CHAPTER 3

Influence of Animal Species, Strain, Age, Hormonal, and Nutritional Status

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1 Introduction

The use of laboratory animals in toxicology and safety evaluation is based on the assumption that they are models for man. For many potent toxins this assumption is well founded in respect of qualitative findings. For weak toxins, for the purposes of safety evaluation and for quantitative prediction of toxic risk, the value of tests in laboratory animals is much more questionable. This is because the animals used are intrinsically poor models and because tests are undertaken under variable conditions which influence the results. The present chapter addresses these problems.

There is, throughout evolution, a continuity in the mechanisms that underlie life processes, and the extent of similarity in body structure between different mammals is striking. Nevertheless, there exist huge differences between species, particularly in the spectrum of foods which they eat and their lifespan. For example, a three year old rat or mouse is equivalent to a human centenarian and yet, within their relatively short lifespans, those species manage to manifest many of the diseases to which mankind is prone, including some degenerative conditions and a wide array of cancers. Higher metabolic rate and faster turnover of cells have been evoked to explain differences in longevity between species. However, these are unlikely to be complete explanations since cells derived from laboratory rodents, for example, behave similarly in *in vitro* cell cultures, and have similar growth requirements, to human cells.

2 Genetic *Versus* Environmental Influences

There has been much investigation of the ageing process but no satisfactory analysis or explanation of the process yet exists. In man, it is

a matter of common observation that age-associated changes occur earlier in some individuals than others. Moreover, longevity and early and late ageing seem to be familial. Nevertheless, it is not clear whether genetic or environmental factors are primarily responsible for these differences. Ageing is not a single entity. It is, rather, a progressive accumulation of structural faults and defects in bodily functions. Although either genetic or environmental factors may determine the time of onset of these faults and defects, it is more probable that genetic and environmental factors *interact* as determinants of the time of onset of age-associated diseases. Undoubtedly, genetic constitution often determines susceptibility to particular kinds of ageing defect. But environmental influences serve to reveal this susceptibility. Thus, α_1-antitrypsin deficiency renders humans susceptible to emphysema, but if deficient individuals totally avoided exposure to lung-damaging agents they may never develop the disease.

In the past, premature ageing and a wide variety of animal diseases have been attributed to defective genetic constitution but it is now clear that these may have been false attributions. By changing the quality of the laboratory environment, for example, many of these diseases can be made to disappear. The first major step in this direction came with the development of specified pathogen-free conditions of husbandry. Diseases such as ectromelia in mice and debilitating chronic respiratory disease, complicated by bronchiectasis, in rats markedly reduced in incidence or disappeared. (Bronchiectasis was once regarded as a strain-characteristic and as a 'normal' age-associated change by some experimentalists). The second major development has been a growing recognition that many spontaneous and age-associated diseases of laboratory animals may be due to gross over-feeding. This is, for instance, true of the common chronic nephropathy of rats and of many types of tumour of both rats and mice. Unfortunately, this growing recognition has not yet led to any major changes in diet formulation or feeding schedules in experimental laboratories. But it is certain that such changes must be made. The distinction between genetic and environmental causes of diseases in laboratory animals is difficult and unreliable. The influence of environment on the risk of developing various diseases continues to be underestimated.

3 Inbred *Versus* Outbred Strains

Sequential inbreeding serves to reveal genetic faults by progressively increasing the risk that offspring inherit defective genes from each parent. Similarly, first generation hybrids of two pure inbred strains are often more vigorous and longer-lived than their parents because hybridisation reduces the chances of inheriting pairs of defective genes.

The experimentalist seeking a model has to choose between two needs. On the one hand, he will desire to improve the experimental design by

using animals which are genetically identical, since the probability that effects are due to test agents is improved if 'like' is being compared with 'like', except for exposure to the test agent itself. On the other hand, the experimentalist knows that man is not inbred and that if he chooses a particular inbred strain it may, because of its peculiar genetic make up, give quite misleading information concerning the effects of a particular test agent.

For the purposes of evaluating new chemicals for safety for man, it has been argued that it is better to use outbred strains in the hope that at least a few of the animals included in the study will respond in a way that is predictive for man. This is a fallacious argument since there is no way of predicting which observation is pertinent to man. Since different experiments have different objectives, there is no single answer to the problem.

