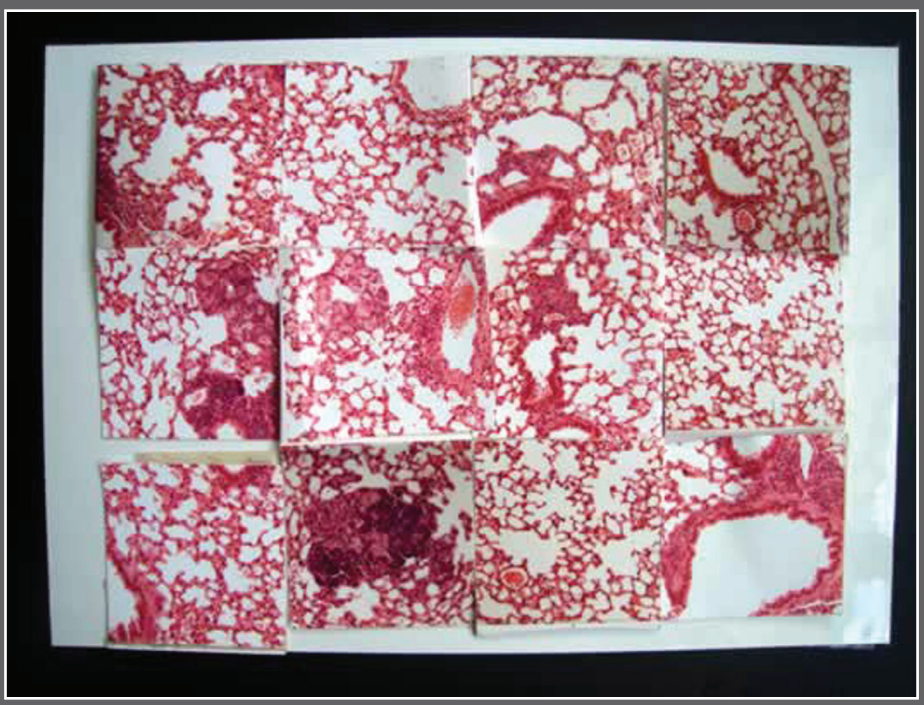
CHAPTER 17

'… Obviously this is a situation set to spiral out of control. Call it what you want, a time bomb or a powder keg, any way you look at it this is a potentially explosive situation….'

WHO Director-General Margaret Chan



Transformation Relief – Katharine Honey
Abstraction from Microscopic Images

'Don't look.
The world's about to break.'

***Don't Look.*. (Poem) – Harold Pinter**

Katharine Honey

Katharine Honey studied painting and stained glass at the Wimbledon School of Art, then taught at the Institute of Education before training with the jewellery designer Miye Matsukata in the United States. Later she returned to London and further consolidated her studies at Sir John Cass and more recently at Morley College. Working mostly in silver and using semi-precious stones, her work emphasizes colour and texture within an abstract form. While some of her work is commissioned, she is now focusing on producing a more coherent range of jewellery, with occasional designs in a limited edition. Her work has been exhibited at the Oxford Gallery; Crafts at Rangers House, English Heritage, Greenwich Tourist Office, Cockpits Arts Morley College, Saltgrass Gallery, and the Greenwich Open Studios.

CHAPTER 17

The Contribution of Diverse Animal Models in the Evaluation of New Vaccines against Tuberculosis

Rogelio Hernandez Pando, Diana Aguilar, and Rafael Hernández

Introduction

The use of animal models to study diverse diseases has been extremely important in the progress of scientific medicine. Indeed, crucial observations on the etiopathogenesis of different diseases in vivo, including infectious diseases, have been done using experimental animal models. Moreover, animal models have been essential in the evaluation of new drugs and their toxicity, as well as in the evaluation of efficacy of new vaccines, representing one of the essential preclinical assays.

The contribution of different animal models on TB research has been quite significant; first with Robert Koch who was the first scientist that used the mouse as an experimental model (1). Then, Julius Cohnenheim used the rabbit's anterior chamber of the eye to study inflammation induced by mycobacteria, describing the earliest inflammatory events induced by mycobacteria in vivo. In fact, Cohnenheim is considered as the father of experimental pathology because of this classical study. More recently, a variety of different animals (mice, rabbits, guinea pigs, rats, cows, fish, and monkeys) have been used to study the immunopathology induced by MTB, particularly in the lungs. Based on the knowledge gained on immunopathogenesis, these animal models have also been used in the design and evaluation of various immunotherapy regimens,

as well as being valuable tools for testing the efficiency of new drugs and vaccines. In this chapter the most significant animal models for the evaluation of new vaccines against TB are described, highlighting their advantages and disadvantages. In fact, any one of these animal models is useful since for each potential vaccine candidate, it is necessary to test its efficacy in several animal models during preclinical study.

The Mouse Model

Mice are the experimental tool of choice for immunological experimentation in vivo because in many respects they mirror human biology remarkably well (2). Recent reports indicate that the human and mice genomes are remarkably similar, and only 300 genes appear to be unique to one species or the other (3). Indeed, the overall structure of the immune system in mice and humans is quite similar (4). However, there are some significant differences such as: the presence of a well-developed bronchus associated lymphoid tissue in the mice (possibly due to the frequent contact with soil antigens since they are much closer to the ground), while in healthy humans this tissue is almost absent, or the predominance of lymphocytes in the peripheral blood of mice instead of the neutrophils in humans (5, 6). These are among the significant differences to be considered in evaluating new vaccines against TB. However, the mouse is the most popular model to study for immunopathogenesis and the testing of new drugs and vaccines (7).

