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# Animal Tests for Carcinogenesis Risk Assessment: Problems and Opportunities

## A REVIEW

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Risk assessment for environmental carcinogens, unless done by epidemiological methods in human populations, involves at least three steps: first, bioassay *in vitro* and/or animals; second, evaluation and interpretation of the data, including statistical analysis; and third, extrapolations to human populations (1, 2). Each of these steps includes opportunities for the commission of errors.

Problems begin with the formulation of protocols. It is a complex and difficult matter to select the most appropriate species for eventual extrapolations from animals to humans. At least the following questions must be considered: Does the proposed test animal metabolize the class of test compounds in a manner similar to their metabolism in man? Is the organ susceptibility to carcinogens of the animal in any way related to the presumed target organs in humans? Will the dose delivered to the susceptible organs of the test animal approach that delivered to the human target organs? It should be remembered that the protocols originated by the Weisburgers (3) and incorporated later into guidelines by others (4), protocols that employed maximal tolerated doses, sometimes as the only test dose, were not intended for quantitative risk assessment but rather for qualitative screening in a search for carcinogens.

The development of protocols for *in vitro* carcinogenesis bioassays raises many questions, some more difficult to answer than are those arising from work with whole organisms.

While the closed systems of *in vitro* bioassays permit closer controls of conditions than is possible in the whole organism, the very closed nature of these systems raises the question of how accumulating waste products may affect the ability of the various parts of the system to handle xenobiotics added to it.

Trosko (5) has formulated other problem areas as follows:

- Not all mutagnic chemicals interact with or damage DNA.
- Agents which damage DNA are usually gene mutagens and chromosomeaberration inducers.
- Agents which cause aneuploidy in yeasts or rodents are probably membrane or spindle fiber-interacting chemicals but do not damage DNA.
- All *in vitro* assays designed to detect mutagens are based on phenotypic, not genotypic, changes, hence can be misinterpreted because some phenotypic changes are due to epigenetic, not genetic factors.
- Phenotypic changes depending on absence of enzyme activities (such as thymidine kinase and 6-thioguanidine mutation assays) can in theory be due to either inactivating mutation or epigenetic gene repression. DNA repair assays are very prone to artifacts: agents which alter plasma membrane permeability or nucleotide pools can yield false positives. The same is true of chemicals which are mitogenic or which suppres cell division.
- The Ames-type assay is a poor predictor of mammalian mutagenesis and of

carcinogenic initiators and promotors, due to differences between mammalian and bacterial cells. The DNA in the bacterial cell can interface with the plasma membrane. The DNA in mammalian cells is protected in the nucleus, far from the plasma membrane. Chemicals which generate oxygen radicals at the membrane interface in bacteria can, in bacteria, damage DNA and be classified as genotoxic. In a mammalian cell these same chemicals, even though they might generate the same toixc metabolites, could not damage the DNA since they do not survive long enough to damage the chromosomes. If the cell were swamped by these chemicals they could damage DNA; the cell, however, probably dies from membrane damage, not DNA damage. This would explain why many non-DNA-damaging chemicals in mammalian cells are mitogenic-type tumor promoters (but not initiators) *in vivo*, yet may be genotoxic in Ames-type assays and be inducers of aneuploidy in some types of eukaryotic cells.

Finally, Trosko sees as a major problem the use of bad *in vivo* results as bench marks for the evaluation of *in vitro* data. He regrets the lack of emphasis on the development of assays to study nongenotoxic, noncytotoxic and cytotoxic chemicals and the roles which they play in toxicology. He suggests that , without a very critical re-evaluation of the whole *in vivo/in vitro* bioassay program, harmful chemicals will continue to reach public environment and useful chemicals will not be developed because of continued erroneous use and interpretation of data from existing systems.

To return to *in vivo* bioassays: Evaluation of the data is quite straightforward and reviews have been written on the statistical methods that are available (6, 7).

