Replacing Primates in Medical Research

An expert report by: Dr Hadwen Trust / FRAME / St Andrew Animal Fund

MEMBERS OF FOCUS ON ALTERNATIVES

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Replacing Primates in Medical Research

Introduction

There have been many reports on the subject of experiments on non-human primates, mirroring the level of concern about this issue. Why another report now?

An important feature missing from most previous publications is a detailed analysis of the extent to which primate experiments have already been replaced by advanced non-animal alternatives such as cell and molecular methods, computer simulations and ethical studies with human volunteers (see panel).

This report aims to fill that gap. It is important because knowledge of these successes will foster a more widespread sense that primate research can be replaced with valid techniques that do not use laboratory animals. We selected five areas of medical research into important human conditions – malaria, cognition, stroke, AIDS and hepatitis C – which have used significant numbers of primates over many years and yet have had very limited success in translating to human benefit. They illustrate where notable progress has been made in replacing primate experiments with non-animal techniques, and where greater progress is achievable.

Around the world many thousands of primates, such as chimpanzees, macaque and squirrel monkeys, baboons and marmosets, are used in research, drug development and safety testing:

- **In the EU, 10,451 primates were used in 2005**
- **In the USA, 62,315 primates were used in 2006**
- **In Japan, an estimated 2,802 primates were used in 2004**
- **In Great Britain, 3,125 primates were used in 2007**

Surprisingly, very few Cochrane-style [1] systematic reviews of primate research have been conducted. Without such reviews – which would use internationally agreed comprehensive and transparent methods to find, select and determine the quality of primate research – there is little independent evidence of the value of primate experiments for human medicine.

Because of the suffering they cause, primate experiments engender serious and long-established concern to the general public. For example, over the last 10 years public opinion polls in the UK have consistently shown that more people are opposed to experiments on primates than support them. In fact, the results of the 2005 European Union (EU) official opinion poll showed that 82% of EU citizens believe we have a duty “to protect the rights of animals whatever the cost” [2]. A resolution to end the use of primates in research and testing, presented at the Fifth World Congress on Alternatives and Animal Use in the Life Sciences, also in 2005, was signed by world-renowned primatologist Dr Jane Goodall and 57 individuals and organisations from 19 different countries.

Politically, the ground is shifting too. In 2007, the European Parliament accepted a resolution to end the use of great apes and wild-caught monkeys in experiments and for a timetable to replace all primate experiments with non-animal alternatives in the EU [3].

In 2008, as part of the revision of EU legislation to protect animals used for experimental and other scientific purposes (Directive 86/609/EEC), the Scientific Committee on Health and Environmental Risks (SCHER) was asked to issue an expert scientific opinion on the perceived need for primate experiments in biomedical research, including an assessment of the latest status of possibilities to replace their use [4].

Discussions about the ethics and the validity of using primates in research feature in the scientific literature [5] and in reports from national ethical and advisory committees [6, 7]. In the UK, Dr Vicky Robinson, Chief Executive of the government’s National Centre for the Replacement, Reduction and Refinement of Animals in Research has said [8]:

“...There is growing recognition in UK science and industry that looking critically at research using animals can benefit the scientific outcomes as well as the animals, and also a greater willingness to think innovatively about where the use of primates can be reduced. The Government and the funders should be seeking to develop a national strategy that is not just about the continued use of primates, but which has the clear aim of replacing, refining and reducing that use wherever possible.”
Examples of non-animal research techniques

- **Gene-hunting tools to pinpoint and understand, in human populations, the importance of different genes in a range of illnesses**

- **Cell and molecular studies to understand disease mechanisms and the effects of vaccines and pharmaceuticals**

- **Ultra-sensitive analytical techniques, such as accelerator mass spectrometry, allowing safe, ethical, microdose studies of medicines in volunteers**

- **Advanced microscopic techniques for imaging and analysing human cell functions in health and disease**

- **Biosensors that synergise cell research with microelectronics, to study metabolism, toxicity and disease biomarkers**

- **High-powered computer models that realistically simulate the human body and its component systems and organs, and their reactions to medicines**

- **Novel gene-silencing approaches to study specific gene functions in human tissues in the test tube**

- **Studies of post-mortem tissues bequeathed by patients to gain insight into cell-level changes in human illnesses**

- **Computational analysis of human data to understand the lifecycle of disease viruses in the human body**

- **Tissue engineering re-creates three-dimensional human tissues in the test tube, for disease research, drug development and safety testing**

- **Computer predictions of medicinal effects based on the structures of pharmaceutical molecules**

- **High-technology, safe imaging of the human brain to understand neurological disorders**

Primates attract particular concern because their advanced cognitive skills and high-level social and behavioural repertoire add significantly to the case against using them in experiments. It remains impossible to capture and breed them, transport them halfway across the world in some cases, and keep and use them in laboratories, without seriously compromising their physical and psychological health [9].

Focus on Alternatives represents all the leading British non-governmental organisations that specialise in developing or promoting approaches to replace animal experiments. This report, authored by member groups Dr Hadwen Trust, FRAME and St Andrew Animal Fund, reflects a range and depth of scientific expertise. We hope that in providing some detailed case studies of how and why primate research is being replaced by non-animal methods, the scientific, research funding, political and regulatory agendas to fully replace primate experiments will move to a higher and more pro-active level.
References and notes

1. Further information from www.cochrane.org
2. Special Eurobarometer 225, on Social values, Science and Technology, 2005.
3. European Parliamentary Written Declaration 40/2007 which "Urges the Commission, the Council of Ministers and the European Parliament to use the revision process of Directive 86/609/EC as an opportunity to: i. Make ending the use of apes and wild caught monkeys in scientific experiments an urgent priority; ii. Establish a timetable for replacing the use of all primates in scientific experiments with alternatives."
Primates have been used in malaria research for three decades. As will become clear from the evidence presented below, malaria research is an example of a field in which there is a strong possibility for the replacement of some, if not all, primate models. Given the human health value of malaria research and vaccine development, not only are primate models of malaria infection critically reviewed, but positive suggestions are made as to the ways in which alternatives can be developed and applied.

Introduction

Malarial infections by one of four species of *Plasmodium*, namely, *P. falciparum, P. vivax, P. ovale and P. malariae*, cause 1-2 million deaths per year, with the first of these being responsible for the majority of them.

The parasite is passed to humans by the *Anopheles* mosquito. The complex lifecycle of the parasite (figure 1), and increasing resistance to current drug regimes, mean that the development of an effective vaccine, though imperative, is very difficult. There are two lines of evidence to suggest that a malaria vaccine is, nonetheless, achievable: i) immunity can be acquired as a result of natural exposure to infection, and ii) various immunisation strategies have been shown to induce protection against experimental infection in animal models and human volunteers.

However, the development of an effective vaccine is hampered by the fact that it would have to induce immunity against multiple species and strains of the parasite, and act at specific points of the parasite's lifecycle. In addition, the mechanisms of immunity observed in the laboratory setting may not provide protection from natural infections. This is particularly true for animal research, as animal models are artificial hosts often with naive immune status and can have very different susceptibilities to infection by malaria strains. Perhaps the greatest limitation is the fact that the malarial strain or species under investigation is not necessarily one that would normally infect the animal model used as the host [1].

For instance, Stowers and Miller [2] proposed that the New World monkey challenge model (NWMCM) is essential, if a vaccine that targets the blood-stage infection pathway is to be developed. This is because the model provides efficacy data for the production of clinical grade vaccine, obviates the need for extensive field trials, and might provide information useful for the validation of *in vitro* assays. Thus, the authors clearly support the use of the NWMCM as a pre-field test model.

However, Heppner *et al* [3] questioned whether the NWMCM will ensure that the best vaccine candidates are advanced to human studies, arguing that the NWMCM consists of an unnatural host (NWM), challenged by an unnatural route of administration (intravenous injection), by using an unnatural inoculum (parasitised red blood cells), which can result in variable infection rates. Instead, they advocate the use of a human sporozoite challenge model in field trials, in which natural human hosts are challenged with their natural vectors (*Anopheles* spp.), so that insect bites to the skin deliver sporozoites into the bloodstream. This results in a consistent pre-patent period (the time between infection and the parasite being detectable in the host blood) and a reliable infection rate in human volunteers.

Moreover, Heppner *et al* [3] questioned the general relevance of the NWMCM as a surrogate for the study
of either human malaria or immunity, cautioning that: “interpretation of the NWMCM must consider that there are intrinsic differences between the relationships that NWMs and humans have with *P. falciparum*” (p. 421). Other studies suggest there is little correlation between the efficacy of vaccines in NWM models and results in human trials [4], presumably because of more fundamental differences between the immune systems of humans and other primates.

Many of the problems arise because of host/parasite evolution. Under normal circumstances *Plasmodium* species are usually adapted to one of a tight group of hosts, so do not in general induce severe illness and do not trigger the host’s immune defence too strongly. This is due to compatible antigens between the host and parasite. However, when the parasite is introduced to an unnatural host compatibility is sub-optimal and the number of molecular targets for a vaccine increased [5]. Thus, protection is far easier to achieve in experimental rather than natural host/parasite combinations and may explain why vaccines shown to be effective in mice and primates fail to prevent infection in humans [6]. Indeed, Chatterjee et al [5] argue that “Since we do not understand well-enough human immunity to Plasmodia, it is not possible to determine which model if any may best reflect the desired pattern of immune responses” (p.322).

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**A historical perspective of 30 years of malaria research**

Practical difficulties with the creation and mass production of a live attenuated vaccine have precluded this more traditional vaccine strategy. In 1973, it was shown that humans could be protected against malarial infection via the bites of around one thousand infected, irradiated mosquitoes [7], but for obvious reasons, this was not deemed to be a suitable means of vaccination. Similarly, Clyde et al [8] observed that inoculums containing irradiated sporozoites conferred some protection against malarial infection in human volunteers. Again, there are logistical problems with producing a vaccine by using this approach.

In the late 1970s, promising vaccines against the primate malarial parasite, *P. knowlesi*, were discovered from research in rhesus macaques (*Macaca mulatta*) [9, 10]. However, this work for reasons which are unclear does not appear to have been translated into vaccines for the prevention of human malaria. The vast majority of research conducted in the 1980s focused on preventing the proliferation of sporozoites in the liver by using antibodies against sporozoite antigens. It was found that malaria-exposed individuals had high titres of antibodies against the circumsporozoite protein (CSP), a sporozoite coat protein [1]. In 1980, mice immunised with a CSP-specific monoclonal antibody were successfully protected against malarial infection [11]. Later research implicated the involvement of CD8+ and CD4+ T-cells in protecting mice inoculated with CSP-specific antibodies against malaria [12].

In 1983, the cloning of CSP from *P. knowlesi* revealed a set of repeat sequences on the molecule that could form the basis of a vaccine [13]. However, despite encouraging results in mice, this sporozoite vaccine was much less promising in human trials, and the researchers concluded that, since antibodies do not optimally prime parasite-specific T-cells in humans, more-potent adjuvants would be needed to enhance the immunogenicity of the vaccine [14].

The first malaria vaccine to be extensively tested in humans was SPf66, a synthetic protein with amino-acid sequences from three proteins derived from CSP. Despite initially
being tested in owl monkeys (Aotus spp.) [15], this vaccine failed to show convincing protection in humans during Phase III clinical trials [16]. It was suggested at the time that this was due in part to human population genetics, since variations in genes and commensurate variations in cellular and humoral immune responses to malaria exist. Another possible explanation for the somewhat disappointing outcome to this study is that SPf66 itself contains a component that corresponds to a variable region of the malaria antigen, MSP1 and, thus, the parasites also exhibit different infectivity. This could explain why the primate model did not predict the lack of efficacy of SPf66 later seen in human studies. More recently, a multiple antigen peptide vaccine, composed of hepatitis B surface antigen and the central repeat and C-terminal non-repeat regions of the CSP (RTS,S/AS02A) that showed partial efficacy in human clinical trials, has now been reformulated to enhance its immunogenicity. Safety and immunogenicity testing was performed on the enhanced formulation in the rhesus monkey, and on the basis of the primate T-cell response, was then introduced into human trials [17]. The outcome of the clinical trial that followed is as yet unpublished.