Mice (usually C57BL strain) can be infected via aerosol with a low dose of organisms, or by intratracheal injection (usually BALB/c) using a high dose of bacilli. In both cases bacteria (usually the reference strain H37Rv) multiply in the lungs and spread to the liver and spleen. The infection is controlled but not eliminated by CMI, especially activated macrophages and Th1 response. In the case of the BALB/c model, due to the high infecting dose, it corresponds to a progressive disease model which during early infection exhibit limited pathology (perivascular, peribronchial, and interstitial inflammation with granuloma formation) and predominance of Th1 cells with activated macrophages that efficiently control the bacilli growth. This is followed by a progressive phase characterized by progressive lung consolidation (pneumonia), extensive lung fibrosis, emergence of Th2 response with high production of anti-inflammatory factors like TGFβ, IL-10, and prostaglandin E2 among others, in coexistence with high bacilli loads and mortality (8–10). In contrast, the low dose infection in C57BL mice produces a well-tolerated infection dominated by Th1 response which may last for more than one year (11), with no evident contribution of Th2 cytokines and other anti-inflammatory or CMI suppressor molecules.

Thus, this is actually a model of slow progressive disease, and animal death is produced by excessive inflammation or immunopathology. The mice immune response against mycobacteria has been shown to have good correlation with the infection in humans, including the participation of CD4+ T cells, IL-12, and TNF-α (12–16).

The BALB/c model is useful for evaluating the level of virulence in vaccines based on mutant attenuated mycobacteria (17) or in recombinant BCG (19), determining the rate of survival, pulmonary bacilli loads, and the extent of tissue damage following intratracheal challenge (20). Studies on the immunogenicity of new vaccines before challenge can be easily performed in this model by the quantification of IFN-γ production by stimulated lymphocytes with various mycobacterial antigens (Ag85, ESAT6, hsp65, whole mycobacterial antigens from liquid culture, or from sonication). These cells are collected from regional lymph nodes, spleen, and lungs after subcutaneous vaccination in different time points. When recombinant BCG or mutant attenuated live mycobacteria are tested, part of these organs are homogenized and used for the determination of bacilli loads via measurement of CFU, which will reflect on the extent of bacterial dissemination and local growth in selected lymphoid organs. These studies are usually performed with at least three different doses to select the best dose that induces the highest and most consistent IFN-γ production. Following the immunogenicity study, challenge experiment is performed by vaccinating BALB/c mice via the subcutaneous route and, two months later, animals are challenged via the intratracheal route with a high dose of H37Rv, and another group of vaccinated mice challenged with the highly virulent Beijing genotype clinical isolate (17). One group of at least 10 animals is left unvaccinated to record animal deaths as a function of time to construct survival curves, while another group of animals are sacrificed two and four months post-challenge (usually five mice per time point) to determine bacilli loads by CFU quantification in five lung lobes (either right or left), and the extent of tissue damage in the other lung lobe by determining the percentage of lung area affected by pneumonia using histology and automated morphometry (21). Controls are represented by a group of non-vaccinated mice and another group of animals vaccinated with BCG substrain Phipps, which, in this animal model, confers the best protection (21). Potential new vaccine candidates which confer significantly better protection than BCG are considered for further testing in other animal models, such as guinea pigs or non-human primate models.

We consider histopathology as one of the most valuable techniques to evaluate the efficiency of new vaccines. For this, it is absolutely necessary to ensure that the lung samples are well perfused with the fixer solution. Immediately after killing the mouse, the ribs are cut and the lungs exposed; one lung is removed after the occlusion of the extrapulmonary bronchus with

surgical forceps. The other lung is then perfused by intratracheal injection with 1ml of the fixer solution using a syringe. The perfused lung is immediately immersed in a plastic tube with 5ml of the same fixer solution for 24 hours. For immunohistochemistry, the use of absolute ethyl alcohol is preferred as the fixer solution since the tissues are fixed by dehydration which preserves the proteins better than formaldehyde, which fixes the proteins by formation of molecular bridges that can produce significant modifications to the three-dimensional structure, which may compromise antibody recognition. Three parasagittal sections are obtained from the whole lung with a distance of 100 microns between them. They are stained with haematoxylin/eosin and analysed using automated morphometry.

The most valuable information obtained by morphometry is the lung surface affected by pneumonia. To obtain this information, we measure the whole surface area of the lung using the Leica Q-win 500, which was specifically designed for histopathology. After determining the whole surface of the lung, the consolidated areas are measured avoiding the big blood vessels and principal bronchi. Then, the percentage of the lung surface affected by pneumonia is determined in each of the three sections per mouse and the mean is obtained. Usually three or four mice per group are used, the mean of the group is obtained and compared with the control non-vaccinated and BCG-vaccinated groups. Other important information, such as granuloma size, can be obtained using the same equipment by measuring the area in square microns of all the granulomas observed in the sections. Necrosis, fibrosis, and the type of inflammatory infiltrate are also important histopathological data that are carefully evaluated in the lung sections.

Subcutaneous, intraperitoneal, and intravenous routes of infection were used in the past (7), but they are artificial and at present the low dose aerosol or the high dose infection by the intratracheal route are currently used in the preclinical screening of vaccines. We prefer the high dose model in BALB/c mice because the number of bacilli that go into the lungs is more easily controlled, and the development of progressive disease and animal death is more rapid, while the low dose approach actually produces a slow form of progressive disease which is consequently more easily controlled by vaccination. Furthermore, the BALB/c mouse model has been considered to more closely represent the human condition in endemic countries (18). Besides the normal mouse model, the natural immunodeficient mouse model like SCID or nude mice have also been used to determine the level of attenuation when recombinant BCG or mutant mycobacteria are tested as potential vaccines (22–24).