Difficulties arise from the high incidence of spontaneous tumors in test animals, which, in rats and mice, are often the same tumors as those induced by known carcinogens. The existence of these neoplasms in untreated controls my make interpretation impossible and render the test equivocal.

By far the gravest problems arise when attempts are made to extrapolate to human populations those conclusions arrived at in animal tests. Conclusions drawn from data obtained in one species are not necessarily valid for the members of another species. Results obtained in mice in carcinogen bioassays, for example, often differ from those observed in rats. The high doses employed in most bioassays designed to detect carcinogenic activity may interfere with the ways in which the test organism would deal with the test compound if lower doses were given. Nevertheless, conclusions as to the consequences of exposure of human populations to low doses are often drawn from animal experiments at high doses. When regulatory agencies publish estimates, based on animal experiments, of the exact numbers of cancers likely to arise among humans exposed during their lives to low doses of the compounds tested in animals at high doses, it is a travesty of science and would be a joke if the consequences of such projections were not so serious.

There is a consensus that some way must be available to evaluate the safety of chemicals about to be released into the environment and to assess their risks. What can be done to improve the methods available to achieve such safety evaluation?

Weisburger and Williams (8) have suggested a step-by-step approach to carcinogenesis testing which employs both *in vitro* and *in vivo* methods in a seemingly rational way.

It is possible through basic research at the molecular level to gain additional insight into the metabolic fate of various classes of xenobiotics in various species and in various *in vitro* systems. This may even-

tually lead to information needed to make possible fact-based species comparisons and, hence, extrapolation.
Our own approach is to sharpen the *in vivo* bioassay method by using additional species and their genetically controlled inbred lines to render more significant the test results obtained in the whole animal. Obviously, many species of animals could and should be studied to detect some that might lend themselves to the improvement of carcinogenesis bioassays. We have concentrated on the Syrian hamster (Mesocricetus auratus, Waterhouse) (9). This required three steps:

1) Development and production of inbred strains and determination of their spontaneous tumor incidence.
2) Determination of the carcinogen-susceptibility of the inbred strains and development of carcinogen-susceptible first-generation hybrids.
3) Evaluation of the performance of large groups of such hybrids in lifetime carcinogenesis bioassay and the ascertaining of the incidence of spontaneous diseases and neoplasms in the untreated control animals.

1) *Development of inbred strains, comments on animal husbandry:*

Each animal species requires special husbandry procedures to assure maintenance of good health and optimal breeding performance. The requirements for hamsters have been outlined before (10). There are certain pitfalls which tend to befall those who for the first time employ hamsters and who treat them as they would rats or mice. Longterm hamster studied cannot be conducted using wire-mesh cages. Bottoms must be solid. At the very minimum, the the animals must have a corner area with a solid bottom to rest on. When circular metabolic cages have to be used, an open rectangular metal enclosure without bottom should be provided which gives the animal the opportunity to huddle in a corner, thus avoiding the stress of cornerless confinement, which might otherwise falsify results obtained in circular walled cages. Cage size is absolutely critical to assure reproducible growth rates and survival and must conform to the minimal sizes suggested in the *Guide for the Care and Use of Laboratory Animals* (11).
The effects of diet composition on the spontaneous pathology of hamsters has been described by Birt and Pour (12).
Hamsters must be handled daily, preferably with bare hands or, at the most, with rubber-gloved hands. This frequent handling will keep them gentle and easily manageable.
Large litters are obtained under good husbandry conditions and inbred lines may readily be developed by documented brother vs. sister mating for at least twenty generations. Many such lines exist and have been recorded by Festing (13) and Altman (14).
In order to obtain reproducible experimental data, inbred lines or their first-generation hybrids must be used. Studies employing non-inbred (random-bred) animals can never be reproduced exactly since hereditary traits continually fluctuate in such populations.