4 Choice of Species

Absolute qualitative differences in the way species respond to chemicals are uncommon but quantitatively very big differences are well documented. For the most part such differences are due to differences in absorption and metabolism, dependent on the presence of particular enzymes and on other conditions which pertain in the gut, the liver, the kidneys, and other organs. Where there is adequate information from studies in several species, which is rarely the case, it is common to find that different species share a spectrum of metabolic pathways but that the predominant pathway in one species is different from that in another. The reasons for such differences are not necessarily genetic. Lead acetate can be fed to rats throughout their lives at dose rates well above ones that would be lethal for man (Van Esch, Van Genderen, and Vink, 1962). This is because the proportion of lead absorbed from the gut is very low in rats fed a standard chow. Changing to a high milk diet multiplies the extent of lead absorption 40-fold and renders lead almost as toxic for the rat as for man (Kostial and Kello, 1979).

Ideally, for safety evaluation purposes, one should choose the species that most closely mimics man in the way that it handles a test material metabolically. In practice, this ideal may be quite unattainable. Firstly, metabolism studies can be difficult, time-consuming, and costly, even if interest is confined to a short list of regularly used laboratory species. Secondly, the studies undertaken may show that man is seemingly unique and that there is *no* good laboratory animal model. Thirdly, it may simply not be known whether other strains behave similarly or how much the available observations are dependent on factors peculiar to the set of conditions of the experiment. Regrettably, much of the literature on comparative metabolism is inadequate and, in addition, the information available for man is usually scanty and not comparable with that for the

laboratory species studied. Thus, for ethical reasons the only human studies performed are often for single, very small doses, whereas there are data for animals which have been exposed to larger and repeated dosage.

Furthermore, in tests for chronic toxicity and carcinogenicity, the choice of species is limited if there is to be a reasonable chance of detection of more than the strongest effects. To achieve sensitivity, relatively large numbers of animals (*e.g.*, groups of 50 males and 50 females) are required, particularly if effects in animals exposed to at least 2, and preferably 3, different dose levels are to be compared. This results in huge experiments that cannot even be contemplated except for small animals such as rats, mice, and hamsters. Since weak toxic and carcinogenic effects may not become manifest until animals are well through their available lifespan, it is usually necessary to choose short-lived species. In practice this tends, again, to limit the choice for chronic toxicity testing to rats, mice, and hamsters.

Finally, there is yet one further important consideration. Meaningful interpretation of tests on laboratory animals depends on the existence of adequate information on the array of 'spontaneous' lesions to which the test species is prone. The pathologist trying to evaluate what he sees at necropsy and later during the histological examination of tissues, may not know whether particular appearances are a result of exposure to the test material or manifestations of a spontaneous disease too uncommon to have appeared in the small number of controls.

Thus, the choice of species for the ideal experiment can be very difficult. Undoubtedly, man is the best model for man, but even here, intraspecies variation in response plus interference from environmental factors prevent really confident extrapolation from one man to another. These sources of uncertainty are magnified by species difference but somewhat reduced by the use of large numbers of animals which is possible in the case of small rodents. Comparative metabolism studies are of little practical value except to identify an animal species as *inappropriate* as a model for man.

5 Numbers of Animals: Safety Evaluation as Distinct from Mechanism Studies

Most toxicologists would regard it as more important and worthwhile (and certainly more interesting) to study mechanisms of toxicity than to screen new chemicals for possible toxicity. Regulatory authorities on the other hand (under pressure from an inadequately informed public) require extensive safety testing which utilises most of the available resources. To make matters even worse, it can be very difficult to persuade a regulatory authority that a substance which is in some way toxic for laboratory animals is safe for man, even if some studies of mechanisms have been completed.

The experimental requirements for mechanism studies and safety evaluation differ substantially. The former may require no more than a few animals in which specific measurements are made using sensitive methods. Arguably, studies of mechanisms are by far the most reliable way to characterise underlying changes that result in toxicity and to establish the basis of reliable prediction of human effects. The field is one that is ripe for fruitful expansion. As an example, there is in progress rapid expansion in our knowledge of regulatory peptides (Bloom, Polak, and Lindenlaub, 1982) and the mechanisms involved in homeostasis. This new knowledge is being turned to good effect by the pharmaceutical industry but has yet to be assimilated by toxicologists. Most of the observations routinely made by toxicologists (*e.g.*, haematological, urinalysis, the examination of haematoxylin and eosin-stained sections of tissues, *etc.*) are no more than fairly crude and insensitive screens for possible effects of xenobiotic agents.

