SECTION 1
Introductory Material

CHAPTER 1
Potential Advantages of Using Biomimetic Alternatives

Jamie Davies

Introduction

Animal experimentation has long been one of the cornerstones of biological and biomedical research. In fields from surgery to physiology and from pathology to pharmacology, *in vivo* models have been dominant for well over a century. It can be argued that many of the successes of modern medicine have been based on animal work. Examples include the use of dogs in the discovery of insulin and its use as a treatment for diabetes mellitus[1,2], the use of cats for the invention of the heart–lung machine[3], the use of mice in the development of penicillin as a clinical antibiotic[4], of rats in the identification of the first drugs effective against psychiatric disorders[5] and of mice in the development of clinically-useful antiviral compounds[6]. In recent decades, the rise of transgenic technology has meant that even fields such as molecular biology, that traditionally used cells rather than animals, now involve a significant number of *in vivo* studies. Current enthusiasm for transgenic mice has meant that a previously gently declining rate of use of vertebrate animals in science has reversed to become a steady rise (Figure 1.1).

With the apparent historical success of *in vivo* investigations, it may seem surprising that so many scientists are now putting so much effort into developing alternatives. There are, however, good reasons for this development, some based on avoiding or reducing the problems that have always been associated with animal work, and some aiming to maximize the opportunities that new technologies make available. The purpose of this short introductory chapter is to give an overview of some of the reasons to consider developing culture-based alternatives or, where a move to an entirely culture-based programme of work would be inadvisable, to consider ways to combine culture and whole-animal approaches.

The main reasons for considering alternatives can be divided, albeit with some room for debate about precise boundaries, into wholly scientific reasons connected with the quality and usefulness of the experimental data that may be obtained, and non-scientific reasons connected with costs, time, ethics, law and public image. Naturally, in most situations scientific progress is itself highly dependent on these non-scientific considerations, for the rate of scientific progress is limited by the availability of time and money, and the latter is much influenced by good will. Despite this connection, the reasons are considered separately in this chapter because clear discussion of the advantages of culture-based systems is all too often compromised by a conflation of very different ideas. In particular, sometimes strident presentation of ethical reasons to move to culture systems has tended to obscure strong but quieter arguments for scientific advantages and opportunities that such a move sometimes makes available. This book is written by scientists, for

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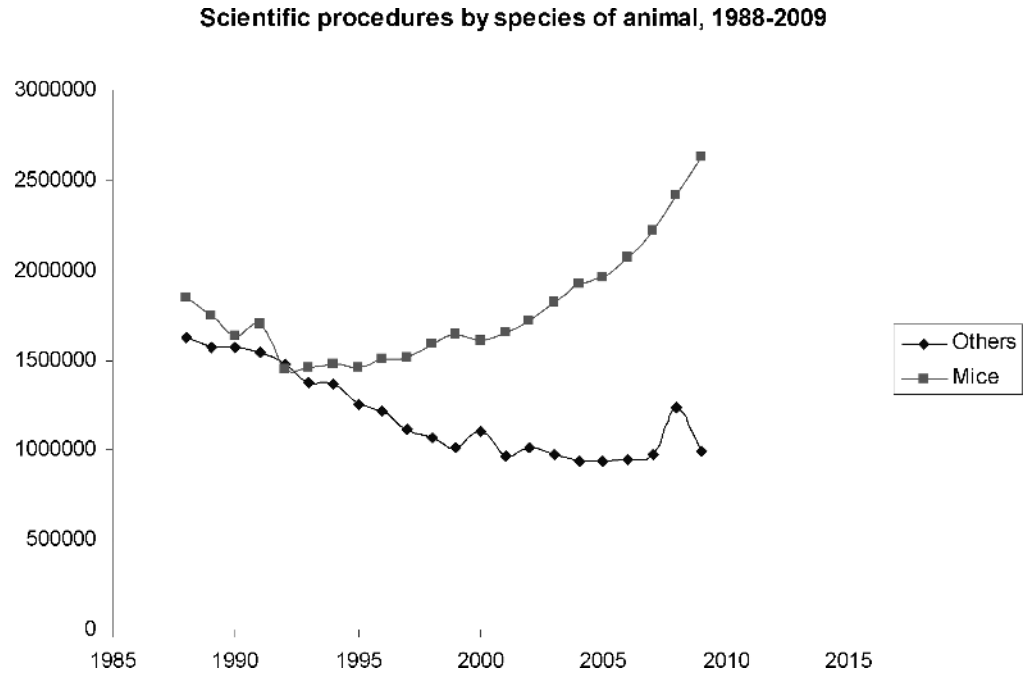