2) *Determining the carcinogen susceptibility of inbred lines, developing first-generation hybrids:*

Since susceptibility and resistance to carcinogens are dependent on inherited characteristics, as has long been established in mice (15), it is necessary to determine whether any strain destined for carcinogenesis bioassay is resistant or susceptible to standard carcinogens. For a number of BIO inbred strains this has been done (16, 17). The incidence of spontaneous tumors and other pathological conditions among animals of inbred lines kept in standard environmental situations have also been recorded (18).

First-generation hybrids are known to be sturdier and more fertile than their parental lines. They provide a more heterogenous genetic pool than inbred animals, hence are a step closer than inbred lines to the complex genetic constitution of humans.
BIO F1D Alexander hybrids are the result of crossing two carcinogen-susceptible lines: males of the BIO 15.16 line versus females of the BIO 87.20 line. These hybrids have been tested and found to be as susceptible as the parental lines to administered carcinogens.
The paradox of high carcinogen susceptibility and low spontaneous tumor incidence, first described by Van Hoosier (19), prevails in these animals. The males survive to 115 weeks, the females to 90 weeks, and by that time the incidences of spontaneous malignant tumors are as shown in Table 1 (spontaneous tumors in % as observed in several groups of approximately 100 animals each).

*Table 1.*

| | Males | Females |
|---|---|---|
| Lymphomas | 6.7—18.4 | 3.3—4.6 |
| Adrenal Cortical Adenomas | 12.6—13.3 | 2.3—3.3 |
| Uterine Corcinoma | — | 4.6—5.6 |

Other tumors, such as sarcomas, colonic carcinomas, pancreatic carcinomas, salivary gland carcinoma, melanoma, stomach adenocarcinoma, heptatoma, occurred in fewer than 2.3 % of the animals. None of the malignant neoplasms readily induced by polycyclic hydrocarbons or nitrosamines were found in any of the untreated animals.
Benign neoplasms occur with significant incidence (24–37 % in males, 2.3–7.8 % in females) only in the adrenal; pancreatic islet cell tumors are found in up to 10 % of the animals. All other benign tumors have incidences below 3 %.
The non-neoplastic pathology in these hybrids is much like that observed in other hamsters ond includes tubular dilatation in the kidneys, hepatic cysts, auricular thrombosis, colonic cystic hyperplasia, and testicular atrophy. Amyloidosis was rare in males (1–3.4 %); but in one female cohort it was found in half of the animals, in another in only 4.4 % of the animals. In all cases it was mild to moderate, never severe.
The carcinogen susceptibility of BIO F1D Alexander hybrids was measured by subcutaneous injections of dibenzanthracene and methylcholanthrene. They proved to as susceptible as the parental strains, with incidences of sarcomas at the injection site of 100 % for dimethylbenzanthracene, with a latency period of 11–13 weeks, and an incidence of methylcholanthrene-induced sarcomas of 55 % in males and of 14 % in females, with latencies of 16–18 weeks. Gastric gavage of methylcholanthrene induced primary mammary carcinoma in 88.5 % of the females, with metastases developing in 87 % of the cancerous animals, leading to death in about 25 weeks.

3) *Evaluating the performance of the BIO F1D Alexander hybrids in carcinogenesis bioassays:*

The four lifetime carcinogenesis bioassays which we have conducted on some 2,000 F1D Alexander hybrids (including 400 control animals) have demonstrated the adequate survival in good health of these animals, as predicted in our first publication (8). A report on the pathology of the untreated animals used in these studies is being prepared and confirms the data referred to in this review. First-generation hybrids such as the BIO F1D Alexander possess all of the advantages and disadvantages of their species for carcino-

*Table 2.* Comparison of spontaneous tumor incidence in rats and Syrian hamsters and consequences for statistical evaluation of carcinogenesis bioassay.

| Animal Species | | Percentage of spontaneous tumors in controls | | | |
|---|---|---|---|---|---|
| | | Pituitary % | Mammary gland % | Liver % | Lung % |
| Rats | Male | 19 (37) | | 1 (10) | 4 (16) |
| | Female | 46 (66) | 25 (44) | 2 (13) | 2 (13) |
| Hamster | Male | 0 (8) | 0 (8) | 2 (13) | 0 (8) |
| | Female | 0 (8) | 0 (8) | 2 (13) | 0 (8) |

