity found in rodent tests may be irrelevant to humans if the test agent is capable only of promotional action.

It should not be imagined that tissues with appreciable numbers of enhanceable cells or foci will always demonstrate a high background tumor incidence *per se*. In the ED_{01} experiment, the female BALB/c mouse was chosen because at 24 months it possessed a low rate of naturally occurring liver cell tumor formation (1–2%). Groups of mice kept to 33 months produced a 33% yield of this tumor without carcinogen treatment (*16*).

An improved assessment of the significance to humans of carcinogens that are identified in such circumstances is of major economic importance. Soderman (*17*), in her data base of carcinogens, identified 811 chemicals that were either evaluated by IARC or tested in the NCI/NTP carcinogenesis bioassay program. Of these, 120 induced or enhanced mouse liver cell tumors, and in about 25% (Table 1) this was the only tumor that was increased in incidence. Some of these chemicals have been regulated out of commercial use; others are strictly controlled. The considerations presented here raise serious questions about the validity of their classification as complete human carcinogens.

Agents that affect the endocrine systems of the body present similar but more difficult problems because the human, as well as the rodent, is controlled by hormones and endocrine antagonists. The elucidation of whether any agent is or is not likely to be effective in exposed humans is a difficult problem and decisions may have to be based on whether or not the hormonal effect has a threshold.

High-Dose Toxicity

The maximum tolerated dose (MTD) is used to ensure that a carcinogen bioassay will not miss labeling a chemical as a carcinogen because too low a dose of the carcinogen has been used or because too few animals were used in this study. Haseman (*18*) reported that 18 of 31 NTP feeding bioassays would have missed labeling chemicals as carcinogens if the MTD had not been used. Haseman did not comment on the possibility that use of the MTD could have introduced confounding toxicological factors, which make it highly unlikely that all such results apply to humans exposed to much lower levels of the agent. This possibility is alluded to in the U.S. Environmental Protection Agency's "Proposed Guidelines for Carcinogenesis Risk Assessment" (*19*):

> Positive studies at levels above the MTD should be carefully reviewed to ensure the responses are not due to factors which do not operate at exposure levels below the MTD. Evidence indicating that high dose testing produces tumor responses by indirect mechanisms should be dealt with on an individual basis.

This statement represents a major advance in thinking; however, it is not clear why such high-dose toxicity should become effective only above the MTD, especially as our current attempts at definition of MTD lack precision. It is not difficult to visualize tissue-specific toxicity that would have little effect on the overall clinical condition or body weight of the test animal but might greatly facilitate carcinogenesis in the particular tissue.

Perhaps the most exciting prospect in this area is toxicity-related aberrant methylation. Shank and Barrows (*20*) demonstrated that toxic levels (LD_{50}) of hydrazine or carbon tetrachloride led to the transfer of the methyl group from *S*-adenosyl methionine to the O-6 position of guanine—an effect that leads to genetic errors after DNA replication. Unfortunately, the technical difficulties inherent in this observation have precluded observations on other tissues or dose-response studies in the rat liver. If these technical difficulties can be overcome, aberrant methylation may provide one general route by which high-dose toxicity may act as an initiating carcinogen in rodents.

Hormonal effects similarly may lead to the dubious interpretation of a chemical as a carcinogen. For example, erythrosine appears to induce only follicular cell thyroid adenomas. It appears to act as a goitrogen, a class of chemicals known to induce benign thyroid lesions in rats (*21*).

The most important way in which a chemical at high doses may aid in tumor formation is by inducing cellular proliferation. This may arise through cytotoxicity, resulting in cell regeneration as with chloroalkanes in the rodent liver or by direct stimulation through endocrine

Table 1. Chemicals listed by Soderman (*17*) that lead only to mouse hepatic cell tumors despite bioassay in two (or more) species.

Pesticides and halogenated hydrocarbons	Aromatic amines and nitro compounds	Other
Hexachloroethane	Nitrofen	*n*-Di(2-ethylhexyl)adipate
Tetrachloroethane	*N*-Nitro-*p*-acetophenetide	*n*-Dithiobiurea
Trichloroethane	Nitro-*p*-phenylenediamine	Phenobarbital
Bis(2-chloroethyl)ether	Dichloro-*p*-phenylenediamine	Piperonylsulfoxide
Trichloroethylene	Chrysoidine	Griseofulvin
Benzenehexachloride	Nitrobenzimidazole	
Hexachlorocyclohexane		
(4-Chlorophenyl)-2,2′-dichloroethylene		
1,1-Di(4-ethylphenyl)-2,2-dichloroethane		
Dicofol		
Pentachlorophenol		
Chlorobenzilate		
Chloranden		
Chlordane		
Heptachlor		
Dieldrin		
Aldrin		

or other processes. The work we have been doing with *t*-butylhydroxyanisole (BHA) in the Food Directorate in Ottawa illustrates the point.

