Techniques for Carcinogenicity Studies

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Abstract

Short-term tests to detect genetic, chromosomal, or DNA damage are now required by regulatory agencies for any new compound proposed for commercial production. Furthermore, full-scale carcinogenicity tests may be required for certain compounds. In this circumstance, the compound-related factors, including stability, purity, physical properties, and chemical structure and reactivity must be considered. Animal factors include species and strain of test animal, route of administration, age, sex, diet, and spontaneous tumor incidence. A team of qualified investigators with experience in various disciplines is required to conduct the studies properly. Quality control measures and adherence to the code of good laboratory practice are also necessary during all phases of the study. The investment in a carcinogenicity study therefore becomes fairly substantial in terms of both time and money.

Introduction

One may consider that the process of testing a compound for carcinogenicity is somewhat analogous to a braid or an interwoven composition. One of the strands of the braid comprises the compound factors, another comprises the animal factors, and a third comprises the research team necessary to carry out the testing.

Many treatises have appeared on how to perform carcinogenicity studies (4, 15, 29, 34, 35, 40, 42, 43, 52–54). In addition, various government agencies have proposed guidelines for the conduct of such tests (11, 17, 21). Regulatory agencies, especially the Environmental Protection Agency, have published in great detail the procedures and techniques which are considered acceptable, along with extensive references to the scientific literature (11). This wealth of references and guidelines may become overwhelming to the newcomer in the field.

A full-scale carcinogenicity study on any particular compound is not to be taken lightly. Factors such as possible production, exposure to general or specialized populations, structure, and end use must be considered. Furthermore, since the passage and implementation of the Toxic Substances Control Act (TSCA), any organization desiring to introduce a new compound into commerce is required to perform certain toxicity and guidelines may become overwhelming to the newcomer in the field.

If a decision has been made to proceed, compound-related factors such as stability of the test material, physical properties (nonvolatile or volatile solid, liquid, or gas), chemical reactivity, and chemical structure are important, for all influence the mode by which the compound can be administered. The purity of the test material must also be known, and any exogenous constituents should be identified.

A stable material can be administered by mixing in the diet, or if water soluble, in the drinking water. Volatile solids or liquids, if stable, may possibly be given in the drinking water. Less stable materials can be administered by gavage or injection of freshly prepared solutions. Obviously, mixing in a diet would be unsuitable for such compounds. Finally, gaseous or highly volatile substances should be tested by inhalation.

The mode of application should simulate, if possible, the route by which any compound contacts the exposed population. Early studies on chemical carcinogens relied heavily upon skin painting or cutaneous application, especially with mice. Cutaneous application simulates the route by which cosmetic materials would be applied but requires somewhat more technical effort than administration in the diet (9). If the test material is absorbed, any effect may not be noted at the site of application but in other more susceptible organs. Furthermore, some active carcinogens such as N-2-fluorenylacacetamide or nitrosodimethylamine were not active by this route, although the polycyclic aromatic hydrocarbons such as benz(a)pyrene or DMBA2 were (54).

Administration p.o., either in the diet, in drinking water, by gavage, or by capsule, is representative of the manner by which food additives or similar materials enter the organism. Since inhalation of dusts leads to swallowing much of the material, p.o. administration also simulates exposure of humans to dusty atmospheres.

Injections i.v. or i.p. usually lead to fairly rapid distribution throughout the body and are excellent for testing some drugs. There are some problems associated with interpretation of results from s.c. or i.m. injection into rats since a granulomatous or inflammatory reaction, eventually leading to injection-site sarcomas, occurs. In mice, this route affords a rapid response, especially for polycyclic hydrocarbons, correlates fairly well with other results, and requires only a small amount of compound.