Figures in parentheses indicate percentages of tumors required in test groups of 100 animals to show the significance of tests at the 1 % level (National Bureau of Standards Handbook 91, 1963).

genesis bioassay and, in addition, provide material which is standardized with respect to carcinogen-susceptibility and resistance and which is of defined genetic background, hence reproducible as long as the inbred parental strains are preserved by brother-sister mating. Their behavior in terms of growth, survival and pathology is reproducible and should remain so for a long time to come.

### *Discussion*

Because of the many difficulties involved in extrapolating from *in vitro* bioassays to carcinogenesis risk assessment for human populations, it is likely that for some time to come experimental animals will have to be used as surrogates for humans in such studies. This approach as well is fraught with pitfalls, some of the most serious ones being the high incidence of spontaneous tumors in animal test populations such as rats and mice. It is a mistaken notion to assume that in order to be susceptible to administered carcinogens an animal species must exhibit many spontaneous neoplasms. The seeming paradox of high carcinogen susceptibility yet low incidence of spontaneous tumors prevails among Syrian hamsters and has been conclusively proven for the carcinogensusceptible first-generation hybrids derived from two carcinogensusceptible inbred lines.

Hamsters are not a panacea to result in perfect animal tests for risk assessment, but they could be used in selected instances to improve greatly the sensitivity of test procedures over and above that obtainable with rats and mice. Table 2 (from: 20) illustrates why this is a reasonable postulate based on the low incidence of spontaneous tumors in Syrian golden hamsters.

Those instances where hamsters should be used rather than mice or rats, or in addition to these other rodents, must be carefully selected, based on knowledge of the metabolism of the test compound in these species as well as in humans. It is known that, in general, hamsters metabolize extraneous substances more rapidly than do mice or rats, yielding similar metabolites. For example, this seems to be the case for dioxin, quazepan, and N-butyl-N-(4-hydroxybutyl)nitrosamine. In the case of DDT, hamsters are less effective than mice or rats in producing the carcinogenic metabolite, hence less prone to revealing the carcinogenicity of the parent compound. These considerations and others which could influence the selection of test species have been discussed in some detail in a previous paper (19).

*Sammendrag ved E. Hansen*

Det er almindeligt anerkendt, at det er vigtigt at vurdere den menneskelige kræftrisiko før nye kemikalier, som ikke tidligere er afprøvet, kommer ud i miljøet. Det bliver fra den offentlige sektor, fra kemiske industrier og fra regeringen krævet, at toksikologerne frembringer videnskabelige metoder, der er istand til at forudsige og måle den risiko, som hører til de enkelte kemikalier. De nuværende metoder fejler tit, når det gælder om at forudsige en kræftrisiko i testarten og in vitro metoderne er ofte langt fra valideret med hensyn til deres anvendelighed overfor problemer med hensyn til mennesker.

Ikke desto mindre baserer myndighederne nogle af deres aktioner omkring den kvantitative risikobedømmelse på sådanne undersøgelser, en procedure der er svær at retfærdiggøre.

Ethvert område indenfor toksikologien, som drejer sig om risikobedømmelse, trænger til at forbedres. En måde til at forbedre forsøgsdyrsmodellen er at studere alternative dyrearter i forhold til dem, der almindeligvis anvendes. Sådanne studier, der er blevet udført i Syriske hamster gennem de sidste 25 år, har udviklet en første generations hybrid, som har vist sig at være følsom overfor almindeligt kendte carcinogener. Den lever længere og er fri for de tumortyper, der sædvanligvis fremkommer ved inducering af carcinoger. Anvendelse af sådanne dyr alene eller sammen med de almindeligt anvendte gnavere vil forøge testprocedurens følsomhed, i hvert fald i de tilfælde, hvor teststoffets metabolisme er sammenlignelig hos hamster og mennesker.

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