The major advantage of using a murine model for vaccine testing lies in its ability to screen a large number of vaccines at limited cost and to determine how the vaccine mediates any protective effect. This model has been extensively

used for the evaluation of vaccine candidates (25). This model is also useful in determining how the route of immunization, type of adjuvant, and specific antigen affect the ability of memory T cells to accumulate in lymphoid organs or in the lungs.

The disadvantages of the murine model are that the nature of protection may not be totally extrapolated to the human beings. In fact, the cellular constitution of granuloma and the histology appearance in mice are different than those seen in humans, as well as in other naturally susceptible hosts (e.g. guinea pigs), due to its natural resistance to the disease, which partially control bacterial growth as well as progression of the disease. The granuloma formation in mice is characterized by the aggregation of lymphocytes towards the centre while in humans and guinea pigs, lymphocytes form a peripheral ring with macrophages situated in the centre during granuloma formation (26–28). However, the mouse model is actually suitable for the first screening of vaccine candidates and the efficacy of new candidate vaccines, and those showing good protection in mice are evaluated in other animal models of TB.

Use of the Murine Model for the Evaluation of New Vaccines to Prevent Latent Tuberculosis Reactivation

MTB is a pathogen capable of causing progressive disease or asymptomatic latent infection (29). During latent infection, MTB can survive within the infected host cells for years or decades before disease reactivation (30). Latent infection is characterized by evidence of cellular immune response against mycobacteria (positive tuberculin test), without evidence of active disease. This suggests that the immune system, specifically CMI, successfully controls mycobacterial growth, permitting the presence of dormant bacilli throughout the person's life (29). Indeed, animal models of latent infection have shown that this condition is induced and maintained by IFN-γ mainly produced by Th1 cells, as well as TNF-α and nitric oxide produced by activated macrophages (31, 32). In conditions in which the host immune response is suppressed, disease reactivation can result (30). One-third of the world's population has latent infection and, in most cases of active TB, the disease arises from latent bacilli reactivation. Thus, it is important to design new vaccines to prevent TB reactivation.

We partially reproduced experimentally this latent infection condition in B6D2F1 mice by the intratracheal injection of relatively low numbers of the virulent strain H37Rv (32). This latent infection model is characterized by low and stable bacillary counts (this is a significant difference to the human condition in which it is not possible to culture the bacilli from the lung tissue), with few granulomas and small patches of alveolitis without mortality. Indeed, these mice continue to gain weight and appear healthy for more than two

years. To test new therapeutic vaccines, mice with stable chronic infection at five months postinfection are vaccinated and after one month immunized mice are treated with corticosterone (3mg/kg) dissolved in drinking water to induce disease reactivation. After one and two months, groups of at least six mice are sacrificed bacilli loads are determined by CFU quantification in their lungs and histopathology assessed by automated morphometry (33). The control non-vaccinated group is used for comparison, initially determining survival in a group of 10 mice. Corticosterone induces rapid reactivation, producing high bacilli loads and extensive pneumonia, usually after two weeks.

Hypothetically, during latent infection, a set of specific mycobacterial antigens would be expressed on the cell surface or actively secreted, inducing stimulation of the Th1 response that maintains this condition. The identification of these antigens and their eventual use as subunit vaccines could reinforce the Th1 response and prevent reactivation. With this rationale, we have analysed the expression and vaccine potential of some mycobacterial antigens (protein Rv1759c, a member of the PG_PGRS family). This antigen is highly expressed and located on the surface of phagocytosed mycobacteria during experimental latent infection, eliciting strong cellular immune response that could contribute to maintaining latent tuberculous infection and, when used as a subunit vaccine, it induced good protection by delaying reactivation (33).

The Guinea Pig Model

Currently the guinea pig is used as a model for several infectious bacterial diseases, including pulmonary, sexually transmitted, ocular, gastrointestinal, and other significant infections in humans (34). Guinea pigs have served as a significant model in past and current TB research (35). Robert Koch used these animals to establish his postulates and identified the tubercle bacillus as the aetiological agent of TB. Guinea pigs are peculiar as they develop classical granuloma similar to humans. Another important feature of this model is the presence of Langhans multinucleated giant cells (36), but perhaps the most significant characteristic of guinea pigs is its extreme susceptibility to MTB, permitting rapid progression of active disease with extensive lung tissue necrosis, weight loss, and death due to the disease, like in humans—so this model is an important tool for testing the efficiency of anti-TB chemotherapy and vaccines (37–40). This model has been well standardized and usually animals are infected by the aerosol route with a small number of bacilli, or with a high dose (41). It is considered that only ~unit log reduction in peak lung bacillary load in BCG-immunized guinea pigs provides a wider spectrum for evaluating the efficacies of the vaccine candidates.

Most of the vaccine candidates are first evaluated in mice and the promising ones are subsequently taken up for evaluation in the guinea pigs. However, this strategy should be cautiously used as there is a possibility of losing some good candidates which may not show promise in mouse models but show good protection in guinea pigs which show more resemblance to humans. The guinea pig model has also some significant disadvantages which precluded its use as a first order screening model. The most important disadvantages are the high cost of animals in a biosafety facility compared to low expenses involved in housing mice, and the restricted availability of immunological reagents to assess immunological factors involved in vaccine protection.

The Rabbit Model

The rabbit model has been used as a model for studying human TB since the beginning of the twentieth century (42). Rabbits have some innate resistance to TB, but lung pathology show close resemblance to human TB. This model has been extensively used by different groups (43–45) particularly those working on pathogenesis and therapy evaluation (46–49). Rabbits produce granulomas with caseous centres very similar to the human granuloma. Indeed, there is a remarkable similarity between the spectrum of rabbit TB and that found in humans (43). This model has been used successfully to differentiate between *M. bovis* and MTB because of the remarkable difference in virulence that these mycobacteria have in these animals.