Inhalation is quite relevant for human exposure to many...
Table 1

Environmental Protection Agency recommended short-term tests

<table>
<thead>
<tr>
<th>Test Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene mutations</td>
<td>(3 minimum)</td>
</tr>
<tr>
<td>Bacteria, with and without activation</td>
<td>(Salmonella typhimurium strains)</td>
</tr>
<tr>
<td>Eukaryotic microorganisms, with and without activation</td>
<td>(Neurospora, Saccharomyces)</td>
</tr>
<tr>
<td>Insects, sex-linked recessive lethal test</td>
<td>(Drosophila)</td>
</tr>
<tr>
<td>Chromosomal aberrations, with and without activation</td>
<td>(Chinese hamsters, mouse lymphomas)</td>
</tr>
<tr>
<td>Mouse-specific locus test</td>
<td></td>
</tr>
<tr>
<td>Chromosomal aberrations</td>
<td>(3 minimum)</td>
</tr>
<tr>
<td>In vivo cytogenetic tests in mammals</td>
<td></td>
</tr>
<tr>
<td>Insect tests for heritable chromosomal effects</td>
<td>(Drosophila)</td>
</tr>
<tr>
<td>Dominant lethal effects in rodents, early fetal loss</td>
<td></td>
</tr>
<tr>
<td>Heritable translocation tests in rodents</td>
<td></td>
</tr>
<tr>
<td>Detection of DNA damage</td>
<td>(2 minimum)</td>
</tr>
<tr>
<td>DNA repair in bacteria, with and without activation</td>
<td>(DNA repair-deficient bacteria)</td>
</tr>
<tr>
<td>Unscheduled DNA repair synthesis in mammalian cells</td>
<td>with and without metabolic activation</td>
</tr>
<tr>
<td>Mitotic recombination and/or gene conversion in yeast</td>
<td>with and without metabolic activation</td>
</tr>
<tr>
<td>Sister chromatid exchange in mammalian cells</td>
<td>with and without metabolic activation</td>
</tr>
</tbody>
</table>

Compounds. Animals are generally exposed in large chambers equipped with metering and monitoring devices and scrubbers to deactivate the exhaust. Inhalation toxicology has become a separate specialty in the general area of toxicology. Of all bioassay methods, this is the most expensive because of the need for the special exposure chambers (5, 39).

An example of how the effects of one compound can differ, depending on route of administration, is that of NMU. Although considered a direct-acting carcinogen because of its reactivity, NMU may affect many sites besides those where applied. Table 2 gives some references to typical results in hamsters and rats where, depending on route, skin, brain, peripheral nervous system, breast, kidney, intestinal tract, trachea, and bladder could be affected (25). In mice and guinea pigs, the tumor type may also vary with route of administration (14, 22, 32, 33, 56). NMU is also effective in larger species such as dogs and monkeys (1, 8). NMU thus has become a very useful carcino- gen to induce a wide variety of model tumors. Furthermore, its instability at alkaline pH values minimizes disposal problems with residues from experiments.

The animal factors have a decided influence on the outcome of any carcinogenicity study. Initially noted was the effect of species in response to skin painting with benzo(a)pyrene. These early results showed the mouse was most responsive to the carcinogen, while rabbits, although less susceptible, still had an appreciable response. Guinea pigs, rats, and monkeys were refractory. To obtain skin tumors in monkeys required 6.5 years of skin application of compounds such as DMBA, followed by the promoters UV or dodecylbenzene, and a total of 10 years of treatment (37).

The unsuitability of some species for testing certain chemical compounds should be kept in mind. As mentioned, rats are often not responsive in skin painting experiments with poly-cyclic aromatic hydrocarbons. Guinea pigs are not the preferred species to test aromatic amines and amides, or their precursors, since they generally are poor in the enzymes which activate such compounds.

Within each species, there are strain variations in response to be considered. Some studies of such differences have been: (a) the incidence of breast tumors after a single p.o. dose of DMBA in virgin female rats (44); (b) the varied incidence and multiplicity of lung tumors in different mouse strains after a dose of ethyl carbamate (urethan) (54); (c) the response of different rat strains to N-2-fluorenylacetamide (2-acetylamino-fluorene) (54); and (d) the response of different rat strains to ethionine (12).