In contrast, to establish the apparent safety of a substance for human use it is usually necessary to study large numbers of animals because of the insensitivity of the methods used, and yet the results are regarded as more pertinent than knowledge of toxic mechanisms. In the case of teratogenic and carcinogenic effects, for example, a positive response at any dose level may lead to the test substance being proscribed for human use. Thus the test is essentially designed to demonstrate the *absence* of activity, *i.e.*, to *prove a negative effect*—which is an impossibility. In practice, the negative results required are listed, along with details of the methods to be used, as 'Guidelines' by various authorities (*e.g.*, OECD Guidelines, 1981).

One consequence of the development of new and more sensitive tests, many of them applicable to living animals, will be to highlight the problem of how to distinguish between an 'effect' and a 'toxic effect'.

6 Animal Susceptibility

Rats and mice in the wild carry microbial and parasitic diseases and, like humans, are prey to epidemics of infectious diseases. Before the development of barrier conditions within which disease-free laboratory animals could be given pathogen-free food, endemic and epidemic disease rendered long-term experimentation frustratingly difficult. The cleaning up of animal laboratories has not, however, completely solved the problem of there being an unacceptable level of background disease, although the spectrum of diseases that most commonly occur has changed.

The inability to eradicate natural disease processes raises the question as to how far such conditions render the experimental animal more (or less) susceptible to the adverse effects of chemical treatments and how this affects the process of extrapolation to man.

It is possible that a high incidence of a particular disease in untreated

animals is indicative of susceptibility to the induction of that disease by exogenous agents. This is of particular relevance to specified types of tumour. For example, it is seemingly easier to induce adenomatous tumours of the lung in strains of mice that exhibit a high incidence of such tumours 'spontaneously' than in low 'spontaneous' incidence strains. At one time breeding programmes, aimed at developing high tumour incidence strains of mice (*e.g.*, the $B6C3F_1$ hybrid) in the hope that such strains would prove to be sensitive tools for detecting carcinogenicity, were initiated. There is no good evidence that such efforts improve safety prediction for man.

Ideally an animal model should exhibit the spectrum of morbid and fatal diseases of man (*e.g.*, high incidences of cardiovascular disease and cancers of epithelial cell origin, *etc.*). At present no such animal model is available. Many strains of the laboratory rat show very high incidences of endocrine tumours (*e.g.*, 100% incidence of mammary tumours in females, 100% incidence of Leydig cell tumours in males, up to 80% incidence of pituitary tumours in both sexes, high incidences of adrenal, thyroid, and parathyroid tumours, *etc.*) (Roe, 1981); and many strains of laboratory mouse, including the $B6C3F_1$ hybrid referred to above, exhibit high incidences of malignant lymphoma, liver, and lung tumours. In addition, rats aged two years or more often develop severe progressive nephropathy which affects both glomeruli and tubules to varying degrees, and which is associated with increased low-molecular weight proteinuria. Nor does the hamster offer a way of avoiding these deficiencies because under laboratory conditions, these animals tend to develop very high incidences of amyloid degeneration of the kidney and of septic atrial thrombosis.

Overall, the position is unsatisfactory in that animals in control groups commonly develop high incidences of diseases which are uncommon or even rare in man and very few animals die spontaneously of any of the diseases which most commonly affect humans. There are two serious corollaries of this. First, if the aim is to reduce man's burden of disease, then the tests should aim at detecting environmental factors or agents which induce or exacerbate the common diseases of man (*e.g.* arthritis, heart disease, stroke). Secondly, if a high spontaneous incidence of a disease is indicative of easy inducibility, the animal models we presently use are of little value for this purpose and others should be sought. Moreover, the high incidence of irrelevant spontaneous diseases render animal models unsuitable for detecting subtle manifestations of toxicity of certain kinds. It would not, for instance, be possible to detect a weak chronic toxic effect on the kidney under experimental conditions in which all the untreated control rats develop serious spontaneous renal disease.

7 The Effects of Overfeeding

When the spectra of neoplastic and non-neoplastic diseases that have been encountered in untreated animals in conventional chronic

Table 1 *A few of the many kinds of background pathology encountered in a group of 60 untreated male Sprague-Dawley rats that constituted the controls in a carcinogenicity study of 2 years duration*
(Unpublished data from personal files)

	%
Moderate to severe chronic progressive nephropathy	67
Parathryoid hyperplasia	67
Calcification of aorta	34
Adrenal medullary	
– hyperplasia/neoplasia	32
– neoplasia	20
Chronic fibrosing myocarditis	83

toxicity/carcinogenicity tests (Tables 1–3) are compared with the effects of controlling dietary intakes that have been observed in different studies (Tables 4–6), it is clear that much of the background disease which is presently such a prominent feature of long-term rodent studies is associated with overfeeding and is probably avoidable. At present, the