Figure 1.1 UK data for animal use between 1998 and 2009 illustrates trends that are common across the scientific world: use of most species has gently fallen but the use of mice has risen strongly, driven mainly by transgenic models.

scientists, and therefore leads with scientific reasons for exploring cultured biomimetic assay systems.

It should be noted that, in this book, the word 'animal' is generally used in the context of a species given some form of legal protection, such as by the UK's Animals (Scientific Procedures) Act. These are generally vertebrate species, although some invertebrate animals such as *Octopus* are also protected. Most jurisdictions permit experimentation on 'lower' animals, such as fruitflies and nematode worms, without restriction and these organisms are also generally very cheap to keep and require little space. This book does not therefore address the replacement of experiments performed in 'lower' animals with culture-based alternatives specifically, because there are fewer benefits from doing so and fewer external pressures to make such a transition. Nevertheless, the general principles outlined by later chapters should still apply and should be adaptable to invertebrate systems if anyone wishes to do this.

Scientific reasons to consider alternatives

Accessibilty

With a few exceptions, such as skin, hair, eyes and oral mucosa, most mammalian tissues reside deep inside an opaque animal. That makes them difficult to observe in a living state, and means that studies of the time-course of a natural phenomenon such as development, or of the progress of a disease or of healing, are frequently done by killing groups of experimental animals after a series of time intervals and making some kind of average measurement that can be used to compare the time-course of the process at different times. As well as involving the expense of large animal numbers, this approach throws away details that might be gleaned by following the time-course of events within the same animal. Also, information about real variation, where it is present, is lost as 'noise' in the data rather than appearing as clear

evidence that disease in different individuals might follow a consistently different course.

Modern imaging technologies such as magnetic resonance imaging and ultrasound ameliorate this problem to some extent, allowing non-invasive imaging of objects such as cysts and tumours[7–9]. Unfortunately, their use requires immobilization of the animal, which may induce stress and affect results. The resolution of these techniques is also limited; they do not yet yield information at the cellular level, although labelling test cells with contrast agents can approach this[10]. Transgenic luminescent reporter mice, and luminescent reporter pathogens, allow *in vivo* imaging of anatomy, events or infections[11,12] but the preparatory work can be complicated (for example, engineering the mice) and again the resolution is limited, especially for deep tissues.

In contrast to these problems, cultured organs or tissues can be put under the microscope at any time and can even be filmed continuously with cellular or sub-cellular resolution. Even where a transgenic reporter mouse is used as a source of the tissue, the improved access allowed by culture models can allow much better imaging than could be performed *in vivo*. An example of the power of this approach is provided Frank Costantini's group, who used live imaging of GFP-expressing cells in organ culture to provide a very high-resolution study of cellular dynamics during branching morphogenesis[13,14].

Reduction of confounding variables

Not all biomedical research is intended to measure the effects of some experimental intervention on a whole organism; rather, many experiments aim to determine the direct effect on one specific cell or tissue. Under these circumstances, the presence of other body systems, which might also have their own reaction to the intervention, can make what should be a 'clean' experiment very messy. Classical gene knockout experiments, for example, will remove a gene from all of the tissues that express it. Given that many very important signalling pathways are used for different purposes by different cells, removal of the gene can create a complex whole-body phenotype that only partly reflects the gene's role in the tissue of interest: worse, some of the effects on that tissue might be mediated indirectly from unknown signals from the rest of the body. This can be circumvented to some extent by the use of conditional knockouts[15], although even there it can be difficult to identify driver promoters that are expressed in only one location from only the time of interest. Exactly the same argument applies to small molecule agonists and antagonists that are used to investigate physiology.

