ROE1973P 371

Reprinted from Excerpta Medica International Congress Series No. 311
EXPERIMENTAL MODEL SYSTEMS IN TOXICOLOGY
AND THEIR SIGNIFICANCE IN MAN
Proceedings of the European Society for the Study of Drug Toxicity
Vol. XV, Zurich, June 1973
ISBN 90 219 0248 6

RECENT DEVELOPMENTS IN THE DESIGN OF CARCINOGENICITY TESTS ON LABORATORY ANIMALS*

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We wish that we could report a new, sensitive, reliable, inexpensive and rapid method for testing chemical agents for carcinogenicity. Not only can we not do this, but we feel bound to give reasons for doubting the value of some of the long-term tests upon which we now rely and criticising the way in which such tests are carried out in some laboratories. We feel this criticism is important at a time when European countries are endeavouring to work more closely together and to set standards to which all will in future be expected to adhere. It is also timely insofar as carcinogenicity testing is a rapidly expanding branch of toxicology and the number of commercial organisations that claim to be able to carry out such testing under contract is increasing.

It is an inescapable fact that most of the routine screening of chemical substances for carcinogenicity as practised today is based on completely outdated concepts of cancer and of the mechanisms involved in carcinogenesis.

Carcinogenicity testing became increasingly sophisticated during the 1950's and 1960's, such that no tissue or body orifice of any laboratory animal species was spared the possibility that an ingenious and eager experimentalist would introduce a prospective carcinogen into it.

TABLE I

Some discrepancies between the logical basis of routine carcinogenicity testing and current knowledge of mechanisms of carcinogenesis

Assumptions	Current knowledge
1. Cancers in test group − Cancers in control group = Cancers induced by the test agent.	Cocarcinogens and immune suppressants may increase risk of cancer development.
2. Studies on animals kept *without exercise and under sex-free conditions* are suitable models for assessing whether chemical agents will increase cancer risk in man.	Under laboratory conditions rats of many strains have high incidences of mammary, pituitary, adrenal and other tumours, suggesting a highly abnormal hormonal status.
3. The amount of diet available to animals under test does not matter.	'Spontaneous' tumour incidence may be greatly influenced by dietary intake.
4. The possibility of interference by oncogenic viruses during tests of chemical agents for carcinogenicity can safely be ignored.	The presence of C-type viruses may greatly influence the risk of malignant transformation of cells on exposure in vitro to chemical agents.

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It took the determined efforts of Dr. Leon Golberg and his former colleague, Dr. Paul Grasso, to show that the development of sarcomas may be a non-specific consequence of the introduction of chemical substances into the subcutaneous tissues of laboratory rodents (Grasso and Golberg, 1966; Gangolli et al., 1967). Because of their work, it is now widely regarded as inappropriate to test, say, food additives for carcinogenicity by injecting them subcutaneously or intramuscularly into rats or mice to see whether and how many injection-site sarcomas arise. It may of course be sensible in some instances to test a substance intended for oral administration to man, by giving it parenterally to animals; it is possible, for example, to circumvent poor absorption from the gut by this means. However, in the evaluation of the results of such tests, local tumour induction at the site of administration should be interpreted with the greatest of caution.

Despite the efforts of Golberg and Grasso, however, there remain several discrepancies between the logical basis of routine carcinogenicity testing and current knowledge of mechanisms of carcinogenesis (see Table I).

There is now abundant evidence from studies on laboratory animals that immuno-suppression, particularly of cell-mediated immunity, by thymectomy (Grant et al., 1966) anti-lymphocyte serum (Allison and Law, 1968), drugs (Reiner and Southam, 1966) or other means enhances both the risk of development of so-called 'spontaneous' neoplasms and the risk of development of neoplasms in response to the deliberate exposure of animals to known carcinogens. It is also well-established, from observations on kidney transplant recipients, that immune suppression is associated with a greatly increased risk of cancers of various kinds in man (Penn, 1970; Brit. med. J., 1972). The tumours that appear in immune-suppressed animals and humans are, one presumes, not induced by immuno-suppressant agents per se, but arise from malignantly transformed cells which become free to multiply when the constraints on their doing so are diminished by the suppression of cell-mediated immunity.