The knowledge that immunisations with plasmid DNA were protective against viruses was exploited by the US Naval Medical Research Institute, to investigate whether DNA vaccines could be developed against malaria. The DNA vaccine developed was specific for the rodent malarial parasite, *P. yoelii*, and was tested in mice. Three antigens, PfCSP, PfSSP2 and PfExp-1, along with a fourth component that corresponds to a variable region of the malaria antigen, MSP1 and, thus, the parasites also exhibit different infectivity. This could explain why the primate model did not predict the lack of efficacy of SPf66 later seen in human studies. More recently, a multiple antigen peptide vaccine, composed of hepatitis B surface antigen and the central repeat and C-terminal non-repeat regions of the CSP (RTS,S/AS02A) that showed partial efficacy in human clinical trials, has now been reformulated to enhance its immunogenicity. Safety and immunogenicity testing was performed on the enhanced formulation in the rhesus monkey, and on the basis of the primate T-cell response, was then introduced into human trials [17]. The outcome of the clinical trial that followed is as yet unpublished.

The cloning of other malarial antigens began in 1994-95, including chemically synthesised or recombinantly expressed antigens that might serve as alternative targets for antibody inhibitors of the human parasite life cycle, and thus could enhance understanding of the infection process itself [1]. Indeed, publication of the *P. falciparum* [24] and *P. yoelii* [25] genomes, and the launch of the *Plasmodium* database [26], paved the way for genomics-related technologies, recombinant DNA and cell engineering to reveal the genes and pathways involved in human malarial infections. This, in turn, might yield a number of possible targets for vaccine development. These developments could also lead to new techniques for studying malaria and its interactions.

For example, one research group is developing imaging technologies that will allow visualisation of individual malaria molecules in living cells [27]. Already, information from non-animal studies is being used to analyse the four stages of the parasite lifecycle, and has revealed that most *Plasmodium* genes are expressed in a rigorously regulated cycle. This means that candidate vaccine antigens can be identified based on the known expression patterns of antigens that have already shown some promise [28].

New technologies and post-genomic analysis can also be used to explain species differences that have confounded the development of efficacious vaccines against human malarial infections by employing commonly-used laboratory species, such as the mouse and the rhesus macaque. One comparison of the human malaria parasite, *P. falciparum*, and the commonly-used rodent parasite, *P. yoelii* has been conducted [25]. The rodent model reproduces many of the biological characteristics of human malaria, and many of the techniques applied to the study of *P. falciparum* were initially developed by using *P. yoelii*. This comparison reveals that many of the candidate antigens under study in the human parasite can be identified in the rodent one, including orthologues known to elicit immune responses in a DNA vaccine against malaria. It would be interesting to investigate the reasons that underlay the decision to withdraw the proposal, in order to determine whether it was based on potential problems that were not highlighted during the preclinical studies in animals.
exposure to natural infection. However, while chromosomal gene synteny is high between the two species in respect of housekeeping genes, it is not in regions where genes involved in antigenic variation and evasion of host immune system are found. Thus, it may be that *P. yoelii* is not the best model for studying *P. falciparum*.

Furthermore, SCID (severe combined immunodeficiency) mouse models implanted with human Hep G2 cells are susceptible to infection by the mammalian parasite, *P. berghei* [29], so can be used in the cultivation and understanding of the intrahepatic stages of the parasite’s lifecycle. However, this model, which involved implantation of cells into the mouse kidney capsule, was unable to sustain the primary human hepatocytes required to support infection by the human parasite *P. falciparum*.

On ethical, welfare and scientific grounds, ideally the replacement of primates should involve completely non-animal methods some of which are outlined below.

The development of human hepatoma primary cell cultures that can be infected with *P. falciparum* may have useful applications. This is because infected hepatoma cells can then infect human erythrocytes [30]. Hence, while limited in their applicability to vaccine research due to a lack of immune system components, these cell-based studies can provide a means to improve understanding of the parasite-erythrocyte interactions and for identifying liver stage-specific antigens.

Binh et al [31] reported an *in vitro* system that provides a novel way of studying interactions between the human immune system and the malarial parasite, independently of the influence of human serum. This system involves the successful propagation of *P. falciparum* in a serum-free culture system. The effects of human sera differed depending on whether the serum was immune serum from infected individuals, semi-immune serum from individuals with a history of infection or non-immune serum from individuals who had never been infected. The parasites were cultivated in human erythrocytes, and human peripheral blood mononuclear cells (PBMCs) from the same three serum groups were subsequently added to the cultures.

In *vitro* studies on the human CD4+ T-cell system might prove to be an invaluable correlate of protective immunity to malaria, and could be a key model in developing vaccines. Bergmann et al [32] proposed that a state of protective immunity can be determined based on CD4+ T-cell subsets expressing certain markers and antibodies, which is characteristic of a memory T-cell response to malaria. In addition, Toure-Balde et al [33] demonstrated that certain T-cell niches may react to specific parasite antigens and thus have important implications for the selection of vaccine antigens.

Similarly, in the past, it has not been possible to study the developmental biology of the liver stages of the malarial parasite lifecycle *in vitro*. However, recent improvements in
human liver cell culture mean that this is now achievable. Sattabongkot *et al.*[34] concluded that their *in vitro* model will facilitate comparative studies, and that, with further improvements, it may also provide a means for studying gene expression in sporozoites and identifying novel liver-stage vaccine candidates, as well as acting as a convenient system for screening potential antimalarial drugs.

Hence, as seen from the above discussions, there are several *in vitro* methods that can be used to study the malaria parasite. When such methods are used in combination with other non-animal approaches, the prospects for avoiding the use of primates in malaria research and vaccine development are greatly enhanced.

Human volunteer studies have been conducted to identify a novel *P. falciparum* gene (MB2) the product of which could be exploited as a vaccine target *[35]*. Human volunteer studies can also be used to understand how gene expression in the parasite influences parasitic survival and pathogenesis *[36]*. In order for a subunit malaria vaccine to be effective, it must contain T-cell epitopes capable of initiating an immune response in diverse populations. Thus, stratifying volunteer studies to account for known population differences in immune response can offer a distinct advantage over animal studies where strain differences are less readily distinguishable from variations that correlate with human population differences.

Characterisation of the CD4+ T-cell response in human volunteers led to the identification of the T epitope *[37]*. Mayor *et al.*[38] argue that more attention to the natural history of malaria is needed to understand the dynamics of infection and immune system interactions, so they have used epidemiological studies to investigate the clinical, parasitological and haematological status of adults living in malaria endemic regions. The genetic analysis of humans and the parasites *[5, 39]* are also important alternatives to using primates. Indeed, Chatterjee *et al.*[5] propose that a new approach to vaccine development “would require a strong commitment to gather an improved understanding of essential defence mechanisms prevailing in humans, this leading to improved models that reflect them best” *(p.323)*. This would involve induction of protection using irradiated sporozoites in human volunteers and better definition of naturally occurring immunity, as well as analysis of the immune responses associated with these situations.

To some extent Druihl and Barnwell *[6]* also support a change in emphasis, highlighting that “Investigations in humans are indeed difficult and limited, yet they might be unavoidable as they supply information that cannot be gathered by alternative means” *(p.375)*. They also suggest that the current tide of malaria vaccine clinical trials provides an excellent opportunity to retrospectively address the validity of the initial paradigms/models that they were based upon.

**Conclusions**

Primates are highly sentient beings capable of suffering as a consequence of their use in research. When the aim of such research is to simulate human infection, there is additional concern since infected animals may suffer to extraordinary degrees. Hence, it is imperative that the use of these animals for scientific and experimental purposes must always be challenged, until such time that alternative methods obviate their use in research and testing.

The development of effective anti-malarial drugs and vaccines to protect against malaria has traditionally involved studies in primate models. However, despite 30 years of research, these models have often resulted in inadequate outcomes. The human health implications of not identifying and developing clinically relevant models require that we carefully appraise whether studies in primates have resulted in improved human health.

Based on the scientific evidence presented herein, it is clear that the relentless defence of primates as models for use in the study of malaria and the development of malarial vaccines is scientifically unsound. It is imperative that we retrospectively evaluate primate studies to identify the type of questions that have failed to be addressed using these animals, in particular why primate models often fail to predict the human effects of candidate malarial vaccines.

It is then reasonable to look for other approaches to bridge the knowledge gap and to utilise our existing understanding of species differences, the disappointing outcomes of attempts to develop vaccines and human variation, to develop more useful, non-animal methodologies. This paper highlights the fact that malaria represents a rational example as a target field in which, with appropriate investment in the development and validation of alternatives, primate use could be phased out in the short term.
Replacing primates in cognition research
Nicky Gordon

Introduction

Primates, including humans, share many psychological attributes: the importance of social interaction, strong bonds between individuals, high intelligence levels and good problem solving skills [1, 2]. Fundamental to these psychological abilities are learning and memory, which in turn shape our behaviour.

In humans, abnormalities in psychological processes can lead to disorders such as depression, anxiety, schizophrenia and autism, the causes of which are at present very poorly understood [3, 4], prompting a huge volume of research in this area. These disorders are highly complex to study and treat because there is a large amount of individual variation between patients in the course of the disease and in the effects of drug interventions, as well as in the existence of co-morbidities [5, 6].

The similarities between the various species of primates have led to the widespread use of non-human primates in psychological research, however, it is also this similarity which leads to the deep ethical concern over their treatment. The evidence shows that primate species are able to anticipate and influence other individuals’ behaviour [7], communicate both meaning and emotion in their vocalisations and non-verbal communication [8, 9], understand and use abstract symbols [10], mentally represent numbers [11], comprehend cause and effect [12] and practise deception [13]. All these are considered as building blocks for theory of mind, which entails a knowledge of one’s own mental state and that of others. Whether or not other primates possess a theory of mind is contentious, but a vital component of the debate on the ethics of the use of primates in research.

These advanced cognitive abilities mean that as well as suffering from any pain and distress that may be inflicted during an experimental procedure, primates are likely to suffer due to the anticipation of pain to come [14, 15], confinement in a laboratory setting [16] and lack of control over their lives and their social interactions [17].

The Marmoset Predator Confrontation test is used to measure fear

Primate experiments in cognition

Primates are used around the world in psychology experiments to research memory, cognition, learning and social communication. Some of these studies are fundamental biological research; experiments simply to understand more about the primate brain [e.g.18], or they may involve creating primate ‘models’ of human diseases such as schizophrenia and depression [e.g.19]. Other primates are used in the development and testing of new medicines for similar psychological disorders [e.g.20].

Electrophysiology studies using intracellular or field electrodes are commonly used in primate studies of cognition. Electrode recording and stimulation are used to investigate the function of active areas of the brain or individual neurons [21]. Tracer studies are used to investigate the connections between different areas of the brain [22]. Lesioning techniques involving electrodes, aspiration or neurotoxins are also used to localise functions in particular brain areas [23].

Lesioning can identify which brain regions are critical for certain functions, but does not give any temporal information about activity in various brain areas, or about regions which were engaged during a task but are not essential to it. Below are some examples of primate experiments in various areas of cognition research; see the following subsections for non-animal replacements for this research.
In an experiment in Oxford, UK, three male macaques were subjected to behavioural testing for food rewards, before and after they underwent three separate brain surgeries [24]. In the first their left frontal lobe was ablated to remove the entire frontal cortex except the primary motor cortex; in the second their right inferotemporal cortex (a visual area of the brain) was ablated, and this disconnected the frontal and inferotemporal cortex. In the third they suffered a bilateral transection of the fornix, an area of the brain carrying signals from the hippocampus. Unsurprisingly the researchers inferred that episodic memory (in non-human primates) requires the integration of various types of information about visual objects.