Another 'whole-body' complication is the metabolism of drugs, particularly by liver and to some extent kidney, and their excretion. The opportunity to escape metabolic effects mediated by remote tissues is double-edged. Where the molecule being applied is itself pharmacologically active, escaping the whole-body situation allows experimenters to avoid rapidly changing concentrations and the appearance of new metabolites. Where drug is itself inert and has to be metabolized into an active moiety, on the other hand, the lack of a functioning liver would be a problem (although this can be circumvented somewhat by transfecting cells with constitutively-active genes encoding proteins such as cytochrome p450, which enables them to perform some 'liver-type' drug metabolism[16]). For larger molecules, from high molecular weight drugs to growth factors, antibodies, nucleic acids and other 'biological' pharmaceuticals, the reaction of the immune system can be a particular problem, especially as the magnitude of its contribution may become larger on each injection. Even where the eventual aim of a research programme is to develop a drug that can be used safely in the whole body, initial investigations into physiological mechanisms are often achieved most easily by large biological molecules such a natural growth factors, antibody or nucleic acid, so that the value of a drug target can be confirmed before much effort is expended in developing smaller, non-immunogenic versions.

In all of the these cases, an ability to study only the tissue of interest in culture, free of any other tissues and free of an immune system, can be a great advantage. It allows experimenters to use reagents that would provoke additional effects elsewhere in the body, or even be downright toxic.

Following disease processes to the end

In most countries that have strong research communities, investigations into pathological processes in whole animals are limited by ethical and legal requirements not to keep an animal in serious suffering. Pathologists studying disease processes are therefore prevented from observing the events that take place beyond this point as the animal must be destroyed humanely. In a culture-based alternative, there is no limit to how much destruction an infective agent might be allowed to wreak, and pathological events can be studied to their end. There will naturally be a difference between what is seen in an isolated tissue and what may be seen in a whole body, with its complex feedback systems and a active immune and inflammatory responses, but for at least some questions valuable data can be gained from examination of infected tissue in isolation.

Fidelity and safety

Where animals are being used as a proxy for people, for example in the modelling of a human disease, the testing of a drug or the safety testing of a chemical, there is another problem: while evolutionary homology means that the physiologies of different mammals are generally very similar, it does not imply that they are always exactly the same. Where they are not, there is the potential for two opposite types of error, the 'false-positive' and 'false negative' ('false' meaning, in this case, not giving a result that will be true in human).

For efficacy testing, false negatives do not carry risk of iatrogenic harm but they do result in a missed opportunity. They happen when a drug or other intervention that is potentially very useful in humans is wrongly seen to be ineffective because it does not work in an experimental animal. For safety testing, a false danger result will occur when a drug that is actually safe for human use generates a serious adverse effect in another species. Because of the historical reliance on animal testing, it is difficult to gather statistics on how common this effect is directly, as many compounds with adverse effects on animal models will never have been tested in humans. Some attempts to perform statistical studies using drugs that were finally accepted for human use have been made: an example, by Fletcher[17], focused on a series of 45 drugs assessed during the 1970s by the UK Committee on the Safety of Medicines. The study examined reports of the different specific types of toxic/adverse reaction (vomiting, ataxia, etc.; a total of 26 categories) in all species tested, including human, to determine the extent of correlation between data from humans and from non-human animals: of the 45 drugs, 13 showed no correlation at all and 17 showed only one correlating symptom. The author summarized the data by stating that 'up to 25% of the toxic effects observed in animal studies might be expected to occur as adverse reactions in man'; this implies that 75% will be false indications of danger. A broadly similar study showed that, of 20 compounds that seem to have no carcinogenic activity in humans, 19 were carcinogenic in animal assays[18]. Some very famous medical compounds that are broadly safe in humans have been found to be dangerous in other commonly used experimental species. For example, antibiotics of the penicillin family, such as ampicillin, are safe in mouse and human but show serious adverse effects in guinea pigs[19].