Theoretically, the development of cancers as an indirect consequence of immune suppression by an agent under test might be mistaken for cancer *induction* by the test agent. It could be argued that the distinction is unimportant and that immune-suppressants should be classed as carcinogens anyway. But such a view, if extended to all the many situations in which the risk of tumour development is enhanced non-specifically, becomes counter-productive. A definition of carcinogenicity which embraces co-carcinogenicity and non-specific enhancement by high calorie intake or by change in hormonal status under artificial laboratory conditions may be of little value for distinguishing between agents that will in practice be dangerous or safe for man.

It is not uncommon for mammary, pituitary and other tumours to be encountered in very high incidence in untreated female laboratory rats. Sometimes exposure of rats to a test agent appears to increase or decrease the risk of development of such tumours. The mechanisms involved are usually unknown, but the possibility that the 'extra' tumours seen in one group as compared with another are *induced* by the test agent (or by its absence, where the incidence in untreated control rats is higher) seems remote. We believe it to be ridiculous to regard evidence derived from studies on animals kept under conditions of over-feeding, without exercise and without the opportunity of indulging in sexual activity as interpretable in terms of the human situation.

It is not unreasonable to require that an animal model for use in screening for carcinogenic activity should be known to be sensitive to the *induction* of cancers by known carcinogens, provided that the word *induction* is not misconstrued. It has been falsely assumed that if a strain of animal experiences a high spontaneous incidence of tumours of a particular kind, that strain is suitable for the detection of agents which *induce* tumours of the same kind. Some authorities have even recommended pure-line strains because they have a high incidence of a particular type of tumour. We regard such advice as being equivalent to recommending an analytic chemist to use a dirty test tube.

The discovery of aflatoxin firstly in ground-nut meal and later in a wide variety of cereals allerted us to the fact that the standard diets which are fed to laboratory animals used for carcinogenicity testing may contain potent carcinogens. Apart from aflatoxin, 3,4-benzopyrene, dimethylnitrosamine and other carcinogens have been detected in laboratory animal foodstuffs. The extent to which the presence of these agents in the diet interfere with the

outcome of tests is unknown, but the possibility that interference occurs should certainly not be ignored.

The work of Tannenbaum and Silverstone (1949*a*,*b*) established that the composition of the diet and calorie intake can influence the risk of tumour development; however, the extent of the influence has not been widely appreciated. Data obtained by one of us (MT) from studies in mice (Tables II and III) relate to this point. These data suggest that we are living in an age which is one of affluence for laboratory mice.

TABLE II

Cancers in affluent mice

Group	Number of mice	Number per cage	Weight of diet per day	Survival to 18 months	Number of tumours
1	40	1	4 g	Similar	4
2	40	1	5 g		4
3	40	1	ad libitum		32
4	40	5	ad libitum		23

Mice = Outbred Swiss albino males.
Diet = Standard pelleted.

TABLE III

Cancers in affluent mice

Feeding	Total tumours by 18 months	Liver tumours	Lung tumours	Lympho-reticular neoplasms	Other neoplasms
4 g diet/day 1 mouse/cage	4	1	1	2	0
5 g diet/day 1 mouse/cage	4	2	0	1	1 testis
Diet ad libitum 1 mouse/cage	32	15	2	11	2 testis 1 kidney 1 thyroid
Diet ad libitum 5 mice/cage	23	8	6	9	0

We think that these observations pose problems which are far too serious to ignore, especially as the diet given to the mice was in no way extraordinary (Table IV).

It is difficult to believe that these findings are interpretable in terms of the presence of traces of carcinogens in the diet. An ad libitum-fed mouse eats very little more (5.8 g per day) than a mouse restricted to 4 or 5 g per day. Does this mean that simple overfeeding increases tumour incidence in mice? Does this have direct implications for man? The epidemiologists have yet to provide a clear answer to this question. But irrespective of the answer, the observations depicted in Tables II and III obviously have important implications in relation to carcinogenicity testing. Differences in tumour incidence might arise between test and control groups in long-term feeding studies simply because of differences in palatability of the diet fed to the two groups. At worst, a carcinogenic effect might be obscured because of a concomitant reduction in tumour risk due to inappetence. Valid comparisons can only be made between test and control animals under conditions of isocaloric food intake.