In an experiment at Yale University, USA using a licensed pharmaceutical, 10 rhesus macaques were injected intramuscularly with the drug clenbuterol, a beta2 agonist used to treat asthma, to assess whether the drug had any effect on working memory. The monkeys were then tested repetitively on their ability to remember where a food reward had been placed, over a period of 10 months [25]. Some of the monkeys given the highest dose of the drug vomited, and the results were so variable between different individuals that researchers could not generalise about the effects of the drug, even within the small sample of animals involved in the study.

A spatial learning and memory experiment was performed in China on another three macaques where they were forced to navigate around a maze [26]. Monkeys were sedated and had a collar and chain fitted, then when they regained alertness, they were put into the maze 15 times a day for eight weeks in order to learn what the experimenters required of them. They were then tested on their maze skills in up to four mazes 15 times a day to assess how much they could retain in their working memory. The researchers reported that the monkeys made numerous mistakes on their first day in a new maze but very few mistakes subsequently and that they retained the information in their working memory effectively.

Whether experiments such as these are advancing medical progress is doubtful. There are differences between macaques and human in the areas of the brain used for various tasks. For instance, recording and stimulation experiments using electrodes have identified that in the monkey prefrontal cortex, a region centring on the principal sulcus is important in working memory for spatial locations. However, fMRI studies have shown the location of the spatial working memory area in humans is superior and posterior to that in monkeys [27]. Given this difference, it is questionable what transferable information studies of monkey spatial working memory could provide.

Researchers in the USA used primates to study facial expressions in social communication. This involved three macaques undergoing surgery to attach a plastic head post onto their skulls under anaesthetic. After regaining consciousness, they were then made to sit in a sphinx position in a plastic barrel, where their heads were fixed in place using the plastic restraint and they were forced to stare at a computer screen [28]. The location of their gaze was tracked using infrared pupil tracking while they looked at images of monkey faces showing various facial expressions. The monkeys also underwent scanning procedures where they were injected with a chemical contrast agent into their femoral vein and subjected to fMRI scans. The researchers concluded that perception of facial expressions modulates activity in some brain regions, and that their results confirmed fMRI findings in humans [29–33], indicating that scientific knowledge of perception in humans has not been progressed by the primate study, but it was in fact an unnecessary comparative study.

The Marmoset Predator Confrontation Test is used as a measure of fear in primates, as a surrogate for human depression, fear and anxiety in research and drug testing. Researchers in Brazil used marmosets to test whether the animals habituated to the test over time. They placed marmosets in a figure-8 maze for a 20-minute confrontation session with a taxidermised wildcat predator hidden in the maze [34]. Marmosets were dosed with diazepam or the vehicle control and their fear response measured, to study whether there was any long term habituation to the predator. They found that the marmosets did not habituate over time and still exhibited behavioural responses consistent with fear, including alarm calling.

The researchers in several of the papers described above made no attempt to justify how their experiments could be useful or relevant to humans, leaving the reader to question whether knowledge of a non-human primate’s brain is considered by scientists as a useful goal in itself. Proponents of primate research insist that it is necessary for medical progress, but when it would have been equally possible to conduct an ethical human study, this seems an implausible defence.

Although there are similarities between primate species in the structures and functions of the brain, and it is unquestionable that all species share many cognitive functions, it is abundantly clear that many differences also exist. Sometimes
these are simply a matter of degree, but others represent fundamental discontinuities between humans and other primates, and mean that results from primate experiments are not predictive for humans. Examples include differences in gene expression [35], cerebellum size relative to brain size [36], visual processing in the intraparietal sulcus [37] and executive control (the flexibility to adapt to different situations or tasks) [38]. There is also within-species variation which makes it difficult to generalise about disorders and treatments, particularly in the area of abnormal psychology research where individual differences are so paramount.

Non-animal replacements in cognition research

Over the last few decades, human imaging and related technologies have emerged as a cornerstone approach, advancing our understanding of the human brain in health and disease more than any other single method. Before imaging, scientists actually knew more about certain aspects of monkeys’ brains from invasive studies than about human brains. The range of human imaging techniques is impressive: from magnetoencephalography (MEG) and electroencephalography (EEG), through positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), to magnetic resonance spectroscopy (MRS) and diffusion tensor MRI (DTMRI). These techniques, as well as others using direct recordings (see below), can be used singly or in combination to give a highly accurate, relevant and reliable picture of the human brain in health and disease [41]. In cognition research, the additional advantages that human subjects can describe their thought processes and respond to specific verbal instructions are invaluable in driving forward scientific understanding. The rest of this chapter will be used to look at some of the areas of cognition research and consider how primates can be replaced now or in the future.

Human imaging studies are suitable to replace many primate experiments, when conducted with volunteers in an ethical manner. Studies of human perception of facial expressions are performed using fMRI with human volunteers, simply by asking them to view pictures of human faces [42]. No invasive procedures are necessary and the information is highly relevant to the human brain. Human spatial working memory is studied using human volunteers in a maze-like virtual environment combined with fMRI [43], which gives much higher quality information on human working memory than primate studies, and so is both relevant and ethical. Human imaging studies could therefore replace primate experiments in the areas of perception and memory, and more specifically, the maze and face perception experiments described above.

PET is a very useful technique for studying neuropharmacology in depressed patients, with a much greater relevancy than marmoset fear tests. PET studies have revealed that patients with major depression have reduced availability of serotonin transporters in the...
thalamus of the brain. These transporters are membrane proteins that move the neurotransmitter serotonin from the synaptic space back into the presynaptic neurons. Human studies have shown that low serotonin transporter availability correlates with high patient anxiety [44].

A fascinating technique used with epileptic patients enables recordings to be taken from single neurons in conscious volunteers. Patients with intractable epilepsy sometimes undergo elective surgery to remove the affected brain area. During this surgery the patient is conscious in order to guide the surgeon, and some voluntarily participate in studies involving the recording of direct field potentials from the brain. In this way, researchers have undertaken studies of visual processing for episodic memory using direct recordings from the hippocampus. They have discovered that neurons within the hippocampus are directly linked to visual memory performance [45]. Cognition researchers often have a justifiably great interest in the theory of mind, because it is, arguably, the single best indicator of others’, and thereby self, awareness. Historically it was thought that humans were the only species to possess a theory of mind, and there is currently much debate and little agreement about whether other animals, including the remaining great apes, also have this insight [46,47].

Research into the theory of mind has involved creating brain lesions in primates to study the effects on cognition in the three areas of the brain consistently activated in association with theory of mind. These are the anterior paracingulate cortex, the superior temporal sulci and the temporal poles bilaterally [48]. However, imaging studies in human volunteers are highly relevant and can reveal much richer information than invasive primate studies. One study using PET asked volunteers to comprehend a story requiring the attribution of mental states and compared the resulting brain activity to a neutral story [49]. In this way the researchers were able to localise the brain regions involved in the comprehension which included the medial frontal gyrus on the left and the posterior cingulate cortex.

For studies where brain lesions are thought necessary to identify the function of part of the brain, temporary brain lesions can be created safely in human volunteers using transcranial magnetic stimulation (TMS) [50]. This technique creates momentary, fully reversible brain lesions and can replace some lesion studies in primates where the brain region of interest is near the surface. As TMS creates short-lived, reversible lesions it has the added advantages that the brain does not remodel to compensate for the lesion, as can happen in animal studies, and that the same individual can be studied repeatedly prior and subsequent to brain ‘lesioning’ [51].

Fundamental research into brain networks and connections has historically been performed using invasive and ultimately fatal tracer studies in primates. Cutting-edge techniques combining DTMRI with fMRI now enable studies of brain networks in humans. Functional data from fMRI is used to drive detailed tracing of connections using DTMRI [52]. Sophisticated mathematical techniques are then used to analyse the information gained from imaging and deduce interconnected brain regions. The spatial resolution of the fMRI scans is a limiting factor but is improving: just two years ago the resolution was 2mm³ and it has already improved to 1.5mm³. DTMRI is being used with human volunteers to study the brain connections involved in schizophrenia [53].

**Primate cognition studies vs. human volunteers – a question of relevance**

Studies employing direct recording from primates’ brains using invasive methods are still common today, and are often defended on the grounds that they give information at the single cell level, something that human imaging is currently unable to provide. However, it is vital that research is asking the right questions. The question should not be ‘can we record at the single cell level?’ but ‘what is the most useful information we can obtain?’.
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The neural networks involved in most cognitive tasks function at the level of thousands of neurons, so it is not necessary to record from single neurons in order to understand a brain area's function [39]. One of the strengths of imaging and related methods is that they allow a more global, integrated view of the human brain. Imaging also enables study at a resolution of a few millimetres, and with the opportunities to study the species of interest – humans – improving all the time, invasive primate experiments could and should soon become an archaic technique.

Additional scientific advantages of human imaging over invasive primate studies include its speed: a scan takes around one hour, whereas it takes weeks to get microscopic slides made up from invasive animal experiments. Imaging also gives information on the whole brain, albeit coarser information, it can be useful because it gives a more complete picture than narrowly focused single cell studies. Humans are also able to give and comprehend verbal information, making the data much richer than the basic physiological information from primate studies, whose interpretation requires inferences and assumptions which involve uncertainties.

Furthermore, most cognition questions require human-specific information and there is simply no animal surrogate for these studies as animal models do not effectively represent humans. For example, in memory studies, the way that humans use language means that information may be processed in a very different way to processing by other primate species. It is fairly established that chimpanzees have roughly the same working memory capacity as humans, so could remember a maximum of around five-seven items or units. However, in humans those units could consist of letters, words, sentences or entire stories and therefore could differ considerably from the chimpanzee’s ability to recall five to seven items [40]. Imaging provides a way of studying human cognition safely and non-invasively.

Finally, other animals do not generally suffer from human psychological disorders and so are not appropriate surrogates; imaging enables the direct and ethical study of this vulnerable patient group without the need for animal models.

The way forward

There is a wealth of opportunities to replace primates in research and testing today with ethical, relevant, human-based methods. To drive this, there needs to be a shift in the way scientists approach psychology experiments, away from primate 'models' and towards gaining gold-standard human data that are relevant for improving our understanding of the human brain as rapidly and reliably as possible.

To support this fundamental shift in focus, research funders should be willing to invest in sometimes expensive imaging technologies which constantly need to be updated to remain state-of-the-art. This will facilitate a focus on improving the spatial and temporal resolutions of imaging to enable even more accurate study of the human brain.

It is also essential to conduct systematic reviews of the effectiveness of existing primate research in cognition, particularly the suitability of primate 'models' of human diseases. An objective evidence base of this kind, which is likely to identify many shortcomings, is vital to achieving the quickest possible medical advances for patients and their families by establishing the most appropriate research methods.
References


Replacing primate models of stroke
Christine Brock

Introduction

Stroke is a sudden, focal interruption of cerebral blood flow. Loss of blood supply to a part of the brain is known as focal ischaemia. The most common type of stroke is ischaemic (80%-95%) and is usually caused by an embolism (e.g. a clot carried in the blood from elsewhere in the body) or a thrombosis (a blood clot forming in the brain itself). It results in focal infarction, a death of brain cells, causing neurological deficits in accordance with the affected area in the brain. Transient ischaemic attacks are symptomatic for less than an hour and rarely cause brain damage [1].

Haemorrhagic stroke is due to vascular rupture in the brain, usually from an aneurysm (a ballooning of a weak part of an artery). It accounts for around 5% of strokes but 25% of stroke-related deaths [1, 2]. Neurological deficits due to haemorrhage are a result of pressure of a blood mass on surrounding tissues [1].

Neurological symptoms due to stroke are diverse, usually abrupt and may be unilateral or bilateral. Symptoms may include one-sided paralysis, speech disorder, loss of vision, confusion, memory loss, seizures and urinary incontinence [1].