The rabbit model offers some advantages over both murine and guinea pig models. Because rabbits are outbred species, infection with MTB or *M. bovis* exhibit the spectrum of disease that represents many of the forms related to specific stages observed in humans (50, 51). The outbred rabbits are relatively resistant to intravenous and aerosol infection with MTB and generally recover from infection in four to six months, like humans (52–53). Interestingly, inbred rabbits are more susceptible to TB than outbred animals (50). Inbred animals develop more caseous necrosis with higher number of bacilli and fewer epithelioid cells than outbred rabbits.

There are few reports where rabbits are used for the evaluation of new vaccines. Immunomodulators, like live attenuated vaccines (*M. vaccae*), have been tested in the rabbit model (48). The tuberculous meningitis model in rabbits has been used for vaccination designed to prevent cerebral infection, comparing BCG with new designed vaccines (54).

The most important limitations of the rabbit model are the paucity of commercial immunologic reagents, the difficulty to get inbred rabbits, and variations in results within each vaccination group, which could be partially

resolved by the use of a large group of animals, increasing the cost and the demand for larger high security facilities.

The Non-Human Primate Model

The non-human primate models of TB have been used for several years for vaccine and drug-testing studies (55–57). The evolution of the disease in monkeys is usually progressive with haematogenous and bronchial spread, extensive caseous necrosis with liquefaction and cavitation, so it is quite similar to the human disease (58). The severity of the disease is reduced when monkeys are immunized with BCG (55), which can prevent visible pulmonary lesions in some animals depending on the infecting dose or in the natural resistance of the particular animal, considering that they are also an outbred species. Both rhesus (*Macaca mulatto*) and cynomolgus (*Macaca fasicularis*) monkeys have been used. It is possible to infect monkeys by aerosol (54), with low infecting doses (10–100 CFU) in cynomolgus monkeys, which is totally controlled by the immune system or permit latent infection, while a larger inoculum (3000 CFU) cause progressive disease in the animals (59, 60).

Because of their close phylogenetic relationship with humans, macaques are often used to evaluate the immunogenicity and safety of new vaccine candidates. Several studies have indicated that rhesus macaques are highly susceptible to TB (56, 61), while the closely related cynomolgus macaques are more resistant (62). Moreover, the cynomolgus monkeys are more efficiently protected by BCG vaccination than rhesus monkeys, and therefore offer a good experimental model for the evaluation of new subunit vaccines.

The most important advantage of the non-human primate model in comparison with the other models is the strong similarity to human TB in terms of the spectrum of disease and pathology, as well as the availability of reagents for studying the immunological parameters. Thus, the results obtained from this model are more directly applicable to the human situation. However, these models have some significant disadvantages like high cost, requirement for biosafety facilities, as well as non-availability of inbred animals. Expensive specialized equipment is necessary and standardizing a dose delivered to the monkey is difficult. Thus, in some experiments it was necessary to use flexible bronchoscope (63). Outbred animals give more variability in the experiments, which in fact is more like the human population and a more realistic situation, but it is necessary to use more animals for statistical validation thus further increasing the cost of the experiments. Moreover, monkeys with TB are contagious to other animals, including other monkeys and personnel working in the animal facility and can pose serious risks in the facility. The high cost

involved in non-human primate research is due to many factors not only because of the cost of animals, but also the need for veterinary care and veterinary technicians, expensive equipment and special facilities, as well as the high salaries for well-trained technicians. Thus, although the non-human primate model appears to be an attractive model for TB research, all the disadvantages mentioned above limits its use in the majority of the research institutions. The monkey model is usually used in the last part of the preclinical assays, after the confirmation of good and promising results of the vaccine candidate in the mouse and guinea pig models.

The Cattle Model

Protection of cattle against bovine TB by vaccination could be an important control strategy in countries where there is persistence of *M. bovis* infection in wildlife, and in developing countries where it is not economical to implement a 'test and slaughter' control programme (64). Cattle can be experimentally infected producing a reproducible disease and the vaccine trials can be completed within a relatively short time. There are several advantages of the cattle model; one of the most important is that the experimentally induced disease is studied in the natural host, with infections acquired predominantly via the respiratory route which helps in the vaccine screening. Other advantages include: the similarity of the experimental disease to the human disease with respect to pathology and immune responses, and the fact that there are many immunological reagents commercially available. Interestingly, vaccination in calves is possible because they are immunologically competent at birth, and calves are usually sensitized to antigens of environmental mycobacteria at younger age like humans. Furthermore, BCG vaccination has variable efficacy in cattle like humans which provides an opportunity to detect better vaccines than BCG (65–69). The main disadvantages of the bovine model are the use of *M. bovis* instead of MTB, and the high cost of the facilities and animals.

Low dose BCG vaccination in cattle has produced encouraging results and field trials should now be carried out to determine whether this vaccination strategy will reduce the spread of infection (70). The options for new candidate TB vaccines have recently increased with the advent of attenuated strains of *M. bovis*, subunit protein and recombinant DNA vaccines. New attenuated *M. bovis* vaccines induced greater protection than BCG in cattle, while limited stimulation of the immune response induced by subunit protein and recombinant DNA vaccines have been reported (71–74). Thus, better immunological adjuvants are required for these types of vaccines.