Unfortunately, *general* safety testing is an area in which the use of culture-based alternatives is most problematic because many adverse events stem from subtle problems at the whole-body level that would not be captured in culture. One mode of antibiotic toxicity in guinea pigs, for example, works through adverse modulation of the gut flora[20]. Toxic effects can also be local to rather obscure parts of the body which are not likely to be tested in a culture system unless a previous result obtained in a whole body (animal or human) has already highlighted a potential problem. The inner ear, affected irreversibly by some antibiotics, is an example of such a tissue[21]. Once the danger of such a specific effect is appreciated, it may well be possible for human-derived culture models to be used as a safety screen but it is very difficult, especially for a new class of small molecules, to predict all risks. Culture methods are therefore not a panacea for the major problem of general safety testing.

False positives in efficacy testing, that indicate that a compound or other intervention is effective in an animal model although it does not turn out to be so in humans, are frustratingly common and are the cause of a great deal of wasted money and effort in the development of new medicines. Arguably, many sophisticated genetic manipulations that are designed to give an animal a disease that its species does not normally have may actually result in very poor models, unless the genetic manipulations mimic exactly the mutations that are known to cause the disease in humans. An example is *Rb* mutation, which causes retinal tumours in humans but pituitary tumours in mice[22]. Mouse tumour models, particularly, can behave very differently to the allegedly analogous tumour in humans; to take one example, tumour growth tends to be much faster in mice than in humans, but metastasis is rare, and special techniques have to be used to make the model more relevant to human neoplasia[23]. It is perhaps for this reason that cancer scientists weary of the problems have made comments such as 'We had basically discovered compounds that were good mouse drugs rather than good human drugs'[24].

False indications of safety from animal models are the most dangerous errors caused by assuming that animal models are more similar to humans than they really are. It has been estimated that about 90% of drugs that are promising in animals go on to fail in human trials[25]. This wastes a vast amount of time and money, limiting the number of useful medicines that are introduced and making those that do make it to the market unnecessarily expensive, because their sales have to cover not only their own development costs but also the money wasted on a company's other drugs that seemed misleadingly useful in animal models. Occasionally, a drug that is safe in animals proves to be so spectacularly dangerous in humans that there is a scandal. A recent example was the 'super-agonist' antibody TGN1412. This antibody was designed to bind the T-cell surface protein CD28 and to activate regulatory T cells even in the absence of the normally necessary T-cell receptor-mediated co-stimulation; this activation of regulatory T cells would calm down the immune system in a way that might be useful to patients with autoimmune disease. The reagent was tested in a non-human primate, and found to be safe and effective. The antigen recognized by the antibody, the T-cell surface protein CD28, has an identical sequence in the two species, so researchers had every reason to assume that TGN1412 would have be as safe in man as in monkey. Nevertheless, when applied to humans at only one five-hundredth of the concentration used in monkeys, TGN1412 provoked a cytokine storm and multiple organ failure[26]. The probable explanation for this lies with another subset of T cells, the $CD4^+$ effector memory T cells: these stimulate immune responses rather than calm them down. In most experimental animals, including all of those used in pre-clinical safety screening of TGN1412, $CD4^+$ effector memory T cells do not express CD28 and are therefore 'blind' to the presence of the drug. In humans, however, but they do express CD28 and would therefore be activated by TGN1412[27]. Thus a very subtle difference between the immune systems of related organisms meant that animal safety testing gave a seriously misleading result.

One response to these problems is to perform at least some preclinical safety testing directly in human systems, perhaps simple cell lines for preliminary tests for grossly toxic or mutagenic effects, and then on artificial cultured 'tissues' that mimic the natural human system well enough to yield useful data.

Non-scientific reasons to consider alternatives

Ethico-legal pressures

The ethics of animal experimentation, of causing possible suffering to one set of non-human animals for the benefit of humans, or for the benefit of other animals in the case of veterinary research, has always been contentious on multiple grounds. First, there have always been arguments on whether non-human animals can suffer in the same way that humans can suffer and even now these are unresolved, and possibly unresolvable through scientific investigation ('detection and reaction to

adverse stimuli' is easy to measure, even in very simple organisms: 'suffering' is much harder to define in terms that everyone agrees and has therefore remained more in the realm of philosophy). Second, if the possibility of animal suffering is admitted, there is the question of whether the good of the many can ever justify the suffering of the few, a philosophical debate that is as relevant to how humans treat other humans as it is to how they treat other animals. Third is a debate about whether inflicting pain on non-human animals is psychologically harmful to the human inflicting that pain, and therefore whether it is ethical to employ any human to do that. Different people take different views, and rational argument makes little difference because each view can be a completely logical development from each original premise. That is why highly educated philosophers are as divided on the issue as people who make decisions simply on 'gut instinct'.