Treatment of stroke includes stabilisation and may involve correction of co-morbidities such as hypertension, pyrexia, hypoxia and dehydration. Drug treatment depends upon the causes and pathological sequelae of the stroke and may include anti-thrombotic therapy such as thrombolytics, anti-coagulants or anti-platelet drugs in ischaemic stroke patients, or drugs to prevent cerebral vasospasm (brain blood vessel spasm) in haemorrhagic stroke victims [1].

Primates in stroke research

Researchers believe that the phylogenetic closeness of non-human primates to humans makes them the animals of choice for stroke research in which behaviour and cognition are assessed [3]. According to the Medical Research Council and the Wellcome Trust [4] the rationale for using monkeys in stroke research is the “structural similarities of monkey and human brains”. There is evidence, however, that primate species including humans use different cognitive processes to perform a similar task [3] and that their pharmacokinetic responses also vary [5].

It is the similarities between non-human primates and humans that make the use of monkeys in research particularly unethical. They have complex social structures and experience emotions and stress as humans do. Stroke experiments involve brain surgery with associated post-surgical effects. They are sometimes conducted on conscious monkeys [6], for which monkeys may be restrained in primate chairs. They may be similarly restrained for neurological testing following experimental stroke [7]. Their intelligence and self-awareness would result in such a situation causing them extreme stress.

Marmosets; cynomolgus, Japanese and rhesus macaques; squirrel monkeys and baboons are commonly used in stroke research. The most common approaches to induction of experimental stroke in primates are middle cerebral artery occlusion (MCAO) or occlusion of the internal carotid artery (ICA) by numerous and varied methods.

Vessels may be clipped, cut, or occluded by balloon [8]. Surgical techniques include removal of part of the skull (craniectomy) over the lateral cerebral cortex with permanent MCAO [9] and using a high-speed drill to enter the skull through the eye socket (trans-orbital approach). The latter technique necessitates removal of the eye and cutting of the optic nerve. The membranes covering
the brain are surgically opened and a balloon occlusion device is placed around the middle cerebral artery (MCA) and fixed in position with the end of the device tunneled under the scalp – to be used when the monkey is awake. The empty eye socket is sewn up [8].

In primate models of haemorrhagic stroke to study cerebral vasospasm, a subarachnoid haemorrhage (SAH) is induced over the surface of the brain, beneath the arachnoid layer in the space where blood vessels supplying the brain lie. Autologous (from the animal’s own body) blood clots are placed around branches of the cerebral and carotid arteries concurrently. Access to the arteries is via craniectomy on one side (e.g. fronto-temporal part of the skull); opening the dura (outer membrane) covering the brain and parting the brain lobes at a connecting fissure [10].

Primate models of stroke are used mainly for efficacy testing of putative neuroprotectants and anti-vasospasm drugs in ischaemic stroke and SAH respectively; for basic biomedical research into stroke; and toxicity testing of stroke drugs. Basic research includes studying biological and physical changes in response to ischaemic stroke [8].

Testing putative drugs for stroke

The majority of procedures for efficacy testing of drug candidates for ischaemic stroke employ similar protocols. In the UK, for example, stroke is induced in marmosets (Callithrix jacchus) by blocking off the MCA on one side of the brain, following intensive training and then assessment of cognitive and motor task performance by the monkeys. After a short recovery period a neuroprotectant drug is administered to half the marmosets (with the other half used as a control group) in order to assess its efficacy. Task performance is re-assessed in both groups [4].

There have been at least 1,009 animal-tested putative stroke drug candidates. Although many of these were tested in rats and rabbits rather than primates, it has not been established that primate models are superior [11]. Of these, around 97 neuroprotectants for ischaemic stroke have been both tested in animals and have reached clinical trials [12], yet studies suggest that only two such drugs are clearly efficacious in humans. These are aspirin and tissue plasminogen activator (tPA) [11, 12].

Phase III clinical trials of AstraZeneca’s disufenton sodium (NXY-059) [13], while initially promising, have also been deemed a failure due to lack of efficacy in a repeat trial, leading the trial investigators to question the validity of animal experiments in stroke research [14]. The drug demonstrated unequivocal neuroprotective efficacy in marmosets that had undergone surgical MCAO [15]. According to an editorial in The Lancet [16], NXY-059 had seemed a promising treatment for deliberately induced strokes in rats and marmosets but it failed in patients. The editorial advised: “Translation of positive results obtained in the laboratory into the clinic has been exceptionally elusive, and the stroke [research] community needs to think long and hard about whether these animal models are financially and ethically viable”.

A systematic review by Perel et al [17] examining concordance between treatment effects in animal experiments and clinical trials, found the drug tirilazad, a lipid peroxidation inhibitor [18] improved outcome in animal models of acute ischaemic stroke, including 29% reduction in infarct size and a 48% improvement in neurobehavioural scores. However it was associated with increased risk of death in human stroke patients. Although it is not clear whether the review included primate research, tirilazad significantly reduced infarct volume in baboons three hours after MCAO [18, 19].

It is noteworthy that a meta-analysis of clinical efficacy studies of tirilazad for prevention of vasospasm in SAH patients indicated no improvement in patient outcome [2]. The review by Perel et al [17] also found that although thrombolysis with tPA demonstrated positive benefit both in ischaemic stroke patients and animal models, the animal experiments were of generally poor quality with publication bias and overstated efficacy.

Examples of stroke experiments conducted on primates

**Ischaemic stroke**

*Macaca fascicularis*

Researchers from Shin Biomedical laboratories and Osaka City University School of Medicine in Japan, used male cynomolgus macaques imported from a Chinese breeding facility, to test the efficacy of ginsenoside Rb1 (a saponin from the root of Panax ginseng) as a neuroprotectant following thromboembolic stroke [20].

In order to determine the neuroprotectant effects of ginsenoside Rb1 (GRb1) on delayed and acute ischaemia two experiments were conducted. In the first, to assess the
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Effects of GRb1 on acute ischaemia, thromboembolic stroke was induced in 18 monkeys by injection of an autologous blood clot via a catheter, chronically implanted into the left ICA two days previously under general anaesthesia. Four animals were injected with GRb1 (300 µg/kg) once daily for seven days prior to, and one day following, embolisation. Another group (n = 5) was injected with GRb1 from two days before until seven days after embolisation, with the rest of the monkeys acting as controls.

The monkeys were neurologically tested for deficits in consciousness, muscle coordination and the sensory system, at various time points following embolisation. GRb1 was considered to be neuroprotective in the pre-treated group with some amelioration of neurological deficits in the post-treatment group. On day 7 following embolisation the monkeys were killed under pentobarbital anaesthesia and their brains were removed for study of the infarcts [20].

In the second experiment, conducted to examine the effects of GRb1 on progressive brain injury following brain ischaemia, monkeys were divided into four groups. Six monkeys underwent sham surgery, five underwent saline pre-treatment, seven underwent saline post-treatment and five had pre-GRb1 treatment. The saline and GRb1 groups were injected with blood clots as in the first experiment. Twenty-four hours or seven days after embolisation all monkeys were killed. GRb1 was deemed to demonstrate a protective effect on the monkey brain at 24 hours following embolisation [20].

It should be noted that P. ginseng is traditionally considered a neuroprotectant in humans when taken orally as whole root powder or tincture and it is unlikely that the intact ginsenosides would normally be bioavailable for prophylaxis. Furthermore, it contains a large number of ginsenosides which may be acting in synergy [21]. Injecting a single saponin into an animal is not relevant to determining the efficacy of a plant in humans. Also, considering that stroke cannot be pre-determined, acute pre-treatment for stroke is of little value.

The lifespan of macaques in captivity can be as much as 38 years and the animals used in these experiments were young (five to seven years-old), and presumably healthy, unlike most stroke patients.

Squirrel monkeys (Saimiri spp.)
This species is favoured by some stroke researchers because its relatively flat, unfissured frontal cortex allows direct access to this part of the brain [9].

To assess the neurological mechanisms behind d-amphetamine (d-AMPH) as an adjuvant therapy in stroke, cortical infarct was induced in squirrel monkeys at the University of Kansas Medical Centre [22].

Artificial stroke is induced in squirrel monkeys in laboratories in the USA

Six male and six female adult monkeys were randomly assigned to three groups: a spontaneous recovery group (no treatment or post-infarct training), a training group receiving saline vehicle and a training group receiving d-AMPH. For determination of baseline hand preference and flexion/retrieval prior to infarction, all monkeys were taught to retrieve food pellets from wells attached to their cages [22]. A few days later, the area of the brain in the primary motor cortex relative to each monkey's preferred hand and surrounding areas, including shoulder and face, was identified by microstimulation (with electrodes). Each monkey received a complete infarct by cauterisation of the blood vessels in that area of the brain. The surgical procedure lasted 15-20 hours and involved a craniectomy under anaesthetic in the frontal cortex area relative to the monkey's preferred hand, so the dura could be removed from this area. For electrostimulation the exposed area was probed with a glass micropipette filled with a sodium chloride solution until movement was detected in the monkey's hand. After removal of the
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Common marmosets (Callithrix jacchus)
According to an electronic database search there has been no published UK research involving primate models of stroke since 2004. The Home Office website also suggests there are no ongoing project licences for stroke research in marmosets. It is hoped that UK investigators are realising that primate research in this area is futile, unethical and should cease and that others, internationally, will follow their lead.

A recently published study [25] conducted at the University of Caen, France, used 10 marmosets to develop a stroke model using an intra-luminal approach to MCAO. The animals were divided into two groups and underwent either transient or permanent ischaemia. Ischaemia was induced by insertion of a nylon filament into the external carotid artery which was pushed up to the MCA. Behavioural testing and magnetic resonance imaging (MRI) were carried out. The marmosets were killed eight days following MCAO for removal and examination of their brains.

Baboons (Papio anubis)
Complement, a group of proteins in the blood which play a pivotal role in the immune response, is believed to exacerbate neuronal injury following ischaemic stroke. Therefore a complement inhibitor, sCR1-sLex, was assessed as a potential clinical neuroprotective drug in a baboon stroke model. Twenty-four adult male baboons were used for the experiment, which took place at Columbia University, New York [26].

Rhesus macaques (Macaca mulatta)
Eight adolescent rhesus macaques underwent MCAO. The procedures were carried out in order to develop a primate model for use with a new type of balloon catheter. The investigators at Qingdao Medical College in China had invented the catheter using a method developed in rats, with a view to flexibility in inflicting either reversible or permanent MCAO. The catheter had also been tested in formalin-fixed monkey brains but it is unclear how many monkeys were killed for these tests [7].

Thirteen monkeys were used for the study, including five sham controls (surgery but no MCAO). All monkeys were given a general anaesthetic and then the branches of the carotid artery and the vagus nerve were separated and three of these arteries were tied. The balloon catheter was inserted into the ICA and advanced to the MCA and the external carotid artery was tied. Next, the MCA was occluded for two hours by inflation of the catheter balloon. Local cerebral blood flow was monitored continuously by Doppler flowmetry, with the head of the laser fibre placed into the ischaemic area of the brain. At three and 23 hours following reperfusion, neurological testing was carried out with the monkeys restrained in a primate chair and undergoing EEG monitoring. Twenty-four hours after reperfusion the monkeys were killed for removal of their brains for study [7].

The investigators suggested that inflation of the balloon catheter is best carried out whilst the monkeys are awake and restrained in a primate chair to avoid the inevitable effects of anaesthesia [7]. Such a practice would cause these intelligent animals a high degree of stress, which would also affect results.
Baboons were anaesthetised and their heads were positioned in a frame for transorbital surgery. Animals were randomly assigned either sCR1-sLe^+ or vehicle, administered by intravenous drip. After 45 minutes transorbital craniectomy was performed on the animals with a pneumatic drill, to expose the circle of Willis arteries in the brain. To induce ischaemic stroke, aneurysm clips were placed on the cerebral arteries for 75 minutes [26].