Bioethical Considerations in Animal Experimentation

Historically, diphtheria was one of the most significant infectious diseases in which animal experimentation for vaccine development and bioethics were considered as two interdisciplinary fields. Moreover, diphtheria was also one of the first illness in which society realized the high benefit obtained from the knowledge produced by experimentation in animals, provoking general acceptance to such approach. The increasing use of animals for biomedical research also induced significant attitude changes in the scientific community with respect to the way in which animals should be treated during the diverse experimental procedures, generating the utilitarian ethics philosophy developed basically in the eighteenth century—where its basic principle is to consider the welfare (well-being) of any creature subjected to any procedure performed by men (75). This animal well-being concept evolved in parallel and is complementary to the three 'R' principle—replacement, reduction, and refining—as proposed by William Russell and Rex Burch. Both concepts are the basis for the present bioethical principles of animal experimentation (76).

Recently, from the emergence of bioethics, new concepts have been incorporated in the use of animals for biomedical research. Bioethics constitutes the interdisciplinary dialogue between life (*bios*) and moral values (*ethos*). Bioethics principles as applied in animal experimentation have enabled to mitigate some social perception about the abuse of experiments on human beings and animals, as well as potential biological and ecological risks (77). To ensure that the three 'R' principles or the bioethics principles are always considered in all scientific research projects, a great number of national legislations such as in North America (Canada, USA, and Mexico) have established that every institution carrying out animal investigations must have an internal control committee, which evaluate and accept the use of laboratory animals. This entity is called Institutional Animal Care and Use Committee (IACUC), which is responsible for monitoring the correct development of investigation projects that use animals. This committee evaluates the scientific pertinence (conceptual and methodological) of the research projects, as well as the bioethical principles implementation on animal use, health and well-being, biosecurity, safety, and professional risks (78).

Regarding scientific research related with the development of immunological products, diverse organizations, like the Canadian Council for Animal Care (CCAC), have developed specific guidelines on bioethical considerations that must be followed during the use of laboratory animals for immunological reagent production, like antibodies (79). These guidelines and protocols to produce polyclonal and monoclonal antibodies will ensure acceptable

immunological results with minimum discomfort and ensure the well-being of the animals involved (80).

Another important aspect which is considered by IACUC is that animals must endure the least possible pain during the experimental procedure, both for their well-being and to avoid secondary effects that could affect the experimental results. In this regard, protocols of research, teaching, or biosecurity should ensure this principle (80). Therefore, investigation protocols must foresee and establish the conditions in which it must be necessary to euthanize an animal due to its health deterioration or high level of pain (81). Researchers must prevent animal pain and suffering, setting well-defined parameters which will permit identification of these situations, establishing critical endpoints or indicators to stop or to end the protocol including euthanizing the experimental animals (82). Endpoint indicators must be clearly specified in the investigation protocol, and IACUC must evaluate the validity and feasibility to apply them, placing the responsibility for this decision on the veterinarian in charge of the animal facility and the technicians associated with the research project (83, 84).

Conclusion

For more than 100 years, different animal models have been used in TB research. They have provided valuable information on the pathogenesis, immune response, bacilli virulence, and evaluation of new forms of therapy and vaccination. Indeed, none of the described models completely mimics the human disease. However, each model has its advantages and disadvantages, thus the choice of which model to use depends on the experimental question that needs to be answered. With regard to vaccination, no single model is good enough for vaccine evaluation and the choice of model is mainly dependent on experience, cost, availability, space as well as biosafety requirements. In general, good vaccine candidates should be evaluated in more than one animal model to guarantee their eventual test in clinical trials.

❒ References

1 Collins FM. Immunology of tuberculosis. *Am Rev Respir Dis*, 1982; 125: S42–S49.

2 Mestas J and Hughes CH. Of mice and not men: differences between mouse and human immunology. *J Immunol*, 2004; 172: 2731–8.

3 Waterson RH, Linbladh-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, et al. Initial sequencing and comparative analysis of the mouse genome. *Nature*, 2002; 420: 520.

4 Haley PJ. Species differences in the structure and function of the immune system. *Toxicology*, 2003; 188: 49.

5 Pabst R and Gehrke I. Is the bronchus associated lymphoid tissue an integral structure of the lung in normal mammals, including humans? *Am J Respir Cell Mol Biol*, 1990; 3: 131.

6 Doeing DC, Borowicz JL, and Crockett ET. Gender dimorphism in differential peripheral blood leuckocytes count in mice using cardiac, tail, foot, and saphenus vein puncture methods. *BMC Clin Pathol*, 2003; 3: 3.

7 Gupta GM and Katoch VM. Animal models of tuberculosis. *Tuberculosis (Edinb)*, 2005; 85(5–6): 277–93.

8 Hernández Pando R, Orozco EH, Sampieri A, Pavón L, Velasquillo C, Larriva SJ, et al. Correlation between the kinetics of Th1/Th2 cells and pathology in a murine model of experimental pulmonary pathology. *Immunology*, 1996; 89: 26–33.

9 Hernández Pando R, Orozco H, Arriaga K, Sampieri A, Larriva-Sahd J, and Madrid-Marina V. Analysis of the local kinetics and localization of interleukin-1 alpha, tumor necrosis factor-alpha and transforming growth factor-beta, during the course of experimental pulmonary tuberculosis. *Immunology*, 1997; 90: 607–17.

10 Rangel MJ, Estrada Garcia I, Garcia HML, Aguilar LD, Marquez VR, and Hernández Pando R. The role of prostaglandin E2 in the immunopathogenesis of experimental pulmonary tuberculosis. *Immunology*, 2002; 106: 257–66.