Table 2 *Incidence of certain non-neoplastic and neoplastic diseases in groups of 86 male and female untreated Sprague-Dawley rats*
(Reproduced by permission from Kociba *et al.*, 1979, *Food Cosmet. Toxicol.*, **17**, 205–221)

% of rats with:	Males	Females
Mineral deposition kidney	—	29
Moderate or severe renal disease	65	7
Mineral deposition in gastric mucosa and muscularis secondary to kidney disease	29	—
Multiple foci of hepatocellular alteration	—	17
Focal hepatocellular cytoplasmic vacuolation	40	—
Periarteritis	23	—
Neoplasms of:		
Pancreas – exocrine	33	—
Pancreas – endocrine	16	9
Pituitary – pars distalis	31	62
Adrenal – medulla	51	8
Thyroid – C-cell	8	8
Mammary gland – fibroadenoma	1	76
– adenocarcinoma	3	8
Any site	88	97

Table 3 *Percentages of rats with certain endocrine tumours in the control groups in 3 separate 2-year carcinogenicity studies on a prolactin-releasing drug. (The arrows in brackets indicate the effect of the drug on tumour incidence, if any*)*
(From confidential data in personal files)

Study No.	1		2		3	
Strains of rat	Wistar		Wistar		Sprague-Dawley	
Sex	♂	♀	♂	♀	♂	♀
Pituitary	22(↑)	62	17	53	41	46
Benign mammary	0(↑)	86(↑)	2	11	8(↑)	77
Malignant mammary	0	10	0	0	2	0
Phaeochromocytoma	18(↑)	22	0	0	2	0
Adrenal cortex	10	10	0	0	0	3
Thymoma (endocrine type)	4(↑)	10(↑)	0	0	0	0
Thyroid – follicular	26(↓)	18	0	0	0	0
– C-cell	0	6	9	5	1	0
Pancreas – islet cell	4(↑)	0(↑)	4(↑)	3(↑)	4	5
Parathyroid hyperplasia	25	7	0	0	0	0

* If only study No. 3 had been carried out, the drug would have run into fewer regulatory problems than it did!

simplest way of avoiding overfeeding is to limit the time during which food is freely available. Whether the same effect can be achieved by reducing the nutritive value of the diet provided throughout the 24 hours of the day, is not yet certain. Of special interest is the observation (Ross, Lustbader, and Bras, 1982) that the amount and kind of food an animal eats shortly after weaning has a seemingly indelible effect on its subsequent body growth and the incidence of tumours. Clearly the effects

Table 4 *Effect of restricting the access of Wistar rats to a standard laboratory chow (PRD formula) from 24 hours per day to $6\frac{1}{2}$ hours per day on incidence of chronic progressive nephropathy at 2 years*
(Reproduced from Salmon *et al.*, 1990)

Sex:	Male		Female	
Hours of access to food per day:	24	$6\frac{1}{2}$	24	$6\frac{1}{2}$
% of rats with moderate or severe nephropathy:	65	5	60	0

Table 5 *Effect of 20% diet restriction by weight on tumour incidence before 2 years in groups of 50 male and female ICI Wistar rats*
(Reproduced by permission from Tucker, 1979, *Int. J. Cancer,* **23,** 803–807)

	Male		Female	
	Ad lib.	20% Restricted	*Ad lib.*	20 Restricted
% survival to 2 years	72	88	68	88
% of rats which developed tumours at any site	66	24***	82	57**
% of rats which developed tumours at more than one site	22	2**	26	10*
Total number of all sites of tumours in group of 50 rats	47	14***	59	37**
% of rats that developed mammary tumours	0	0	34	6***
% of rats that developed malignant mammary tumours	0	0	12	2
% of rats that developed pituitary tumours	32	0***	66	38**

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

of diet composition, food-scheduling, and eating patterns on spontaneous disease in laboratory animals requires more intensive investigation.

The data presented illustrate the extent to which environmental factors influence the incidence of diseases which, in the past, many experimentalists considered to be primarily determined by genetic constitution.