The animals underwent an MRI scan (under ketamine sedation) to determine infarct volume on day 3 following surgery. All underwent neurological testing until day 10 when they were killed by a pentobarbital overdose. Those considered so severely brain-damaged that they were no longer “self-caring” had to be killed after the MRI scan [26]. Physiologic parameters of only three animals from each group were ultimately evaluated. It was discovered that sCR1-sLe^+ treated animals experienced low blood pressure. Also the infarct area in the treated group was greater than that in the vehicle group. The drug was therefore deemed unsuitable for clinical trials [26]. However the baboon experiment may not have indicated potential human response accurately.

Haemorrhagic stroke
Perhaps reflecting the difference in occurrence of the two types of stroke, very few SAH-vasospasm studies appear to have been conducted on primates. The following experiments were conducted in the USA:

Cerebral blood flow and cerebral blood flow velocity were compared by transcranial Doppler sonography (TCD) in two groups of cynomolgus monkeys. One group had SAH induced by autologous blood clot, which resulted in vasospasm, and the other group was used as a control [27].

To see if vasospasm in a cerebral blood vessel following an experimentally-induced SAH could be prevented by boosting local nitric oxide, 10 cynomolgus monkeys were divided into test and control groups. All monkeys underwent induction of experimental SAH under general anaesthesia. An incision was made on the right side of their heads, tissues were elevated and a 3 cm by 2.5 cm craniectomy was made with a high-speed drill. An incision was then made in the dura and an autologous blood clot was placed around the cerebral arteries. A vinyl polymer attached to the vessel during surgery delivered nitric oxide to one group and nothing to the other. The dura, muscle and skin were sewn up and the monkeys were allowed to recover. Following surgery the animals in the treatment group exhibited varying degrees of disordered movement and one monkey died after two days. All the other monkeys underwent angiography and were killed seven days after surgery [28].

An in vitro study of cellular mechanisms and pharmacology of a cerebrospinal fluid chemical, believed to instigate delayed vasospasm following SAH, was followed by experiments on 12 cynomolgus monkeys. They were divided into two groups and administered either drug or placebo prior to surgery to induce experimental SAH (as above). The aim was to investigate in vivo the effects of a pharmaceutical (probucol, a cholesterol-lowering drug) that had inhibited the chemical in vitro. All animals developed vasospasm and the drug had no beneficial effects. The results of the primate experiments did not correlate with the in vitro studies on human cells [29].

Critique of stroke research using primates
Differences in pathophysiology between primates used in stroke research and human stroke patients are likely to be reflected in the outcome of an experimental procedure. A confounding factor, for example, is that it is unlikely that monkeys would have similar co-morbidities to humans [30]. For instance, a person with a stroke may have altered blood biochemistry or impaired liver function as a result of medication for treatment of diseases that are known risk factors for stroke. Such diseases include hypercholesterolaemia, hypertension and diabetes mellitus [1].

Other risk factors for ischaemic stroke in humans and not present in experimental primates include cigarette smoking, recreational drug use (e.g. amphetamines, cocaine), hypercoagulability states and long-term heart disease leading to atrial fibrillation. Many of these variables may affect the ischaemic process in humans [31]. In primate models of stroke there is no gradual thrombus formation or embolus as a result of atheromatous plaque as may be the case in humans. It appears obvious therefore that it is not possible to replicate a stroke in a (usually elderly) patient by inflicting focal cerebral ischaemia in an otherwise healthy animal.

Although the major risk factors for haemorrhagic stroke differ from those of ischaemic stroke, with high alcohol consumption and cigarette smoking being the most important, they are nonetheless risk factors not seen in non-human primates [2]. Furthermore, induction of SAH by placing blood clots around arteries in a monkey is not the same as human aneurysmal rupture and bleed.
Species differences between primates and humans are likely to create intrinsic differences in pharmacokinetics and pharmacodynamics following administration of a pharmaceutical compound. Pharmacokinetics – or absorption, distribution, metabolism and excretion (ADME) – are likely to be influenced by differences in genes encoding for the cytochrome P450 series (CYP), the biotransformation and elimination enzymes found in virtually all organs.

Even minor differences between species in amino acid sequences in CYP enzymes (isoforms) can result in major differences in CYP activity in relation to substrate specificity and catalytic activity. Furthermore, certain CYP isoforms are induced or inhibited by certain drugs in monkeys but not in humans, and vice versa [5]. These differences will affect bioavailability and pharmacodynamic responses.

Another possible factor in the failure of primate stroke research is the necessary use of general anaesthesia, which alters brain metabolism. Attempts to conduct experiments without general anaesthesia inflict severe stress, which introduces other confounding variables such as increased stress hormone output [31].

It is without question that nearly 170 years of stroke research in animals, including primates, has failed to produce a safe, efficacious therapy for ischaemic stroke. The use of aspirin for acute stroke was not developed in animal models [32]. Despite their assumed similarity to humans, the value of primate models in stroke research remains dubious. It is therefore appropriate for more funding to be available for the development of research methods that are relevant to human stroke patients, not only in the hunt for novel stroke drugs but in all aspects of stroke research. Perhaps only then will therapies for stroke be clinically effective.

**Alternatives to animal models in stroke research**

An early editorial in Stroke [31] proposed that, due to the failure of animal models in stroke research, rather than continually repeating such experiments there is more potential in the development of techniques that enable in-depth study of stroke in humans. This would include studying basic metabolism and molecular mechanisms of the pathophysiology of stroke and advancing ischaemic stroke imaging techniques. Ischaemic stroke is, after all, a uniquely human affliction [8].

**Functional magnetic resonance imaging (fMRI)**

Functional MRI is being used at the Wellcome Department of Imaging Neuroscience to examine the process of cerebral re-organisation and its relationship to recovery of function in human subjects [33].

To determine the mechanisms of neuronal reorganisation in the brain and likelihood of post-stroke recovery, fMRI was used to demonstrate multiple motor nerve tracts in the brains of human volunteers [34]. The neuronal pathways from motor areas to the base of the brain were mapped out in 12 healthy people during hand-grip sessions. By comparison it was possible to infer damage to these connections in three stroke patients with cortical white matter damage. Similar neuronal mapping of hand function had been carried out previously by others on rhesus monkeys [35] using invasive techniques involving craniotomy and insertion of electrodes and tracer dye injections into the brain.

**Quantitative structure-activity relationships (QSAR)**

This computer-aided drug design technique can potentially predict the activities of new drugs by quantifying the relationship between a compound’s physical, chemical and biological activities. Various parameters such as its toxicological and ADME profiles can be pre-determined in accordance with the unique molecular structure of a compound. As the body of molecular data increases so will the ability of QSAR to predict properties of new compounds [36].

QSARs have already been used with a view to developing neuroprotectants. For example, using human neuroblastoma cells in an in vitro model of ischaemia, researchers in South Korea tested the neuroprotective activity of 13 plant compounds. They then developed a QSAR model, eliciting three-dimensional structures of the compounds, to predict their neuroprotective behaviour [37].

**Human microdosing**

Many stroke drug candidates that appear promising following in vitro and animal studies fail to show sufficient safety during clinical trials and never reach the market. There are even racial and gender differences in pharmacokinetics and pharmacodynamics, not to mention drug-drug interactions [38]. These widely recognised problems have stimulated increasing interest in the development of technology that can help bypass preclinical testing, while shortening the time it takes for a drug to reach clinical use.
Microdosing involves the administration of a minute amount of a pharmacological substance to a human. The European Medicines Agency defines a microdose as 1/100th of a dose of a pharmaceutical calculated to produce a pharmacological effect and not exceeding 100 µg. Sensitive analytical techniques such as accelerator mass spectrometry (AMS) or positron emission tomography (PET) are employed to assess the pharmacologic profile of a drug following dosing [39]. Microdoses can be delivered by any route (e.g. orally or intravenously).

Based on the knowledge that the best model for human drug development is the human, Xceleron, a leading company using this technology, asserts that microdosing ‘de-risks’ drug development [40]. Primates are often used for testing the ADME characteristics of new drugs at a fairly late stage of development prior to clinical trials. Human microdosing can help prevent novel drug candidates from failing later in the development and testing process, by providing human data early on and so identifying inappropriate drug candidates before they reach the stage of primate testing. It could potentially spare many primates from pre-clinical testing.

Proteomics and genomics

Proteomics looks at many proteins at the same time and can identify tissue markers for stroke, such as those involved in inflammation. Genomics identifies cell-cycle gene pathways in disease by looking at the gene products. Used together the techniques can be applied to the structural, developmental and functional relationships of nerve and vascular cells in the brain, i.e. the ‘neurovascular unit’. They can therefore be used to provide insights into predetermining drug responses and development of stroke drug candidates by, for example, identifying neurotoxic and neuroprotective pathways [41, 42, 43]. Proteomics and genomics can be used alongside other non-animal techniques e.g. in vitro to provide additional information.

In vitro stroke studies

Although in vitro testing in cell cultures alone cannot mimic the complexity of the neurobehavioural deficits associated with stroke, it can provide a sound adjunct to other non-animal experimental approaches in stroke research.

Of particular value in addressing the molecular and mechanistic paradigms of stroke are the three-dimensional co-cultures using human cells and human brain slice models. In the co-culture system, the different cells of the neurovascular unit, consisting of glial cells (nervous system supporting cells), neurons and vascular cells are cultured together [43].

Anoxic and ischaemic damage have been studied in human brain slices. Marcoli et al [44] induced hypoxic ischaemia in brain slices obtained from 18 male and female patients undergoing neurosurgery. As glutamate efflux from neurons is an early event in nerve damage following ischaemia, the purpose of the experiment was to understand the efflux mechanism and to determine whether inhibition of glutamate could have neuroprotective effects in the human brain. Ischaemia was induced by glucose- and oxygen-deprivation. The experiment provided insights into a putative neuroprotectant [44].

Bicker et al [45] have developed a cell culture model to investigate hypoxic-ischaemic cell damage and for studying the effects of neuroprotective drugs. Using a human teratocarcinoma cell line (NT-2) they induced the cells to differentiate into neurons by treating them with retinoic acid. Anoxia was created in the neurons by keeping them in an atmosphere of argon and carbon dioxide. Cell injury and the production of free radicals were induced by the presence of glutamate, released from neurons when they become ischaemic.

The team, at the University of Veterinary Medicine in Hannover, Germany, had previously demonstrated protection of NT-2 neurons against ischaemic damage by a licensed cardiovascular drug, diltiazem. This cell line is particularly suitable for screening neuroprotective drugs as the cells are derived from humans and not animals. The team believe this system has the potential to replace some animal models in basic stroke research [45].

Vasospasm is the leading cause of death from haemorrhagic stroke but its aetiology is unknown. Loftspring et al [46] developed an in vitro model of aneurysmal subarachnoid haemorrhage (aSAH) by incubating blood from healthy volunteers with cerebrospinal fluid from hydrocephalus patients, plus an enzyme (HO-1) to liberate and metabolise the red blood cell pigment haem. The model was used to investigate the hypothesis that vasospasm occurs as a result of oxidation of bilirubin, a pigment produced from haem following lysis of red blood cells, and it could replace some primate SAH models.

Glutamate plays an important role in brain function but is toxic to nerve cells if its concentration is too high, as occurs in ischaemic stroke. Astrocytes are cells in the
A number of observational epidemiologic studies support the hypothesis that anti-oxidants, such as carotenoids, flavonoids, vitamin C and vitamin E found in plant materials, exert a protective effect against cardiovascular disease. Anti-oxidants may help prevent oxidation of low density lipoprotein, therefore preventing atherosclerotic plaque formation with risk of associated thrombi [52]. These clinical studies can provide valuable insights into human stroke.

**Conclusions**

It is evident that primate models of stroke are unethical and have proved to be scientifically unsound. Despite decades of using primates in stroke research there has been no demonstrable advance in our understanding of best practice in therapeutic intervention for this common and devastating human condition.