It is well known that the sex of the animal also influences the response to many xenobiotics, for hormone levels influence to some extent the activating or detoxifying enzymes. Treated male mice had both a higher skin tumor incidence and more tumors per mouse than did females after cutaneous application of DMBA (3). Male rats often are more susceptible to liver carcinogens than are females as demonstrated several times (48).

Although standard bioassays use animals a few weeks post-weaning, there have been many studies which demonstrated the greater sensitivity of neonatal animals, especially mice (7, 49). Although older animals apparently are less sensitive to many carcinogens, this may not always hold true. Usually, if a bioassay is started with old animals, the animals may die of age-related disorders before tumors can develop.

Another influence, that of diet, will be discussed at this workshop. Dietary restriction in experimental animals on a basis of either calories or required nutrients may decrease the response to a chemical carcinogen (45). In certain cases, vitamin deficiencies enhanced the carcinogenicity of specific compounds (28). Conversely, a diet with high levels of vitamins or similar factors may lead to such rapid detoxication of some compounds that no carcinogenic effect is noted. This was especially true for the azo dye 4-dimethylaminoazobenzene, where a diet with optimum levels of riboflavin blocked the action of the carcinogen.

Also to be considered in animal diets are such factors as traces of mycotoxins, endogenously formed N-nitroso compounds, pesticides, vegetable matter, and antioxidants. The first 2 of these are likely to be dangerous and carcinogenic in their own right. The latter 3 are more apt to induce enzymes which often detoxify the test compound rapidly. Pesticides such as dichlorodiphenyl-trichloroethene and especially tetra-chlorodibenzo-dioxin, often found in trace quantities in herbicides, are potent enzyme inducers. Therefore, animal diets should be carefully formulated and checked for the presence of possible toxic or carcinogenic materials which may cause an increase in the spontaneous tumor incidence or a promoting effect on any putative carcinogen being tested.

Another animal-related factor is the spontaneous tumor incidence in a particular strain. A high tumor incidence at any one site leads to complications in evaluation of bioassay data. As an example, the AKR mouse develops a high spontaneous incidence of leukemia, and most of the animals die by the age of 9 to 12 months. Furthermore, these animals die too early to be of value in a bioassay system. The C57BL/6 × C3H F, (hereafter called B6C3F,) mouse now used in the National Cancer Institute program is prone to show liver tumors in males and lymphomas in both males and females (Table 3; Ref 51).

The widely used Sprague-Dawley rat often becomes fairly large. Both sexes develop tumors of the endocrine organs, while females have a high incidence of breast tumors (38). Another strain, the Fischer or F344 rat, does not become so large, but old males have a high incidence of testicular tumors. Thus, compilations of tumors in various strains (30, 38, 41)
Table 2
Effect of route of administration of NMU on tumors in rats and hamsters