In plural democracies, there are pressure groups pushing in each direction. Groups campaigning politically against animal experimentation include The American Anti-Vivisection Society (USA), the British Union for the Abolition of Vivisection (UK), the European Coalition to End Animal Experiments (EU), the Irish Anti-Vivisection Society (IRL), the National Anti-Vivisection Society (UK) and People for the Ethical Treatment of Animals (USA). Organizations whose work involves what is euphemistically called 'direct action' – which may include violence against persons or property – include the Animal Liberation Brigade (USA), the Animal Liberation Front (UK) and the Animal Rights Militia (UK). On the other side of the argument, pressure groups defending animal experimentation (within the current legal limits applied to that work) include the National Association for Biomedical Research (USA) and Understanding Animal Research (UK). The response of democratic governments to this plurality of opinion has generally been the compromise of passing legislation that controls, licenses, restricts and inspects animal work, but does not ban it altogether. In some countries, the legislation controls with a light touch while in others, such as the UK, the administrative burden imposed by legislation can be onerous and time-consuming. In addition to the legislation that controls how animals are looked after when they are used, there is the impact of legislation that controls when they can be used at all. One of the most dramatic examples is the recently revised European Union directive 86/609/EEC, which, amongst other things, outlawed the animal testing of cosmetic products from 2013 (that practice has been outlawed in the UK since 1998). Since new cosmetic products are still required to be demonstrably safe, this legislation is a strong driver for the development of alternative methods.

There are various reasons that ethical and legal concerns may create a push towards finding alternative approaches. Many scientists have direct ethical concerns themselves, irrespective of pressure groups or laws, and would be glad to escape or avoid *in vivo* work. A recent study[28] in the journal *Nature* suggests that about 16% of working scientists report significant ethical concerns. Others, while being ethically content with using animals, feel pressure either from the ethical concerns of other people in their lives, or possibly fear of violence. For everyone working in a country with a substantial legal and inspection framework, the time and delays involved in having a new experiment licensed, and involved in attending compulsory training and refresher training sessions, can be a substantial incentive to find another way, particularly if a competitor might be doing exactly that.

Economic pressures

Compared to cells, experimental animals can be very expensive to keep. They require skilled supervision, carefully controlled accommodation, cleaning, feeding and inspection, and relatively spacious cages with facilities for behavioural enrichment, especially in the case of larger animals. All of this is very expensive. Within the European Union, the chemical industry in particular now has to perform a great deal more health and environmental safety testing of substances under the stringent requirements imposed by European Union REACH legislation (EC 1907/2006)[29,30]. Performing all of this by animals would be hugely expensive, in terms of money and possibly also in terms of public

image, and alternative methods are appealing on both grounds. Developing such methods is being encouraged by initiatives such as CRACK-IT, run by the UK's National Centre for the 3Rs, which allows industry to call easily on academic expertise to solve specific problems (see Appendix 1).

Limits to the use of biomimetic alternatives

As there are both scientific and non-scientific reasons to consider using alternatives to whole-animal experiments, so there are both scientific and non-scientific limitations to what can currently be done in this direction.