A deeper understanding of human cerebrovascular disease can only be developed through investigational approaches that are relevant to humans. This obviates the use of non-human animals of any species and necessitates a battery of diverse techniques, encompassing clinical, in vitro, analytical, imaging, mathematical and human pharmacological approaches, as outlined above.
References


Replacing primates in the search for an AIDS vaccine
Gemma Buckland

Introduction

A disease first reported in 1981 amongst a group of homosexual men in Los Angeles became known as Acquired Immune Deficiency Syndrome (AIDS) and in 1983 the retrovirus responsible for this illness was found to be Human Immunodeficiency Virus (HIV) [1]. In 2007 there were 33.2 million people living with HIV, 2.5 million people had been newly infected and 2.1 million people died. While most people with HIV live in sub-Saharan Africa, since 2001 eastern Europe and central Asia have seen a 150% increase in incidence [2].

Since its discovery, 25 years of research have failed to yield an effective vaccine against human AIDS. In 2007, Merck’s large-scale clinical trial of an HIV vaccine was halted because it actually boosted the risk of infection in some patients [3, 4]. There is a need to show the public that progress is being made but the routes currently being pursued are proving highly unsuccessful. The complexity of the virus, including the rapid mutational ability used to escape immune attack, has hindered vaccine development; but also the continued use of primates as a failing and misleading model for the disease contributes hugely to the delays and disappointments so far.

Despite this, an increase in primate research is likely to be seen in the United States, as Anthony Fauci, the director of the National Institute of Allergy and Infectious Diseases (NIAID), announced the primate model will have a resurgence of interest to answer fundamental questions about the virus and form part of the new HIV-initiative of transmission research [5].

This chapter addresses the use of primates in AIDS vaccine research, highlighting the need for a shift in focus away from primate experiments towards techniques, increasingly being explored by researchers, which offer greater relevance and reliability.

AIDS and vaccine research

HIV is a retrovirus which attacks CD4 cells, also known as helper T-cells, responsible for coordinating the immune response. The virus then replicates itself by integrating into the host cell DNA. In the blood of infected people there is a steady fight between viral replication and the production of new CD4 cells to replace those that are lost by the virus. If left untreated, HIV will eventually win the battle and the immune system will collapse.

Of the two main strains, HIV-1 and -2, HIV-1 remains the most common and most virulent. However, there are now many different subtypes of the virus in different parts of the world, which vary genetically from each other by 30-35% [6]. This poses serious problems in vaccine development, since a vaccine which may be effective against one type of HIV may not protect against other types.

It takes on average nine years before a person who has become infected with HIV begins to feel symptoms and can be said to have developed AIDS. Once AIDS has started, opportunistic infections, such as pneumonia and TB, will eventually cause the death of a patient. It is unknown why...
it takes so long for HIV to progress to AIDS, but it is known from human studies that during this time the virus ‘hides’ in the lymph nodes of patients causing structural changes [1, 7].

The most successful treatment for HIV currently available is called highly-active anti-retroviral therapy or HAART, which uses cocktails of drugs that can tip the balance in the blood in favour of the immune system. Protease inhibitors, fusion inhibitors, nucleotide and non-nucleotide analogue reverse transcriptase inhibitors are the four types of drug used. These are not available to all HIV-positive people around the world because of their cost. Also, there are serious side effects to treatment, including liver disease [8, 9].

The vast majority of CD4 cells, some 94%, are in the mucosal tissues, probably because pathogens enter the body through these tissues. Only about 2% of CD4 cells circulate in the blood. The number of T-cells in the gut falls by 60% after the first few weeks of infection, and HAART does little to reverse this damage. Some of the T-cells self-destruct and some are killed by other T-cells. An effective AIDS vaccine will have to trigger ‘mucosal’ immunity in the gut lining, in addition to immunity in the blood and ideally, also stimulate antibodies capable of knocking out the virus before it enters CD4 cells as well as directing T-cells to destroy infected cells [10, 11].

Despite initial failures to infect chimpanzees, mice, rats, hamsters, guinea pigs and rabbits with HIV-1, scientists persisted and turned to other species such as gibbons, macaque monkeys, baboons, sooty mangabeys and genetically modified animals including rabbits and mice [12]. Researchers injected animals with human infected blood, artificially suppressing their immune systems and even injecting human HIV-infected brain tissue directly into their brains [13, 14, 15].

The currently favoured model for the disease is the rhesus macaque monkey. Rhesus macaques can be experimentally infected with various simian immunodeficiency virus strains of differing virulence, many of which cause simian AIDS. However, because HIV-1 does not productively infect macaques, it cannot be used as a challenge virus to assess whether a given vaccine can prevent or ameliorate infection. Hence, pre-clinical AIDS vaccine studies rarely test the identical vaccine constructs that are planned for human use. In efforts to increase the relevance of the macaque ‘model’ to human vaccine trials. But SHIV still differs in important ways from the HIV-1 strain; for example, it may be more manageable than HIV for the immune system to control [11].

Two-thirds of the UK scientific community working on developing an AIDS vaccine believe that a vaccine will not be developed within the next 10 years (after a survey by The Independent newspaper [4]) and therefore this poses a serious need for a reassessment of current AIDS research strategies and how the research is focused and continued from now on.

The use of primates

The failures both of the recent vaccine trials and of much AIDS vaccine research have been unexpected to some in the science community, as results in primates had appeared promising. However results have continually been non-transferable between these species and humans. In addition to the scientific criticisms of primate usage in the quest for an AIDS vaccine there are also ethical reasons that make research on these animals inappropriate.

Current vaccine research involving primates includes both fundamental HIV/AIDS research and the testing of candidate vaccines for toxicity and immune reactions. HIV pathogenesis and viral behaviour are studied in primates infected with the virus [16]. In post-mortem conditions and on live animals, primates are regularly used to provide samples of infected blood and are also used in other areas of HIV prevention research such as the study of protective gels [17]. The majority of primate work, however, is focused on the testing of HIV candidate vaccines to assess the immune response and detect the likely success of the vaccine for treatment potential in human patients.

Scientific limitations

A basic, important difference between humans and non-human primates is that although monkeys can be infected with HIV, they do not develop AIDS [6]. There are species differences between primates, including humans, in immune system function. The immune systems of non-human primates react only in a minor way to HIV infection. HIV also multiplies less actively in non-human primates. The simian form of the HIV virus (SIV) also varies from the human form; there are differences in how the two viruses are transmitted and their mode of action [18, 19]. The human virus HIV-1 can infect cells using several cellular receptors (such as CCR5 and CXCR4 as well as CD4) but most...
strains of SIV cannot use CXCR4 receptors [6]. The critically important ‘envelope’ proteins in the outer shell of the viruses are different in HIV and SIV. For example, in HIV-1 the important V3 loop is enormously variable, but does prompt an antibody response in humans. However, in SIV the V3 loop is more consistent and does not generate effective antibody activity in monkeys. Vaccines based on virus envelope proteins appear to activate the immune systems of monkeys, but have largely failed to prevent disease in humans [1, 11].

SIV-viral accessory proteins are not found in HIV, and even with the creation of the SHIV hybrid viruses that include human accessory proteins, differences still remain and form a stumbling block for research progression. Variations in genetics between humans and other primates are also involved. It is now known that non-human primate cells contain a protein called TRIM-5a which stops the virus from replicating [20]. Humans do not have this protein.

These differences demonstrate why SIV is a poor model for HIV and AIDS studies and this view is shared amongst researchers: “...efficacy of HIV-1 based vaccines cannot be directly evaluated in the SIV model”, according to Dr Hu, a researcher at the Washington National Primate Research Centre [21].

When infected monkeys do become ill their symptoms are not always the same as humans: for example, monkeys do not get the characteristic Kaposi’s sarcoma [22]. Additionally, viral infection and disease development are affected by a combination of features of the host individuals, the virulence of the virus and environmental factors. None of these are the same in humans as in monkeys artificially infected with a different virus in the laboratory.

By the year 2000, more than 6,000 healthy volunteers had participated in 60 different phase I or II trials of 30 different candidate AIDS vaccines [23]. By 2006, this had risen to more than 35 candidate vaccines tested in phase I or II trials, involving 10,000 volunteers; and two phase III trials were completed, involving an additional 7,500 volunteers [24]. All these clinical trials, and subsequently the Merck vaccine study, failed. Repeatedly, experimental vaccines have been found to prevent infection in monkeys but been insufficiently effective in humans [3]. There have also been candidate vaccines tested in primates that failed before the clinical trial stage. Despite the failures of animal-based research, monkeys are still subjected to invasive, lengthy and terminal AIDS experiments [25].

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A focus on replacements

Many new and exciting non-animal techniques and also more classical methods are now being harnessed to provide insight into HIV and AIDS. A greater focus on these humane research approaches, and a redirection of funding away from primate experiments towards these, may present the most promising ways of gaining fundamental insights into AIDS and developing effective vaccines and medical treatments. Indeed, the most crucial breakthroughs in understanding and treating HIV infection and AIDS have come from studies not involving laboratory animals.

Population studies

Human population studies (epidemiology) continue to inform policy makers and doctors on preventative programmes, modes of viral transmission, drug efficacy and risk factors [26, 27, 28]. Using the large-scale population databases of HIV sequences and the approach of molecular phylogenetics, estimations have been made on the extent of ‘clustering’, or grouping of similar data sets, in HIV transmission – a key issue in the epidemiology of sexually transmitted infections [28].

A large research coalition is screening the genetic make-up of hundreds of HIV sufferers in order to identify patterns that might explain why some people succumb to the virus more easily than others [29].
Clinical research – In vivo and ex vivo

Unlike experiments with primates, the study of healthy and HIV-infected volunteers and their tissues is the gold standard approach to understanding HIV and AIDS and to developing a vaccine. This is because the approach uses the relevant species (humans) and the relevant sub-populations of the species, some of whom are infected with the virus of interest and experience the illness under research.

A recent study of HIV patients revealed that the production of chemokines (messanger molecules) was able to delay and even block onset of AIDS [30, 31, 32]. The activation state and compartmentalisation of different dendritic cells during HIV-1 infection has been poorly understood. Some studies have used dendritic cells, which function as antigen-presenting cells in the immune system, taken from infected patients’ blood samples. Using these cells and ex vivo lymphoid tissue, test-tube studies revealed that activation and depletion of dendritic cells in blood, with accumulation in the lymphoid tissue, may contribute to HIV-associated chronic immune activation and T-cell dysfunction [33].

Studies using human lymphoid tissue, acutely infected ex vivo with HIV, were used to examine the effects of HIV on chemical messengers called cytokines. The lymphoid tissues were either cultured in vitro as blocks or as aggregate cultures of tonsil and lymph node cells. This research demonstrated the ability of some cytokines to enhance HIV replication in certain clinical settings, which may be a mechanism HIV relies on during acute infection [32]. Further human donor studies made use of colorectal tissue explants and cultured them to develop a useful screening tool to determine the effectiveness and toxicity of microbial gels in preventing HIV transmission [33].

A robotic machine called Akubio that analyses samples with sound waves is helping scientists at St George’s Hospital, London, to develop vaccines for HIV. The Akubio detects sound waves emitted by vibrating crystals, known as resonant acoustic profiling (RAP), to examine how molecules interact in samples such as serum, urine and cell cultures. It enables scientists to analyse molecule interactions, strength of binding, and quantities of active molecules. The new robotic machine can analyse hundreds of samples automatically and is currently being used to examine the immunoglobulin composition of HIV-positive patients, (immunoglobulins are antibodies expressed to ward off infections) [34].

Resonant acoustic profiling is also being used at St George's Hospital in multiplex assays to measure the immunogenicity of candidate vaccines in phase I HIV vaccine trials. Finding and using reliable biomarkers of immune response to candidate vaccines in human volunteer studies offers a more reliable way of assessing potential HIV vaccines than tests on primates.

