Up to now, experiments with animals have been indispensable in the development and evaluation of vaccine candidates.

‘…the flowers that it left, wouldn’t want to talk to me.’

Blue Unicorn
Silvio Rodriguez

In This Turning Environment It Will be Difficult to Find My Place
Raul Santoserpap
Oil on canvas; 170 × 256 cm
Collection of the National Museum of Fine Arts,
La Habana, Cuba
CHAPTER 4.1

THE CONTRIBUTION OF DIVERSE ANIMAL MODELS IN THE EVALUATION OF NEW VACCINES AGAINST TB

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Only animal models can indicate to us which vaccine candidates should continue to be evaluated, and which should be rejected.

‘Snow falls, leaving the world diminished.’

December 24, 1986
Joseph Brodsky

Autumn in Vedado
Ileana Mulet
Oil on canvas; 120 × 100 cm
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 \textit{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 \textit{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 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 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 neutrophils in humans (5, 6). These are among the significant differences to be considered in evaluating new vaccines against TB. However, 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 lungs and spread to the liver and spleen. The infection is controlled but not eliminated by CMI, especially by 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 exhibits 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 TGF-β, 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) and recombinant BCG (20) or determining immunogenicity and protection after priming with BCG and booster with subunit
or DNA vaccines (18), measuring the rate of survival, pulmonary bacilli loads, and the extent of tissue damage following intratracheal challenge (21). 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 (22). 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 (22). 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, ribs are cut and 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 proteins better than formaldehyde, which fixes the proteins by
formation of molecular bridges that can produce significant modifications to the three-dimensional structure, compromising 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 (19). 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 (23–25).

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 (26). It 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 themselves 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 controls bacterial growth as well as the 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 (27–29). However, the mouse model is actually suitable for the first screening of vaccine candidates and evaluate 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 TB reactivation

MTB is a pathogen capable of causing progressive disease or asymptomatic latent infection (30). During latent infection, MTB can survive within the infected host cells for years or decades before disease reactivation (31). 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 (30). 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 (32, 33). In conditions in which the host immune response is suppressed, disease reactivation can result (31). 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 (33). 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 (34). 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 analyzed 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 (34).

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 (35). Guinea pigs have served as a significant model in past and current TB research (36). Robert Koch used these animals to establish his postulates and identify 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 (37), 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 (38–41). 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 (42). 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 (43). 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 (44–46) particularly those working on pathogenesis and therapy evaluation (47–50). 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 (44). 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 exhibits the spectrum of disease that represents many of the forms related to specific stages observed in humans (51, 52). 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 (53–54). Interestingly, inbred rabbits are more susceptible to TB than outbred animals (51). 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 (49). The tuberculous meningitis model in rabbits has been used for vaccination designed to prevent cerebral infection, comparing BCG with new designed vaccines (55).
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 (56–58). 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 (59). The severity of the disease is reduced when monkeys are immunized with BCG (56), which prevents 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 (55), 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 (60, 61).

Because of their close phylogenetic relationship with humans, macaques are often used to evaluate immunogenicity and safety of new vaccine candidates. Several studies have indicated that rhesus macaques are highly susceptible to TB (57, 62), while the closely related cynomolgus macaques are more resistant (63). 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 (64). 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 this 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 (65). 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 (66–70). 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 (71). 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 (72–75). 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 (76). 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 (77).

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 (78). 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 (79).
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 (80). 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 (81).

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 (81). 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 (82). 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 (83). 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 (84, 85).

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. Regarding 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.
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