Scientific limits

Most working scientists would agree that there are limits to what can be done in cultured biomimetic systems. For example, it may be possible to explore thoroughly the developmental biology of axon guidance during neural development using a cultured mimic of brain tissue, but it would be much more difficult to use cultured systems to study higher-level functions of the brain such as attention, emotion and cognition. The cell and tissue biology of a viral infection can be studied very easily with the correct *in vitro* tissue substitute, but the effect of the disease on the well being of a whole individual cannot. Similarly, much can be done in culture to test the safety and efficacy of a compound on isolated tissues or on developing embryonic organs, but there will still be the possibility of an unexpected effect on whole-animal physiology. In the cycle of drug development, therefore, human-based biomimetic alternatives to whole animal experiments are likely to be used mainly as a pre-screen, that will confirm that potential drugs to at least have their desired activity in human cells and that they and other compounds do not have unexpected toxicity across a range of target tissues that have proved problematic with broadly similar compounds in the past. If compounds fail these tests, there is no point in their moving to the financially expensive, ethically expensive stage of *in vivo* experiments. Wasted effort will therefore be reduced and the animals will be treated only with compounds that have already shown themselves to be non-toxic in *in vitro* tests, reducing the probability of an adverse reaction that would induce suffering. This means less effort wasted on dead-end compounds, and fewer animals needed per hundred medicines that finally make it to clinical use.

Non-scientific impediments to the use of alternatives

The heading to this paragraph uses the word 'impediments' rather than 'limits', because the problems discussed here are essentially cultural and can, and should, be solved. One cultural impediment operates at the time of publication of original research in academic journals. Although many journals 'sign up' in their statements of aims and values to the principles of refining, reducing and replacing animal use, and many require clear ethical statements for use of animals as well as humans, researchers who use alternative methods often experience problems when publishing (it is a very common topic of coffee-time conversation in conferences devoted to the development of alternatives). The problem stems from peer reviewers who accept the internal quality of culture-based experiments without any criticism of how they were done, but then say they will only recommend publication when the result is also seen in a whole animal. For some studies, ones that claim to have described something at the whole-animal level, this may be appropriate. For other studies, that claim only to have described something at the level of an isolated tissue and are clear about the system used, it may not be appropriate at all and often seems to be given as some sort of reflex reaction at the level of 'four legs good, test tubes bad'. As a developmental biologist, the author of this chapter has frequently had to argue against the idea that a result on local cell interactions, obtained in a 'clean', limited, well-controlled and characterized organ culture system, must be 'validated' in a knockout animal subject to all of the interfering complexities of other tissues and body systems reacting to change, and in which only very limited time-course and end-point observations can

be made. Journal editors who do their job properly will assess the content of reviewers' reports and question the rigour of their logic as they question the rigour of the manuscript itself. Alas, many editors seem to require every peer reviewers' wish be met without question, rather than remembering the dictum 'Reviewers advise, Editors decide'. Collectively, journal editors can make an immense difference to the uptake of alternative methods simply by taking the time to make an independent judgement about whether a reviewer's demand for whole-animal validation is reasonable. The editor of a typical scholarly journal does not have much time to devote to these things, typically being a full-time academic as well as an editor, but the occasional half-hour spent engaging fully with this kind of argument, when it arises, can do a great deal of good. Editors must feel strongly about the development of their field (why else would they take on the job?), and since the issue is one including alternative systems that deliver *better science*, the investment of time is surely worth the effort.

A second impediment, more applicable to industry, is the requirement that a culture-based alternative test for safety (or a set of tests taken together) be proven to be as effective as the traditional animal test. This seems entirely reasonable and is indeed so if the effectiveness of the original animal-based test is itself supported by data, for example on its ability to predict human toxicity. In reality, many animal tests were introduced by educated guesswork, without the support of clear statistical performance data, at the beginning of widespread safety testing, although retrospective data do now exist for many common ones. The bar for introducing alternative methods is therefore set rather higher than it was for many original animal tests: this is to the good of the public, who will benefit from safety testing being done using assays whose performance is known from the start. It does, though, require investment in testing the new tests, and negotiation with regulators to have them accepted. The resources required for this can be a significant impediment, research organizations being trapped in the problem that they know that moving to new methods would be financially and scientifically better in the long term, but never having the spare cash to make the move right now. Fortunately, enterprises that are competitors in the market place have shown laudable initiatives in working together, and with academics, to pool efforts to gain industry-wide and regulatory acceptance for new methods. Some government-funded and charity-funded bodies can also help with this: examples are listed in Appendix 1.

Summary

In summary, culture-based biomimetic alternatives are not a panacea, but used for the right purposes, they can be cheaper, quicker, better controlled and more relevant than traditional animal models. In short, considering such as system may enable a researcher to do more science and better science.

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