Case Report

BHA and other phenolic antioxidants are added to food to inhibit the development of rancidity during transportation and storage. Until Ito and his colleagues (*22*) demonstrated that 2% BHA in the diet induced rat forestomach squamous cell carcinoma and papilloma, BHA was thought to be toxicologically the safest of these additives. The immediate response to Ito's discovery was that man does not have a forestomach, so why worry? This argument is specious unless there is supporting evidence, as there are many examples of carcinogens that affect different tissues in different species.

At the start of our studies we knew (a) BHA was effective in inducing forestomach tumors when fed at 2% but apparently not at 0.5% in the diet for up to 2 years (*22*); (b) BHA appeared not to act as a genotoxic agent in the various mutagenicity and clastogenicity tests in which it had been studied (*23*); and (c) at levels well below the carcinogenic 2% level, Wattenberg (*24*) had conclusively shown BHA to be anticarcinogenic.

My colleagues and I decided to feed 2% BHA in the diet to rats for a short period and observe the consequences by light microscopy and radioautography using tritiated thymidine, a specific DNA precursor, to measure the proportion of cells in DNA synthesis prior to cell division. Since there was some temporary food refusal with 2% BHA, the first observation was made after 9 days and showed that: (a) BHA at 2% induced the highest level of proliferation along the line of lesser curvature of the forestomach, the area in which most tumors subsequently developed; (b) the effect of BHA was similar whether it was presented in a pellet, ground into the diet, or dissolved in corn oil and then ground into the diet; (c) further, dose-response studies suggested a no-apparent-effect level at 0.25% in the diet, a concentration considerably above the human use level; and (d) the effect at 27 days was little different than at 9 days (*25*).

In the second series of studies, rats were fed a range of concentrations of BHA (2%, 0.5%, 0.25%, 0.1%, and 0.0%) for 91 days, when groups of rats were killed and the remainder transferred to basal diet and killed at intervals thereafter (*26*). This experiment showed that: (a) the apparent-no-effect-level noted at 9 days was still present after 91 days; (b) the major effect along the lesser curvature of the forestomach was still present, although there was now greater proliferative activity in the midregion of the forestomach; (c) this proliferative activity was dependent on the continued presence of 2% BHA. Within 1 week of removing the BHA from the diet, proliferative activity, as measured by ^{3}H-thymidine radioautography, had returned to control levels; (d) the induced pathological changes were much slower to regress; minor changes were still apparent 63 days after removal of BHA from the diet; and (e) from the appearance of the body weight/time curve for the first 91 days of treatment, 2% BHA in the diet exceeded the MTD in Ito's original carcinogenicity study (*22*).

We have initiated a further study to ensure that after 2% BHA is withdrawn from the diet and extensive proliferation ceases, the tumors do not appear or reappear. At least 6 months more must elapse before these results are complete. We have also done quite a large number of 9-day assays on different phenols to determine the uniqueness of the effect of BHA on the forestomach epithelium. Other phenols react similarly, but to different degrees as exemplified in the paraben series in which the methyl ester is apparently without effect but the *n*-butyl ester approaches BHA in its effect (*27*).

The importance of these rat studies is threefold. They strongly suggest that the important action of BHA on the rat forestomach is a direct or indirect result of inducing epithelial cell proliferation. It appears that the effects of 0.5 to 2.0% dietary BHA are an example of high-dose toxicity that does not cease completely at or about the MTD but may be present at exposure levels

below the MTD. These studies also suggest the sort of changes that must be looked for in short-term studies in species without a forestomach. A high level of induced forestomach proliferation after a few days of dosing with a chemical may indicate later tumorigenicity.

Four subchronic studies have been or are being undertaken in alternate species without a forestomach on an international basis. Olsen (*28*) has reported negative studies in the minipig from Denmark. We have reported virtually negative studies in the Cynomolgus monkey (*29*), while the U.S. has found negative effects of BHA in dogs (*30*). A Japanese dog study is awaited. The completed studies, each with very careful pathological examination, have all proved negative. In other words, BHA's effects in rats, and recently in hamsters, do indeed appear to be forestomach specific. Although more detailed knowledge of the mechanism of how BHA exerts its effect and the importance of high-dose levels in exerting this effect are still needed, current evidence tends to support the view that BHA is high-dose and forestomach specific, and may not therefore affect *Homo sapiens*. This system provides a model for defining effects that other high dose level toxicity may have on tumorigenesis.