An interesting article raised issues of medical ethics in vaccine development in the Journal of Medical Ethics after a group of activist clinicians offered to volunteer for clinical trials of live attenuated HIV vaccines. The generous, yet provocative offer explored complex issues about process in research ethics, risk-benefit analysis, consent, volunteering, disclosure as well as the implications of event failure [35].

Tissue, cell and molecular research

The most successful drugs to date, the protease inhibitors, were initially developed by non-animal molecular and cell techniques [36, 37]. These included using human infected cells in vitro which can further be used to understand drug resistance and drug interactions. Genetic techniques can reveal how the virus mutates and molecular techniques, such as polymerase chain reaction (PCR), have already helped to find signs of the presence of HIV in various tissues [7]. Indeed it was molecular methods that originally revealed how HIV gets into cells and how it is structured.

Researchers in Paris have used real-time imaging techniques to monitor the virus-host interaction of HIV in human cells [38]. By tagging the virus with a fluorescent compound, the movement of HIV within cells and its integration into host DNA has been tracked in three dimensions over time (4D), with the kinetics of the virus being observed by automated particle tracking. This work gave new insights into the movements of HIV-1 complexes within infected cells and provides a useful tool for the study of virus-host cell interactions during infection.

Langerhans cells are dendritic cells which, on infection of the skin, take up and process viral antigens and become fully-functional antigen-presenting cells. Langerhans cells are abundant in the epidermis of the skin. They are thought to be likely targets for HIV following sexual exposure to virus and may provide a means for the virus to gain entry to lymph node T-cells. Researchers co-cultured HIV-infected Langerhans cells with different subsets of CD4 T-cells, and used multicolour flow cytometric analyses to reveal the T-cell preference of infected Langerhans cells.
They successfully demonstrated that close interactions between Langerhans cells and specific subsets of CD4 T-cells are important for optimal HIV replication. A disruption of this cluster formation may be a novel strategy to interfere with sexual transmission [39].

The University of Edinburgh, in collaboration with EPiVax of the United States, has used a multiplatform in silico/in vitro approach including highly sensitive ELISpot (enzyme-linked immunosorbent spot) assays to select and evaluate a broad range of stable HIV epitopes as potential vaccine candidates [40]. These epitopes are peptides from the invading virus that are recognised by the immune system, and comprise stable elements in the otherwise rapidly mutating HIV genome. It is known that genetic variants of MHC I molecules (the structures on cells that recognise and display antigens), in particular MHC-B7, in humans determine the susceptibility to HIV infection. Therefore discovering the epitopes of HIV for these molecules presents a potential target for treatment. The most immune response-provocative epitopes discovered will go forward and be included in a multi-epitope vaccine.

A very promising and innovative technique touted as a “clinical trial in a test tube” has been developed by VaxDesign in the United States. Using white blood cells from human donors, VaxDesign have created the Modular IMMune In vitro Constructs system (MIMICTM). MIMIC is a two-stage microscale model of the human immune system, set in the wells of cell culture plates. T-cells, B-cells and monocytes are separated from human donor blood and added to wells containing a tissue construct comprising endothelial cells and collagen, which creates an environment for the cells to thrive. The blood cells migrate through the endothelial cells into the collagen matrix and differentiate into dendritic cells, which are then able to reverse migrate and respond to added challenge antigens. The antigen-presenting dendritic cells are then removed and added to a lymph node model in a second culture plate where the immune response can be characterised in terms of cytokine production and antibody production. The system enables hundreds of donors with diverse gene pools to be studied on a large scale and at speed, and can predict the human response to vaccines. The company believes “...MIMIC technology will generate valuable predictions of human outcomes for immunopharmaceuticals and vaccines, accelerating the therapy discovery and development process and increasing the probability of success in human clinical trials” [41]. The International AIDS Vaccine Initiative awarded VaxDesign an innovation grant and hopes to begin trials of potential AIDS vaccines in 2009. VaxDesign is also creating functional human mucosal constructs, since many viruses including HIV enter the body through mucosal tissues.

**Mathematical modelling and statistical analysis**

Mathematical modelling played a vital role in the 1990s in understanding the HIV lifecycle in infected patients and helped to develop successful treatment strategies [42]. Mathematical (or computer) modelling can have an impact
on several aspects of research to develop HIV vaccines, including predicting the acquisition of infection, the disease state (viral load) after infection, and likely escape by the virus from immune control. Modelling can be used to simulate these factors in virtual clinical trials of HIV vaccines, improving the design and analysis of actual clinical trials. Modelling in advance of clinical trials can also suggest new biological hypotheses to use with post-trial data [43].

Very recently researchers have discovered what they believe is the first new mechanism in nearly 20 years for inhibiting a common target in all HIV patients, which could eventually lead to a new class of AIDS drugs. Researchers at the University of Michigan used computer models to develop the inhibiting compound, and then confirmed in the lab that the compound does indeed inhibit HIV protease, which is an established target for AIDS treatment. The protease is necessary to replicate the virus, according to the principal investigator of the study [44].

Intelligent and creative development and application of tools for statistical analysis are a key to exploiting and using relevant data of all kinds [45]. There is a great opening for the use of computer modeling and mathematical packages in AIDS research in a similar vein to the work carried out for other viruses such as hepatitis [46].

**Prevention of AIDS**

In 2006 UNAIDS said: “If anything has been learnt from the past 25 years of the epidemic, it is that HIV prevention works.” Recently, it was concluded by a group of Nobel Prize winning researchers that the most effective way to spend $50 billion in saving lives was to invest in AIDS prevention. A sobering thought, when the breakdown of NIAID’s 2007 AIDS research budget reveals $497.1M (33.3%) is spent on vaccine research [5].

Greater spending on prevention now would not only prevent more than half the predicted new infections by 2015 but would actually produce overall financial savings, as future costs for treatment and care are avoided [12]. Funds currently being diverted into ethically and scientifically dubious animal experiments would be far better diverted into modern non-animal methods and into promoting and educating about methods of prevention of disease.

The main causes of HIV transmission are already known to be sexual, through blood and mother-to-child. Therefore, ideal prevention methods include social education programmes to explain the transmission of AIDS, to discourage multiple sexual partners, and to encourage the use of the condom and delaying sexual activity in young people [47].

The proper use of the male condom has been shown to reduce the risk of HIV infection by 80-90%, an efficacy far in excess of that offered by any potential vaccine. Brazil, Uganda and Thailand have all shown a reduction in AIDS spread due to these methods. The supply of free condoms and sterile needles for drug users are the most cost-effective ways to prevent AIDS, with proven results [48, 49, 50]. Transfusion of infected blood or blood products is the most efficient of all ways to transmit HIV but this can be greatly reduced by screening all blood supplies for the virus, by heat-treating blood products and sterilising equipment used for medical procedures [51].

HIV can be transmitted from a mother to her baby during pregnancy, labour and delivery, and later through breastfeeding. If HIV infection is not prevented, a caesarean section will reduce the baby’s exposure to its mother’s body fluids and lower the risk of HIV transmission. Consideration should also be given to the safest birth and feeding options for the child [52, 53]. The diagnosis and treatment of other sexually transmitted infections that increase susceptibility to HIV infection should also be a focus [54].

Prevention efforts addressing multiple levels have reversed HIV epidemics in Uganda and Thailand, and averted an epidemic in Senegal. Senegal, for example, used prevention programmes on the individual level (HIV counselling and testing), community level (HIV education in schools, condom promotion among sex workers), medical level (treatment of sexually transmitted diseases), and structural/political level (mobilising religious and political leaders to talk openly about HIV) to maintain one of the lowest rates of HIV infection in sub-Saharan Africa [55].
The way forward

The road to eliminating HIV infection requires effort and coordination from many sectors: scientific, political, religious and societal.

There are political, economic and religious factors contributing to the AIDS epidemic which cannot be ignored. Some societies find it difficult to discuss sex openly, there is gender inequality and some authorities restrict which subjects can be discussed in the classroom, or in public information campaigns, for moral or religious reasons. Particularly contentious issues include premarital sex, condom use and homosexuality, the last of which is illegal or taboo in much of the world. Marginalisation of groups at high risk, such as sex workers, can be a major hindrance to HIV prevention efforts; authorities are often unwilling to allocate adequate resources to programmes targeting these groups. It is also often the case that the resources are simply not available for these important activities.

To be successful, a comprehensive HIV prevention programme needs strategic planning based on good quality surveillance, as well as consideration of local societies and cultures. From a scientific perspective the use of non-animal research methods relevant to humans will lead to an improved basic understanding of the virus and disease, essential for the development of effective vaccines and treatments. Focusing on non-animal models with a view to better research approaches should not only have beneficial implications for animals’ lives but also for patients.

There is an urgent requirement for global acceptance that the primate is a failing ‘model’ for HIV research including in vaccine development. There needs to be a culture change, moving away from reliance on these animal experiments and towards alternative approaches embracing current and potential non-animal research methods. A redirection of funding into these alternative channels is an essential part of this culture change and will give the green light to humane, transferable, reliable and high-throughput research.

As with the replacement of any animal research, the key is a multidisciplinary approach combining a range of human-relevant, non-animal methods. These include population studies, clinical research, computer modelling, human cell and tissue research and molecular studies. In the particular case of research into HIV and AIDS and with a focus on vaccine research and development, several highly relevant models are available. Genetic epidemiology, for example, can shed light on why some people succumb to HIV infection and others do not. Clinical research with volunteers has revealed that some individuals may spontaneously produce high levels of chemokines and be relatively resistant to HIV infection. The discovery of new and more reliable biomarkers of immune function and vaccine effect in humans will permit safer, earlier and more predictive clinical trials.

Ex vivo studies using blood and lymphoid tissue samples donated by healthy volunteers and by people infected with HIV are unravelling the complexities of HIV infection mechanisms (e.g. virus lifespan and behaviour) and the human immune response to the virus. These experiments are providing crucial insights into the potential for manipulating HIV. Multiplatform in silico, in vitro approaches and in vitro models of the humane immune response are being used to select and evaluate a range of HIV vaccine candidates. Many of the human cell-based techniques are amenable to high-throughput robotic processing, providing results for multiple compounds in hours rather than the weeks or months that animal research requires. Human cell and tissue studies are accelerating the discovery and development process itself, and increasing the probability of success in human clinical trials.

Mathematical modelling can be used to predict the likely successes and failures of candidate HIV vaccines in simulations of clinical trials and can improve the design and analysis of actual clinical trials. Modelling can also generate and test new hypotheses about HIV and AIDS. New statistical tools are key to exploiting and using relevant data of all kinds.

A decreasing reliance on misleading research with primates and an increasing focus on these techniques should offer a brighter future for progress against HIV and AIDS.
References


Towards replacing primates in hepatitis C research
Gill Langley and Nicky Gordon

Introduction

The use of primates in hepatitis C virus (HCV) research covers a number of aspects, from basic infection models of the virus in chimpanzees to expression of reconstructed virus in primate species, such as the common marmoset and tamarin monkey.

The initial progress of research was complicated by the lack of an in vitro replicative model for the wild type virus and the numbers of different genotypes of the virus. However, despite recent advances in the development of in vitro infection models, chimpanzee experiments are still reported [e.g. 1, 2]. In one review it was argued that the use of chimpanzees was preferred as it was unethical to collect frequent tissue samples from infected human patients [3]. One often overlooked cost of these studies is the number of chimpanzees used who are now chronically infected with hepatitis C and need to be cared for.

Hepatitis C as a distinct virus was not identified until 1989 [4] but was assumed to be the causative agent in up to 90% of cases previously diagnosed as non-A and non-B hepatitis. To date it is estimated that 200 million people worldwide are infected, with a prevalence of 1-2% in Europe and the US to 10% in countries such as Egypt [5]. The virus is a blood-borne infection acquired from the usual risk factors, contaminated blood products, intravenous drug administration and unprotected intercourse.