11 Flynn JL and Chan J. Immunology of tuberculosis [Review]. *Annu Rev Immunol*, 2001; 19: 93–129.

12 Caruso Am, Serbina N, Klein E, Triebold K, Bloom BR, and Flynn JL. Mice deficient in CD4 T cells have only transiently diminished level of IFN-γ, yet succumb to tuberculosis. *J Immunol*, 1999; 162: 5407–16.

13 Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J, et al. Depletion of Cd4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon-γ and nitric oxide synthatase 2. *J Exp Med*, 2000; 192: 347–58.

14 de Jong R, Altare F, Haagen IA, Elferink DG, de Boer T, Vriesman Pva Breda, et al. Severe mycobacterial and Salmonella infections in interleikins-12 receptor deficient patients. *Science*, 1998; 280: 1435–8.

15 Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, et al. Tumor necrosis–alpha is required in the protective response against Mycobacterium tuberculosis in mice. *Immunity*, 1995; 2: 561–72.

16 Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, et al. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. *Infect Immun*, 2001, 69: 1847–55.

17 Aguilar LD, Infante E, Bianco MV, Cataldi A, Bigi F, and Hernandez Pando R. Immunogeniicity and protection induced by Mycobacterium tuberculosis mce-2 and mce-3 mutants in a Balb/c model of progressive pulmonary tuberculosis. *Vaccine*, 2006; 24: 2333–42.

18 Rook GAW, Dheda K, and Zumla A. Immune responses to tuberculosis in devoloping countries; implications for new vaccine. *Nat Rev Med*, 2005; 5: 661–7.

19 Castañon Arreola M, Lopez Vidal Y, Espitia PC, and Hernàndez Pando R. New vaccine against tuberculosis shows greater protection in a mouse model with progressive pulmonary tuberculosis. *Tuberculosis*, 2005; 85: 115–26.

20 Aguilar D, Infante E, Martin C, Eamonn G, Brigitte B, and Hernàndez Pando R. Immunological responses and protective immunity against tuberculosis conferred by vaccination of Balb/C mice with the attenuated Mycobacterium tuberculosis (phoP) SO2. *Clin Exp Immunol*, 2007; 147(2): 330–8.

21 Castillo-Rodal A, Castañón-Arreola M, Hernández-Pando R, Calva Mercado J, Sada DE, and López-Vidal Y. BCG sub-strains confers different protection against Mycobacterium tuberculosis infection in BALB/c model of progressive pulmonary tuberculosis. *Infection and Immunity*, 2006; 74: 1718–24.

22 Sampson SL, Dascher CC, Sambandamurthy VK, Russel RG, Jacobs WR Jr, Bloom BR, et al. Protection elicited by a double leucine and pantothenate auxotroph of Mycobacterium tuberculosis in guinea pigs. *Infect Immun*, 2004; 72: 3031–7.

23 Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russel RG, Morris SL, et al. Long term protection against tuberculosis following vaccination with severely attenuated double lysine and pantothenate auxotroph of Mycobacterium tuberculosis. *Infect Immun*, 2005; 73: 1196–203.

24 Sambandamurthy VK, Derrick SC, Hsu T, Chen B, Larsen MH, Jalapathy KV, et al. Mycobacterium tuberculosis Δ RD1 Δ panCD: A safe and replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. *Vaccine*, 2006; 24: 6309–20.

25 Gupta UD, Katoch VM, and McMurray DN. Current status of TB vaccines. *Vaccine*, 2007; 25: 3742–51.

26 Rhoades ER, Frank AA, and Orme IM. Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent Mycobacterium tuberculosis. *Tubercle Lung Dis*, 1997; 78: 57–66.

27 Timo U and Stefan HEK. New insights in to the function of granulomas in human tuberculosis. *J Pathol*, 2006; 208: 261–9.

28 Turner OC, Basaraba RJ, and Orme IM. Immuno-pathogenesis of pulmonary granulomas in the guinea pig after infection with Mycobacterium tuberculosis. *Infect Immun*, 2003; 71: 864–71.

29 Parrish NM, Dick JD, and Bishiai WR. Mechanism of latency in Mycobacterium tuberculosis. *Trends Microbiol*, 1998; 6: 107–12.

30 Tufariello JM, Chan J, and Flynn JL. Latent tuberculosis: mechanism of host and bacillus that contribute to persist infection. *Lancet Infect Dis*, 2003; 3: 578–90.

31 Flynn JA, Scanga CH, Tanaka K, and Chan J. Effects of aminoguanidine on latent murine tuberculosis. *J Immunol*, 1998; 160: 1796–803.

32 Arriaga AK, Orozco EH, Aguilar LD, Rook GA, and Hernández-Pando R. Immunological and pathological comparative analysis between experimental latent tuberculosis and progressive pulmonary tuberculosis. *Clin Exp Immunol*, 2001; 28: 229–37.

33 Campuzano J, Aguilar D, Gonzalez–y-Merchant J, Salas P, Espitia C, and Hernàndez Pando R. The PGRS domain of Mycobacterium tuberculosis. PE-PGRS-Rv1759 is an efficient subunit vaccine to prevent reactivation in a murine model of chronic tuberculosis. *Vaccine*, 2007; 25(18): 3722–9.

34 Padilla-Carlin DJ, McMurray DN, and Hickey AJ. The guinea pig as a model of infectious diseases. *Comp Med*, 2008; 58: 324–40.

35 Orme IM. Mouse and guinea pigs models for testing new tuberculosis vaccines. *Tuberculosis (Edinb)*, 2005; 85: 13–17.

36 Flynn JL, Cooper AN, and Bishai W. Animal models of tuberculosis. In Cole ST, Eisenach KD, McMurray DN, Jacobs WR Jr, editors. Tuberculosis and the tubercle bacillus. Washington DC: ASM Press, 2005, pp. 547–60.