Conclusions: Qualitative Analysis

The purpose of biological risk assessment advanced here is to identify situations in which animal carcinogens may not be relevant for human safety. Two major situations have been identified: the presence of high yields of tumors in the control animals and the possible critical intervention of high-dose toxicity in the carcinogenic process. At this prevalidation stage, biological risk assessment can only suggest the probability that an animal carcinogen is unlikely to be effective in humans. Other evidence must be carefully weighed to ensure it supports the conclusions of the primary biological risk assessment. For example, the fact that an agent is structurally related to a known carcinogen, as with the aromatic amines and nitro compounds in Table 1, would be strong grounds to continue suspecting its likely effectiveness in man. Although the predictivity of genotoxicity tests is presently in doubt because of their relatively poor performance in reproducing the results of animal bioassays, a consistently positive screen of genotoxicity tests would again be grounds for rejecting the possibility that such a chemical is unlikely to be effective in man.

On the other hand, if carcinogenicity is in a specific instance determined to be highly dependent on some aspects of high-dose toxicity and that toxicity has a threshold, the possibility that much lower doses of the agent will affect humans is more remote. This has been illustrated in the case of BHA, and the approach used is likely to demonstrate that many similar effects will be found with other animal carcinogens.

Quantitative Aspects

The preceding arguments suggest that even a minimal consideration of the biology or mechanisms of carcinogenesis may indicate that individual chemical carcinogens identified in animals may not be effective in humans. Quantitative aspects of biological risk assessment for carcinogens will clearly need greater and more probing use of our mechanistic knowledge. Knowledge of carcinogenic mechanisms is essential if the shape of the dose-response curve for the induction of tumors in a particular tissue in a test animal is to be established. For example, the shape of bladder and liver tumor dose-response curves in the ED_{01} experiment are very different from each other (*16*). Without appreciation of the underlying biology of tumor induction, we must make large assumptions about the probable low dose risk from *N*-2-acetamidofluorene for these tissues. Clearly, no one mathematical model is adequate to account for the different dose-response curves.

Another series of problems is presented by the need to extrapolate the results of many carcinogenesis bioassays quantitatively between species and thence to man. There is very little experimental work in this area, perhaps the most relevant being that of Booth et al. (*31*), who showed that *in vitro*, liver slices from rats, mice, and hamsters bound aflatoxin B_1 to their DNA in the same order as the potency of the mold product for the induction of liver cancer in these species (rat >> hamster > mouse). Human liver slice DNA bound aflatoxin B_1 to an extent intermediate between mice and hamsters, suggesting that humans are not exquisitely sensitive to this chemical as are rats (Table 2).

This approach seems very appropriate for comparing activities of genotoxic carcinogens which, for one reason or another, are not possible to exclude from our societal environment. From the mechanistic viewpoint, the low exposure levels to which humans are to be normally subjected are unlikely to markedly and consistently affect the processes concerned in fixation of DNA adducts or other lesions (i.e., DNA replication or DNA repair systems). Similarly, these levels are unlikely to have a major influence on the processes that lead to tumor development. Therefore the interspecies comparison of the level of DNA adduct formation may provide a suitable method for the more accurate assessment of the quantitative effect of low level genotoxic carcinogen exposures in experimental animals and humans. However, much more work is needed in these quantitative extrapolation areas if their promise of a rational approach is to become fact.

Table 2. DNA adduct formation to liver DNA after incubating aflatoxin B_1 with liver fragments.[a]

	Carcinogenicity	Binding[b]
Rat	+ + + +	31.7
Hamster	+ +	11.3
Mouse	+	1.3
Human	?	6.8

[a] Modified from Booth et al. (*31*).
[b] Nanograms aflatoxin B_1/nanogram DNA.

Overview

At this time a carcinogen is defined as any agent or process that increases the yield of tumors in a population (*32*). It has been suggested in this paper that carcinogens may exert their effects through a variety of different mechanisms and that interspecies extrapolation requires knowledge of, at least, the nature of these mechanisms and their ability to act in individual animal species and their tissues. It is only by the replacement of articles of faith by scientifically based methodology that we will be able to attain knowledge of which of the many presently identified carcinogens are likely to be truly disastrous for individual men and women.

In conclusion, this paper suggests that if the community has the will, the way to a more precise understanding of the risks of carcinogens to humans is almost within our grasp. Overall, the position can be well summarized by a quote from George Orwell's *Animal Farm* (*33*), "All animals are equal but some animals are more equal than others." Biological risk assessment is suggested as a method to separate the "equal" from the "more equal."

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