The last two decades have seen a dramatic increase in knowledge of oncogenic viruses. This increase in knowledge has been largely ignored by those concerned with the carcino-

TABLE IV

Affluent mouse diet

	%
Barley	26
Maize	10
Oats	18
Bran	18
Fish meal	5
Yeast	1
Skimmed milk	13
Meat and bone	8
Vitamins	0.5

Carcinogens possibly present in trace amounts:
3,4-Benzopyrene
Dimethylnitrosamine

N.B. Aflatoxin not present in detectable amounts.

genicity testing of chemical agents. Most experimentalists have not deemed it necessary to determine whether the animals they are using for carcinogenicity tests are carrying known tumour viruses, and when tumours have arisen in animals following their exposure to chemical agents, no attempt has been made to determine whether the tumours are primarily of viral origin or really induced by the chemical under test. We would not wish to imply that such research would necessarily be easy. However, we do most seriously suggest that quicker, more reliable and less expensive test systems for carcinogenicity will not be developed unless and until the role of tumour viruses is taken into account.

For many reasons we do not believe that the time has come when we can rely on observations made on the response of cultured cells to chemicals to tell us whether a substance is a carcinogen or not. Nevertheless, we are mindful of findings such as those reported last year by Rhim et al. (1972) (See Table V). Meier et al. (1973) commented 'These findings suggest that the genome of endogenous type-C RNA viruses is the major determinant for tumorigenesis; although they provide no clues about the factors responsible for the various histological types'. We agree with this comment and find it difficult to believe that these findings should not be influencing the design of carcinogenicity tests using whole animals.

TABLE V

Influence of C-type RNA viruses on malignant transformation of cultured cells by known chemical carcinogens

	Mouse embryo cells + AKR leukaemia virus			Mouse embryo cells without virus		
	BP (1 μg/ml) + Acetone	DMBA (0.01 μg/ml) + Acetone	Acetone only	BP (1 μg/ml)* + Acetone	DMBA (0.01 μg/ml)* + Acetone	Acetone only
Malignant transformation	+++ ↓ Sarcomas on transplantation into newborn mice	+++ ↓	0	0	0	0

* > 1 μg BP/ml and > 0.01 μg DMBA/ml are lethal. (From Rhim et al., 1972.)

Recently we have witnessed several chemicals of established importance to man coming under fire because, under certain conditions, their administration to animals increases the risk of the development of tumours. Examples are cyclamate (Lancet, 1970), DDT (Nature, 1972), saccharin (Hicks et al., 1973) and soon, no doubt, phenobarbitone. We do not propose to discuss the arguments for banning or not banning these substances. However, we do believe that such decisions should not be made on the basis of the laws made many years before modern concepts of carcinogenesis evolved. Nor do we think such decisions should be made solely by lawyers following a standard book of rules. Trained toxicologists, using every ounce of knowledge available to them and every fibre of their capacities for critical judgment should be involved and each case should be considered separately on its merits.

In the early 1960's a pathogen-free colony of random-bred Swiss mice was established by I.C.I. The average overall tumour incidence among 1,000 mice allowed to live out their natural life-span without the deliberate exposure to any chemical agent was around 10%. Ten years later in the same mouse colony, kept under the same conditions and fed on the same diet, the spontaneous tumour incidence was 80%. This is the kind of background information that those responsible for deciding whether substances such as DDT should or should not be banned ought to take into account – especially since, as we have indicated already, the 80% tumour incidence can be reduced again to 10% merely by dietary restriction.

There have been several, usually abortive, attempts to develop completely standard, sometimes semi-synthetic, diets for laboratory animals. Prohibitive cost and the impossibility of preventing traces of possibly toxic chemical contaminants from creeping into such diets have been the usual reasons why such attempts have had to be abandoned. Perhaps in the light of the I.C.I. data we should be a little less concerned about the problems of variation in the composition of diets and more concerned with standardising how much animals under experiment eat.