37 Dai GS, Phalen S, and McMurray DN. Nutritional modulation of host responses to mycobacteria. *Front Biosci*, 1998; 3: 3110–22.

38 McMurray DN. Disease model: pulmonary tuberculosis. *Trends Mol Med*, 2001; 7: 135–7.

39 Orme IM , McMurray DN, and Belislr JT. Tuberculosis vaccine development: recent progress. *Trends Microbiol*, 2001; 9: 115–18.

40 Smith DM, Balasubramanian V, and Wiegeshaus EH. A guinea pig model of experimental air borne tuberculosis for the evaluation of the response to chemotherapy: the effect on bacilli in the initial phase of treatment. *Tubercle*, 1991; 72: 223–31.

41 Williams A, Hatch GJ, Clark SO, Gooch KE, Hatch KA, Hall GA, et al. Evaluation of vaccines in the EU TB vaccine cluster using a guinea pig aerosol infection model of tuberculosis. *Tuberculosis (Edinb)*, 2005; 85: 29–38.

42 Lurie MB. The correlation. of the biological changes and the fate of living tubercle bacilli in the organs of tuberculous rabbits. *J Exp Med*, 1932; 55: 31–54.

43 Lurie MB. Resistance to tuberculosis : experimental studies in native and acquired defense mechanisms. Cambridge, Mass: Harvard University Press, 1964, p.391.

44 Yamamura Y, Ogawa Y, and Yamagata H. Prevention of tuberculosis cavity formation by immuno-suppressive drugs. *Am Rev Respir Dis*, 1968; 98: 339–49.

45 Danenberg AM Jr and Collins FM. Progressive pulmonary tuberculosis is not due to increasing numbers of viable bacilli in rabbits, mice and guinea pigs, but is due to a continuous host response to mycobacterial products. *Tuberculosis (Edinb)*, 2001; 81: 229–42.

46 Bishai WR, Dannenberg AM Jr, Parrish N, Ruiz R, Chen P, Zook BC, et al. Virulence of Mycobacterium tuberculosis CDC1551 and H37Rv rabbits evaluated by Lurie's pulmonary tubercle count method. *Infect Immun*, 1999; 67: 4931–4.

47 Tsenova L, Sokol K, Freedman VH, and Kaplan G. A combination of thalidomide plus antibiotics protects rabbits from mycobacterial meningitis-associated death. *J Infect Dis*, 1998; 177: 1563–72.

48 Converse PJ, Dannenberg AM Jr, Shigenga T, McMurray DN, Phalen SW, Stanford JL, et al. Pulmonary bovine type tuberculosis in rabbits: bacillary virulence , inhaled dose effects, tuberculin sensitivity and Mycobacterium vaccae immunotherapy. *Clin Diagn Lab Immunol*, 1998; 5: 871–81.

49 Manabe YC, Dannenberg AM Jr, Tyagi SK, Hatem CL, Yoder M, Woolwine SC, et al. Different strains of Mycobacterium tuberculosis cause various spectrums of disease in the rabbit model of tuberculosis. *Infect Immun*, 2003; 71: 1733–45.

50 Dorman SE, Hatem CL, Tag SK, Aird K, Molina JL, Pitt MLM, et al. Susceptibility to tuberculosis: clues from studies with inbred and outbred New Zealand white rabbits. *Infect Immun*, 2004; 72: 1700–5.

51 Lurie MB. The fate of human and bovine tubercle bacilli in various organs of the rabbit. *J Exp Med*, 1928; 48: 155–82.

52 Heppleston AG. Quantitative air-borne tuberculosis in the rabbit. *J Exp Med*, 1949; 89: 597–610.

53 Lurie MB, Abramson S, and Heppleston AG. On the response of genetically resistant and susceptible rabbits to the quantitative inhalation of human-type tubercle bacilli in the organs of tuberculous rabbits. *J Exp Med*, 1952; 95: 119–34.

54 Tsenova L, Harbacheuski R, Moreira L, Ellison E, Dalemans W, Alderson MR, et al. Evaluation of the Mtb72F polyprotein vaccine in a rabbit model of tuberculous meningitis. *Infect Immun*, 2006; 4: 2392–401.

55 Barclay WR, Busey WM, and Dalgard DW. Protection of monkeys against air borne tuberculosis by aerosol vaccination with bacillus Calmette Guerin. *Am Rev Respir Dis*, 1973; 107: 351–8.

56 Corcoran KD and Jaax GP. An attempt to predict anergy in tuberculosis suspect cynomolgous monkeys. *Lab Anim Sci*, 1991; 41: 57–62.

57 Good RC. Simian tuberculosis: immunologic aspects. *Ann NY Acad Sci*, 1968; 154: 200–13.

58 Flynn JL, Capuano SV, Croix D, Pawar S, Myers A, Zinovik A, et al. Non-human primates: a model for tuberculosis research. *Tuberculosis (Edinb)*, 2003; 83: 116–18.

59 Barclay WR, Anacker RL, Brehmer W, Leif W, and Ribi E. Aerosol induced tuberculosis in subhuman primates and the course of the disease after intravenous BCG vaccination. *Infect Immun*, 1970; 2: 574–82.

60 Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Vilahermosa LG, Young LJ, et al. The Philippine cynomolgous monkey (Macaca fasicularis) provides a new non human primate model of tuberculosis that resembles human disease. *Nat Med*, 1996; 2: 430–6.

61 Langermans JA, Andersen P, van Soolingen D, Vervenne RA, Foost PA, Laan T van der, et al. Divergent effect of bacillus Calmette-Guerin (BCG) vaccination on Mycobacterium tuberculosis infection in highly related macaque species: implications for primate models in tuberculosis vaccine research. *Proc Natl Acad Sci USA*, 2001; 98: 11497–502.