The virus causes a two-stage infection; the initial acute stage may last up to six weeks during which the individual may appear asymptomatic. Between 20-50% of infected individuals may naturally clear the virus during this stage and may become non-infectious. The remaining 50-80% of infected individuals will progress to chronic hepatitis [6, 7, 8]. Within this 50-80%, approximately one third will show no physical effects of the infection, some will suffer from mild fibrosis and liver scarring, whereas in the final third some will progress through to severe liver fibrosis or liver cancer, potentially resulting in end-stage liver failure usually requiring transplantation. Recently Pham et al [9] have demonstrated the persistence of viral replication within patients’ white blood cells up to five years after spontaneous or antiviral therapy-induced resolution of hepatitis. There is no vaccine for HCV and available antiviral drugs are toxic, expensive and only partially effective.

Primates in HCV research

The types of procedures to which primates are subjected are only briefly described in the literature. These include blood sample collection, and in earlier studies liver resections to remove samples for pathology and molecular diagnosis of disease progression [10]. Liver resection is a major operation causing considerable post-surgical pain, with risk of haemorrhage, infections and kidney failure, damage to bowel or bile ducts, and scar tissue causing bowel obstruction. In later studies needle biopsies of the liver have been performed. These are usually conducted under sedation, and there is a risk of trauma to adjacent organs or major blood vessels, bleeding, infection or even sepsis. Post-biopsy pain is common and can be moderate to severe. In the case of smaller primates, blood collection requires restraint of the animal.

These would seem to be traumatic experiments if they happened only once during the study, but to follow the progression of the viral infection some procedures can be repeated as frequently as every two days [1] or monthly over a period of several months [11]. In addition, these intelligent animals are suffering a lifetime of incarceration in a laboratory environment, where every aspect of their lives including their social interactions and diet is out of their control.

Initial studies of the virus were complicated by the lack of an in vitro replication model. It was discovered that the virus would replicate in chimpanzees although the onset and severity of disease differ from that observed in humans. Chronically infected chimpanzees only suffer mild hepatitis that is of transient onset; however from these initial experiments some information about the lifecycle and the structural basis of the virus could be elucidated leading to potential targets for therapy.

References to the numbers of animals used in these studies are limited but there are mentions in the literature of only two to three animals per study, probably because of the costs of acquiring and maintaining chimpanzees in the
laboratory. HCV in serum from infected patients was shown to infect and replicate in chimpanzee hepatocytes but not in baboon hepatocytes. Replication was also demonstrated to be inhibited by the presence of alpha-interferon [12]. In these studies primary hepatocytes were isolated from liver resections from chimpanzees and baboons.

A more recently developed animal model involves the use of human hepatocytes transplanted into immune-deficient mice. In these mice their own liver cells are engineered to die and are replaced by human cells [13]. After infection of the mice with a strain of HCV that replicates in in vitro systems, viable virus particles were found in the blood of the mice after one week. The virus was also used to infect chimpanzees [14] who showed positive infection within two weeks of inoculation and continued production of HCV particles for up to 17 weeks. However, this method of research did not effectively mimic the natural disease: none of the chimps used in the study showed any clinical signs of hepatitis, even at the minimal level seen previously in this species, which is again different in onset and severity to that seen in human patients.

The cloning of the HCV genome led to the development of model recombinant viruses using GB virus, which is known to be of similar structure but infects tamarin monkeys. These recombinant viruses were later shown to replicate in tamarins and in common marmosets [15, 16]; however, there was still a considerable need to develop an in vitro model of replicative virus to enhance antiviral therapy programmes and to replace experiments on marmosets and tamarins.

### Replacing primates in HCV research

In 1998, an elegant mathematical model was developed that materially advanced understanding of the dynamics of HCV in human patients [17]. The model was fitted to data on viral load acquired from volunteer patients randomly assigned to receive different doses of the antiviral drug interferon-alpha. Neumann and colleagues deduced the mechanism of action of the drug and calculated its efficiency in decreasing viral load. They also quantified HCV production and clearance in carriers, and their findings overall had significant implications for designing effective treatments.

Subsequently, mathematical modelling based on human studies has continued to play an important role in HCV research. A key finding has been that even though hepatitis C disease develops over one or more decades, very rapid dynamic processes occur in infected humans over timescales of hours to days, as well as slower processes that occur over weeks to months. These findings strongly influenced the way in which patients were treated with antiviral drugs [18].

Another application of mathematical modelling with potential to replace chimpanzee and other animal studies of novel drugs is physiologically based pharmacokinetic modelling. This uses computer simulations that predict the absorption, distribution, metabolism and excretion of substances such as novel drugs. Using physico-chemical and in vitro information about the drug itself, melded with standard species-specific human physiological data, the simulations predict how the medicine will behave in the human body and over what time course.

In 1997 Dash et al reported the first case of infection by and replication of the virus in human HepG2 cells, by gene transfer of in vitro transcribed HCV RNA [19]. The aim was to develop a reproducible in vitro system to study the lifecycle of HCV and to test anti-HCV agents. Intracellular levels of HCV RNA were measured from 10 to 50 days after transfection and were stable over this period at fairly high levels. This discovery paved the way for increased research using in vitro cell lines for studying replication and potential inhibitors of the viral lifecycle.
In the late 1990s and early 2000s the HCV replicon model was developed further, allowing replication of viral proteins in human cell cultures and leading to an increased investigation of anti-HCV compounds [20, 21]. Sequences encoding HCV genomes were cloned and transfected into human hepatoma cells to analyse quantitatively HCV RNA replication and protein transcription/translation. This system was used to study the mechanisms of action of interferon-alpha and to study other potential antiviral drugs. Much of what is known about HCV replication came from these studies, but still there was little knowledge of other important mechanisms and host functions, such as viral entry into cells, viral uncoating, trafficking, assembly and exit from cells [22]. In 2005, three teams of researchers separately developed robust in vitro HCV-infection systems, finally making possible and practical laboratory studies of these processes [23, 11, 24]. One of the most effective of these new models was based on in vitro transcribed genomic HCV RNA, derived from a Japanese patient with fulminant hepatitis.

Immediately, these cell-based models led to the discovery that a receptor called CD81 on human cells plays a crucial role in entry of HCV into cells. Cells that did not express CD81 were immune to virus entry [11]. Subsequent use of the new in vitro models has led to dramatic progress. For example, an association of cholesterol and sphingolipid with HCV particles has recently been found to be important for virion maturation and infectivity. This suggests that altering the lipid composition of HCV particles might be a useful target for anti-HCV therapy [25].

It has also become possible to screen existing drugs for other indications, to see if they have anti-HCV effects. Griseofulvin, an anti-fungal agent, was recently found to suppress HCV-RNA replication and protein expression in a dose-dependent manner [26]. Screening existing drugs quickly in human in vitro systems offers two advantages: it provides rapid data, and on drugs that have already undergone pre-clinical and clinical studies for safety, so that if anti-HCV activity is discovered fewer animal tests would be needed to bring the drug to clinical trials.

In vitro human models are now being devised and implemented for a range of purposes and at a breathtaking pace. For example, a system has been developed to analyse determinants of HCV genome packaging and virus-particle assembly. It is being used to decipher mechanisms of HCV assembly and to identify RNA elements and viral proteins involved in particle formation. The model is expected to be valuable in vaccine research [27]. The new availability of high-throughput methods, such as cell-based reporter assays using green fluorescent protein, offer time- and cost-saving benefits for quantitative screening of anti-HCV reagents [28].

Although cell-based models provide a simulated HCV infection process, still the only way to fully test antiviral compounds against the actual HCV infection is considered by some to be in primate infection models. Research into early markers of vaccine or drug efficacy and of early toxicity in human volunteers is a way to tackle this, overcoming the need for liver biopsies and facilitating safer and more effective clinical trials. Reliable biomarkers should also enable a reduction in the size and costs of clinical trials required to prove efficacy or detect early toxicity, making the replacement of chimpanzee studies a reality. Work is already ongoing in these areas with some promising quantitative serum markers of liver fibrosis being studied [29].

In December 2000 the USA passed the improbably named Chimpanzee Health Improvement and Protection Act known as CHIMP. The main impact of this act was to prohibit the euthanasia of chimpanzees for anything other than humane health reasons [30]. Many of the ex-research chimpanzees are now infected with human HCV virus and the passing of this act means that between 1,600-2,000 former laboratory chimps in the US need to be cared for and maintained.
There has also subsequently been an imposed ban on chimpanzee breeding programmes in the USA. The population of research chimps in the USA has been decreasing since 1995 [31] and without any further breeding there are projected to be none left by 2035. Laboratory chimpanzees surplus to current research use in the USA are being retired to facilities throughout the country with an estimated lifetime cost of up to $500,000 per animal, most of the cost is being met by the US government. One independent organisation the Hepatitis Research Foundation has established its own chimpanzee sanctuary in Liberia for retirement of its own population of HCV-infected chimpanzees [32].

Worldwide, the movement to end experiments on chimpanzees has enormous support. In 2008 a commission of the Spanish parliament declared support for the Great Ape Project, a proposal to grant rights to life, liberty and protection from torture to all the great apes. This is the first time that a national parliament has supported these rights for non-human animals. Considering these developments, the time is right for a coordinated research drive to develop missing non-animal techniques, so that all chimpanzee and other primate research into HCV can be replaced with methods that have the potential both to save animals and benefit people.
References


32. Hepatitis Research Foundation http://www.heprf.org/vilab2chimpsanctuary.htm
Conclusions and recommendations

In this report we examine five case studies according to the need and potential for replacing non-human primates in medical research. We show how vaccines and drug treatments have continually failed to translate from primate ‘models’ to humans in these medical fields. Numerous AIDS vaccines and stroke treatments have shown promise in primate tests but consistently failed in clinical trials. We also discuss the types of procedures to which primates are subjected in medical research, and highlight widespread ethical concerns about these experiments.

This analysis supports the growing consensus that more scientifically robust methods are urgently needed in order to improve patient care, prevent disease and raise ethical standards in research. In order to achieve this aim, a coordinated effort is required from policy makers, funders of research and scientists themselves to bring about strategic change. To establish and maintain focus and direction, these stakeholders should develop a targeted, timetabled strategy for replacing primates in medical research.

This may also require a conceptual shift in scientific thinking, to acknowledge that compared with primate experiments, non-animal studies may take different routes to finding information of an equally or more valid and relevant kind. Primate experiments need to be rigorously assessed by the same objective measures used to judge any other scientific technique. Focus on Alternatives hopes that this report will encourage major funders of primate research to review their funding policies and emphasise the development non-animal methods.

For research into AIDS, malaria, stroke, cognition and hepatitis C, we suggest areas where greater scientific effort and funding is needed in order to accelerate the replacement of primates with non-animal methods. For example, studying infected human cells in vitro is showing great promise in understanding infection mechanisms in AIDS and hepatitis C vaccines research. Primate experiments have previously been misleading in these areas because the diseases in primates are fundamentally different from the human forms. In cognition research, recent advances in human imaging have led to huge leaps forward in our understanding of the human brain in health and disease, which was not previously possible because researchers were using invasive methods to study primate rather than human cognition.

It is clear that there is a high level of public concern about the use of primates in research, and if policy makers want to pay genuine heed to this, replacement techniques urgently need to be properly funded and taken seriously as centrally important research methods, not just as adjuncts to primate research.

The current review of legislation governing animal experiments in the EU, Directive 86/609/EEC, provides a unique opportunity to implement a targeted and time-tabled strategy for replacement of primates in European research. This will help to avoid large-scale animal suffering, to enhance medical progress and to accelerate the development of novel, enabling technologies with wide applicability throughout the scientific world. It would also provide a lead to other national authorities to implement similar policies and drive forward change on behalf of patients and primates throughout the world.
Focus on Alternatives brings together representatives from British non-profit organisations funding the development or promoting the acceptance of methods that replace the use of laboratory animals in research, education and testing.

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