More important still, there is an urgent need to study the particular constellations of factors involved in the causation of the more commonly occurring tumours of animals used for carcinogenicity tests. The art and science of toxicology is debased by those of us who are content merely to count the mammary tumours in rats, or lung or liver tumours in mice, without ever taking any steps to find out how they are caused. Unless we know the list of factors involved in their causation, we cannot design a clean experiment and we cannot know whether their occurrence in increased incidence in animals exposed to test chemicals has significance for man. A corollary of this approach is that special attention should be paid to finding out why exposure to a particular chemical increases the risk of tumour formation in one species and not another (e.g. effect of DDT on mouse liver and its lack of effect on hamster liver). Unless such research is undertaken there may be little chance of finding out which of the two species is the appropriate model for man.

TABLE VI

Liver tumours in CF_1 mice given phenobarbitone in diet

Concentration of *Phenobarbitone* in diet (ppm)	Number of mice	Type A nodules	Type B nodules	Lung secondaries
Males				
0	24	2	0	0
1000	24	8	15	8
3000	24	2	19	7
5000	24	7	9	0
Females				
0	23	3	0	0
1000	23	4	16	5
3000	24	6	16	5
5000	24	6	10	2

(From Dr. E. Thorpe, Shell Research Ltd.)

Dr. E. Thorpe of Shell Research Ltd. (personal communication) has kindly allowed us to refer to the results of an experiment in which mice were given diets containing phenobarbitone in various concentrations. Before the experiment was undertaken it was postulated that phenobarbitone might increase the risk of liver tumour development because it resembles certain other agents, which give rise to liver tumours in mice in being an active inducer of microsomal enzymes in liver parenchymal cells.

As shown in Table VI, the results were positive. Dr. Thorpe regards the Type A nodules as benign, and the Type B nodules as probably malignant. Several of the latter exhibited metastases in the lung and several of them grew on transplantation into other mice without the aid of immuno-suppressant agents.

It would be premature at this stage to conclude that phenobarbitone favours liver tumour development in mice *because* it is an inducer of microsomal enzymes. All that can be said at the moment is that the tumours that arise in phenobarbitone-fed mice are identical to those seen in response to DDT (Hart and Fouts, 1963).

One of the problems of assessing whether DDT is hazardous for man is that there is no unexposed population, at least in the western world. The position is different with phenobarbitone. It would be possible prospectively, if not retrospectively, to compare cancer incidence in humans exposed and not exposed to large daily doses of this drug since many epileptics will take it all their lives.

TABLE VII

Carcinogenicity testing: Common design faults

1.	Inadequate randomisation
2.	Unintended variation – Position on racks – Room differences – Operator differences – Observer differences
3.	High loss of animals without post-mortem examination
4.	Poor records of necropsy findings – position and size of lesions not recorded
5.	Non-standard post-mortem technique – e.g. procedure less rigorous on Saturdays and Sundays than on weekdays
6.	Failure to match microscopic with macroscopic findings
7.	Failure to take survival differences into account in expressing results

There is one other matter which we feel bound to mention. At this meeting there are representatives of companies who achieve very high standards in their conduct of carcinogenicity tests. We apologise to them for what we are now about to say but the plain fact is that there are other companies and organisations where the standards of testing leave much to be desired. We see, therefore, an urgent need for a general raising of standards of carcinogenicity testing. Poorly designed and poorly executed tests provide little protection for humans and are a waste of valuable resources.

In Table VII, we list some faults which are encountered all too frequently both in the open literature and in submissions to Regulatory Bodies.

Note added in proof

Since this paper was prepared for publication, C. Peraino, R. J. M. Fry and E. Staffeldt have confirmed (*J. nat. Cancer Inst., 51*, 1349, 1973) that dietary phenobarbital enhances spontaneous hepatic tumorigenesis in mice (of the C_3H strain), and Dr. Thorpe's findings have been published (E. Thorpe and A. I. T. Walker – *Fd. Cosmet. Toxicol., 11*, 433, 1973).

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