<table>
<thead>
<tr>
<th>Species</th>
<th>Route</th>
<th>Dose</th>
<th>Tumor site</th>
<th>Tumor type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrian golden</td>
<td>i.g.</td>
<td>0.5% solution 3 times/wk (180 mg/kg total dose)</td>
<td>Skin</td>
<td>Carcinoma</td>
<td>14</td>
</tr>
<tr>
<td>Syrian golden</td>
<td>i.p.</td>
<td>1 mg/wk for 4—5 mos.</td>
<td>Small intestine</td>
<td>Adenocarcinoma</td>
<td>19</td>
</tr>
<tr>
<td>Syrian golden</td>
<td>i.v.</td>
<td>2.5 mg/mo. (7.5—12.5 mg total)</td>
<td>Small intestine</td>
<td>Adenocarcinoma</td>
<td>19</td>
</tr>
<tr>
<td>Syrian golden</td>
<td>i.t.</td>
<td>5 mg/wkly or 2 times/wk for 10—30 wk</td>
<td>Larynx</td>
<td>Carcinoma</td>
<td>55</td>
</tr>
<tr>
<td>Syrian golden</td>
<td>s.c.</td>
<td>0.5—1.0 mg/wk (5—9 mg total)</td>
<td>Injection site</td>
<td>Sarcoma, Papillomas</td>
<td>18</td>
</tr>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>Cutaneous 0.5% solution 3 times/wk (180 mg total)</td>
<td>Skin</td>
<td>Carcinoma</td>
<td>14</td>
</tr>
<tr>
<td>Wistar</td>
<td>i.g.</td>
<td>Single dose, 90 mg/kg</td>
<td>Kidney</td>
<td>Interstitial tumor</td>
<td>26</td>
</tr>
<tr>
<td>CD-Fischer</td>
<td>i.r.</td>
<td>1—2.5 mg 3 times/wk for 10 wk</td>
<td>Large intestine</td>
<td>Adenocarcinoma and</td>
<td>32</td>
</tr>
<tr>
<td>BD, CD, CDF</td>
<td>i.v.</td>
<td>5 mg/kg/wk</td>
<td>Aggressive tumor</td>
<td>adenoma Lymphoma</td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>i.v.</td>
<td>50 mg/kg, 3 injections at 4-wk intervals</td>
<td>Mammary gland</td>
<td>Adenocarcinoma or</td>
<td>16</td>
</tr>
<tr>
<td>F344, female</td>
<td>Intravesicular</td>
<td>1.5 mg at 4 biweekly intervals</td>
<td>Bladder</td>
<td>Carcinoma</td>
<td>20</td>
</tr>
</tbody>
</table>

a i.g., intragastric; i.t., intratracheal; i.r., intrarectal.

and other background information should be consulted before the decision is made to use one particular strain in a long-term program. Furthermore, spontaneous tumor incidence in an inbred strain may shift over a period of years, a compelling reason for always having control animals in any experimental design (54).

Current National Cancer Institute protocols for a carcinogenicity study calls for the following stages: (a) an acute toxicity study, with at least 3 dose levels and 5 males and 5 females of each species per dose level; (b) a repeat dose study over 14 days, with 5 dose levels and 5 males and 5 females at each dose level; (c) a 90-day subchronic study, again at 5 dose levels, but with 10 males and 10 females per dose level; (d) a 2-year study, using 50 animals of each sex and species, generally at a maximally tolerated dose and one-half that level as a minimum number of dose levels. Other fractions of the maximally tolerated dose should be tested if possible. Appropriate untreated controls and vehicle controls are also indicated. If sequential histopathological or biochemical studies are to be done, the animal groups should be larger to accommodate such efforts. In some cases, a small group of positive controls, animals given a known carcinogen, should be included to check the response of the species and strain.

Facilities for such studies should be constructed according to various guidelines now suggested. The clean-dirty corridor system, with small animal rooms so that only animals receiving one compound are kept together, is desirable. Proper ventilation and temperature controls are mandatory. A 12-hr light-dark cycle system is needed. Surfaces in animal rooms and corridors must be impervious and easily cleaned; entrance should be through a shower system to decrease contamination. Proper clothing, respirators, and gloves are necessary to prevent exposure of the personnel to any putative carcinogen.

Animals should be monitored carefully over the experimental period. Individual weights and notes on clinical condition should be entered into a data system on a predetermined regular basis (27). Quality control measures should be initiated to check entries and to detect errors (36).
At the end of the experimental period, animals should be killed. A thorough necropsy involving removal of almost 40 tissues plus any lesions or masses should be done. Tissues should be fixed in buffered formalin and processed for microscopic slides, and finally reviewed by the pathologist. Confirmation of the pathological diagnoses by a team or review group of other pathologists is necessary (36, 50).

Although a statistical group should be involved in the experimental design, statistical evaluation of the data to determine whether there are any significant differences in tumor incidence between the controls and the experimental animals is now necessary. A useful technique for estimating survival probabilities is the "life-table" method of Kaplan and Meier (23). The Fisher exact test for comparing the tumor incidence of the controls with that of the dosed animals is a general technique (46, 47).

### References


24. Ketkar, M., Reznik, G., Haas, H., Hilfinger, J., and Mohr, U. Tumors of the heart and stomach induced in European hamsters by intravenous adminis-
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