62 Ribi E, Anacker RL, Barclay WR, Brehmer W, Harris SC, Leif WR, et al. Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the rhesus monkeys. *J Infect Dis*, 1971; 123: 527–38.

63 Capuano SVIII, Croix DA, Pawar S, Zinovik A, Myers A, Lin P, et al. Experimental Mycobacterium tuberculosis infection of cynomolous macaques close by resembles the various manifestations of human M. tuberculosis infection. *Infect Immun*, 2003; 71: 5831–44.

64 Buddle BM, Skinner MA, Wedlock DN, de Lisle GW, Vordermeier HM, and Glyn Hewinson R. Cattle as a model for development of vaccines against human tuberculosis. *Tuberculosis (Edinb)*, 2005; 85: 19–24.

65 Buddle BM, Wards BJ, Aldwell FE, Collins DM, de Lisle GW, and Skinner MA. Influence of sensitization to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine*, 2002; 20: 1126–33.

66 Vordermeier HM, Cockle PJ, Cockle AO, Rhodes WS, Chambers MA, Cliford D, et al. Effective vaccination of cattle with the mycobacterial antigens MPB83 and MPB70 does not compromise the specificity of the comparative intradermal tuberculin skin test. *Vaccine*, 2000; 19: 1246–55.

67 Wedlock DN, Skinner MA, Parlane NA, Vordermeier HM, Hewinson RG, de Lisle GW, et al. Vaccination of cattle with DNA vaccines encoding the mycobacterial antigens MPB70 and MPB83: protein boosting induces antibody and does not enhance vaccine efficacy. *Tuberculosis*, 2003; 83: 339–49.

68 Buddle BM, Aldwell FE, Skinner MA, de Lisle GW, Denis M, Vordermeier HM, et al. Effect of oral vaccination of cattle with lipid-formulated BCG on immune responses and protection against bovine tuberculosis. *Vaccine*, 2005; 23: 3581–9.

69 Wedlock DN, Vordermeier HM, Denis M, Skinner MA, de Lisle GW, Hewinson RG, et al. Vaccination of cattle with a mycobacterial protein vaccine and BCG induces better protection against bovine tuberculosis than vaccination with BCG alone. *Infect Immun*, 2005; 73: 3540–6.

70 Van Rhijn I, Godfroid J, Michel A, and Rutten V. Bovine tuberculosis as a model for human tuberculosis; advantages over small animal models. *Microbes Infect*, 2008; 10: 711–15.

71 Skinner MA, Wedlock DN, de Lisle GW, Cooke MM, Tascon RE, Ferraz JC, et al. The order of a prime boost vaccination of neonatal calves with Mycobacterium bovis BCG and a DNA vaccine encoding mycobacterial proteins Hsp65, Hsp70 and Apa is not critical for enhancing protection against bovine tuberculosis. *Infect Immun*, 2005; 73: 4441–4.

72 Buddle BM, Wedlock DN, and Denis M. Progress in the development of tuberculosis vaccines for cattle and wildlife. *Vet Microbiol*, 2006; 112: 191–200.

73 Wedlock DN, Vesosky M, Skinner MA, de Lisle GW, Orme IM, and Buddle BM. Vaccination of cattle with culture filtrate proteins and interleukins -2 against bovine tuberculosis. *Infect Immun*, 2000; 68: 5809–15.

74 Wedlock DN, Keen DL, Aldwell FE, Andersen P, and Buddle BM. Effects of adjuvants on immune responses of cattle vaccinated with culture filtrate proteins from Mycobacterium tuberculosis. *Vet Immunol Immuno-pathol*, 2002; 86: 79–88.

75 Audonnet JCh, Lechenet J, and Verschuere B. Animal experimentation in the discovery and production of veterinary vaccines. *Rev Sci Tech*, 2007; 26(2): 443–9.

76 Balls M and Straughan DW. The three Rs of Russell & Burch and the testing of biological products. *Dev Biol Stand*, 1996; 86(1): 1–8.

77 Botovchenco-Rivera EA. Ética na Experimentaçâo Animal e Alternativas ao Uso de Animais em Pesquisa e Testes. En: Ética e Bioetica aplicadas à medicina Veterinaria. eds. Botovchenco-Rivera EA, Amaral MH e Pinheiro do Nascimento V UFG Goiânia, Brasil, 2004.

78 SAGARPA. NOM-062-ZOO-1999. Norma técnica para la producción, cuidado y uso de los animales de laboratorio, 1999.

79 CCAC. Guidelines on antibodies production. Otawa ON: CCAC, 40 pp. http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/ANTIBODIES/.pdf, 2002.

80 European Vaccine Manufacturers. Animal welfare and vaccine development. 2008. *www.evm-vaccines.org/pdfs/vaccines_and_animal_welfare_fin.pdf*

81 Coenraad F, Hendriksen M, and Steen B. Refinement of vaccine potency testing with the use of humane endpoints. *ILAR Journal*, 2000; 41(2).

82 CCAC. Guidelines on choosing an appropriate endpoint in experiments using animals for research, teaching and testing. Ottawa ON: CCAC, 30 pp. http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/ENDPTS/g_endpoints.pdf, 1998.

83 Morton DB. A systematic approach for establishing humane endpoints. *ILAR Journal*, 2000; 41(2).

84 Morton DB. Humane endpoints in animal experimentation for biomedical research: ethical, legal and practical aspects. In Hendriksen CFM and Morton DB, editors. Humane endpoints in animal experiments for biomedical research. London: Royal Society of Medicine Press, 1999, pp. 5–12.