Table 6 *Effect of diet restriction on longevity and tumour incidence in mice*
(Reproduced by permission from Conybeare, 1980, *Food Cosmet. Toxicol.,* **18,** 65–75)

	Ad lib.	Restricted (75% of *ad lib.*)	*Ad lib.*	Restricted (75% of *ad lib.*)
Number of mice	160	160	160	160
% survival to 83 weeks	58	66	62	77*
% Lung tumours	19	12*	15	5**
% Liver tumours	29	8***	4	0.6*
% Any tumour at any site	44	23***	31	11*
% Any malignant tumour at any site	11	4	14	4**

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

However, it is clear that the problem of high incidences of background disease cannot be fully resolved by attention to diet. Although diet restriction has been shown to have major beneficial effects on the incidence of kidney disease, endocrine changes, and neoplasia of various kinds, *etc.*, there remains in the restricted-fed animals an unacceptably high incidence of such lesions (particularly hyperplasia and neoplasia of the anterior pituitary gland in rats, and malignant lymphoma in mice). This suggests that the contributions of other aspects of the laboratory environment to the causation of disease in untreated animals should be explored. The suggestion that enforced celibacy may be detrimental (Roe, 1981) has not been supported by a recent small-scale study (Salmon *et al.*, 1990). It is probable that lack of exercise or general boredom will be found to be important. A study of the effects of providing an exercise wheel on the development of ageing and other diseases in mice was reported by Conybeare (1988). Behavioural scientists are probably best placed to devise protocols for testing the effect of general boredom!

8 Future Development in Long-term Testing

At present, the laboratories that carry out tests and the regulatory authorities who judge the results are understandably reluctant to use, or recommend the use of, diet restriction in the design of chronic toxicity/carcinogenicity tests. A fundamental change of this kind might devalue the banks of accumulated data derived from earlier conventionally-designed tests, and there is the fear that by reducing background disease, the model will be made less sensitive to the induction of those diseases relieved by dietary control. It is of critical importance that these objections are examined by properly conducted scientific experiment. It may then be possible to devise conditions which maintain laboratory animals in good health, free of infection, obesity, endocrine disorder, and many forms of neoplasia, and which are all properly monitored and controlled. It will then only be a short step for regulatory authorities to *require* that all tests for toxicity and carcinogenicity shall be undertaken in *hormonally normal animals* rather than in animals that display a mixed medley of laboratory artefacts.

9 Summary

This chapter has addressed a number of inter-related aspects of basic toxicology.

(a) There is no clear understanding of the 'ageing' process because of widespread confusion between diseases that are simply more common in old age (*i.e.*, intrinsically due to an ageing process) and diseases which are caused by avoidable environmental exposures.

(b) Genetic constitution influences longevity where inbreeding results in offspring receiving defective genes from both parents. Hybrid vigour illustrates an escape from this handicap.
(c) Many so-called 'strain characteristics' are wholly or partly environmentally determined.
(d) The advantages and disadvantages of using inbred as distinct from outbred strains must be considered in relation to the type and purpose of the experiment.
(e) The use of a species and/or strain of animals that handles the chemical to be tested in the same way as man is usually impracticable, either because the comparative metabolic data do not exist, or because no species which it is feasible to use is sufficiently akin to man.
(f) The principles underlying the investigation of mechanisms of toxicity are quite different from those underlying the design of safety evaluation tests. More studies of mechanisms are necessary to improve interspecies extrapolation.
(g) The spectrum of diseases which afflict man is quite different from the spectra which afflict rodent species. The relevance of rodents as models for the more common diseases of man merits further study.
(h) The high incidence of obesity and of many kinds of background disease in the laboratory rodents used in chronic and carcinogenicity testing today and, in particular, the high incidence of various endocrine disturbance and neoplasia of endocrine glands are unacceptable. Over-feeding appears to be a major factor in the causation of chronic progressive nephropathy, periarteritis, cardiomyopathy, and various endocrine tumours in rats and of malignant lymphoma, liver, and lung tumours in mice.
(i) Further research into ways of maintaining laboratory animals in normal endocrine status throughout their natural lives is urgently needed.

10 1993 Update

Since the above chapter was written for the first edition of this book two major developments have occurred in relation to the role of non-genotoxic mechanisms in carcinogenesis.

In relation to the first of these developments, the reader is referred to two books: Weindruch and Walford (1988) and Fishbein (1991). In the latter, there is a report of the results of an experiment involving 1200 rats exposed to various dietary regimes (Roe, 1991) and some of the findings in this experiment have been summarised in Roe (1993). Particularly noteworthy are the highly significant correlations between body weight at the age of 29 weeks and risk of premature death and/or malignant tumour development before the age of 133 weeks.

The second development concerns the growing recognition of the role of endogenous mutagens and the importance of increased cell turnover rates as determinants of premature ageing and increased cancer risk. This development is also discussed briefly in Roe (1993).

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