Animal Models of Tuberculosis: Guinea Pigs

Simon Clark, Yper Hall, and Ann Williams

Microbiology Services, Public Health England, Porton Down, Salisbury SP4 0JG, United Kingdom

Correspondence: ann.rawkins@phe.gov.uk

The progression of the disease that follows infection of guinea pigs with *Mycobacterium tuberculosis* displays many features of human tuberculosis (TB), and the guinea pig model of TB has been used for more than 100 years as a research tool to understand and describe disease mechanisms. Changes in the bacterial burden and pathology following infection can be readily monitored and used to evaluate the impact of TB interventions. Demonstration of the protective efficacy of vaccines in the low-dose aerosol guinea pig model is an important component of the preclinical data package for novel vaccines in development, and there is a continual need to improve the model to facilitate progression of vaccines to the clinic. Development of better tools with which to dissect the immune responses of guinea pigs is a focus of current research.

Guinea pigs are synonymous with experimentation and this animal has been used to model several diseases of man (Padilla-Carlin et al. 2008). From the studies performed by Robert Koch to identify the causative agent of tuberculosis (TB) to the present day, the guinea pig has played its part to understand disease processes, to define important components of *Mycobacterium tuberculosis*, and to test the potency of vaccines and therapies. In general, researchers have favored the outbred Dunkin–Hartley strain of guinea pig, although inbred strains are available. It is possible to induce disease with very low doses of *M. tuberculosis*, particularly when delivered by the aerosol route (Smith and Harding 1977). Guinea pigs are therefore more susceptible to TB than other model species and this has both advantages and disadvantages. Infection with *M. tuberculosis* can be established following injection into

various body compartments or instillation to the lungs via the trachea or intranasally but the most relevant and well-characterized guinea pig model of TB uses the aerosol route of challenge. There have been many excellent reviews written about the aerosol infection guinea pig model of TB and these are referenced frequently to provide the reader with further details. The aim of this article is to give an overview of the main features of the model, highlighting what has been learned through using the guinea pig to study TB, and to suggest areas for refinement and development in the future.

WHAT IS THE MODEL?

Clinical Picture

Although guinea pigs are susceptible to TB, outwardly the signs of infection are only apparent

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in the latter stages of disease. Weight loss is a sensitive indicator of progressive infection, and it is usually the most significant component of clinical scoring systems that are used to monitor animal welfare and trigger a humane end point, whereby the animal is killed prior to suffering. General behavior, condition of the coat, and changes to normal feeding and drinking patterns are other components of the humane end point, which is sometimes referred to as a modified Karnofsky score (Williams et al. 2009). Temperature changes have been suggested as an early indicator of disease outcome (Grover et al. 2009), but temperature monitoring is not adopted widely.

Infection invariably leads to death of the animal, but the time taken to reach the humane end point varies according to the initial dose and virulence of the strain of *M. tuberculosis*. Initial studies performed to establish and characterize the guinea pig model of TB infection were based on very low-dose aerosol inoculation. This “rational animal model of TB” was developed by Smith and colleagues and is expertly described by McMurray (1994). Unsensitized animals infected with less than five bacilli succumbed to disease from week 14 postchallenge, but even after 40 wk, 20% of the animals had survived (Wiegshauss et al. 1970). In contrast, studies performed more recently generally use aerosol doses of 20–50 bacilli and, even

though this is still regarded as low-dose, the progression to severe disease is more rapid and 100% of animals have met humane end points by week 30 (Brandt et al. 2004; Williams et al. 2009; Grover et al. 2012). Even higher aerosol doses (~500–1000) have been used, which result in accelerated times to humane end points of between 8 and 20 wk. This very high-dose challenge model has been used in vaccine evaluation studies to show improved efficacy over BCG (*Bacillus Calmette–Guérin*) (Williams et al. 2005a), but, because of the very acute nature of the disease that develops, high-dose challenge is less clinically relevant.

M. tuberculosis Replication

The pattern or profile of multiplication of *M. tuberculosis* in the lungs and spleens of guinea pigs following aerosol infection has been comprehensively described by Alsaadi and Smith (1973). In the lung, as illustrated in Figure 1, an initial period of logarithmic growth up to ~16 d postchallenge, is followed by an extended period of many weeks during which the bacterial load is stable, neither increasing nor decreasing. This profile of bacterial load is similar in animals infected with different doses, but dose affects the maximum concentration attained in the lung and this is proportional—a 10-fold increase in the dose results in a 10-fold increase in the num-

Figure 1. Bacterial load (mean log₁₀ colony forming units per mL of tissue) in the lungs of guinea pigs following low-dose (10- to 20-CFU) aerosol infection with *M. tuberculosis* H37Rv. Solid circles show unvaccinated animals; open circles are animals vaccinated with *Bacillus Calmette–Guérin* (BCG) (5×10^4 CFU, 12 wk before aerosol infection). Error bars are standard error of the mean.

ber of bacteria at ~ 16 d postchallenge. Ultimately, the control of bacterial replication is lost and a further increase in bacterial load is synonymous with the humane end point (Williams et al. 2009).

In the spleen, there is an initial period in which no bacteria are detected by culturing spleen samples on agar and this is ~ 14 d postchallenge (Alsaadi and Smith 1973). It is entirely possible that viable bacteria are present before this but they are below the threshold of detection. Dissemination to the spleen has been used as a sensitive way to compare the virulence or fitness of mycobacterial strains, with an increased rate of dissemination being associated with higher virulence (Balasubramanian et al. 1992; Williams et al. 2005b). Similar to the profile in the lungs, a stable control of replication occurs from ~ 4 – 6 wk postchallenge, and this is maintained until animals meet humane end points.

Enumeration of bacterial load in lungs and spleens is frequently used to define the burden of disease, and this is usually measured by culturing homogenized tissue on appropriate agar plates. The colonies that arise are counted and the bacterial load is commonly expressed as colony forming units (CFU) per mL or gram of tissue. Culture on solid agar has limitations, and there is a consensus that a subpopulation of bacteria may be viable even if not culturable. Alternative techniques to measure viability have been described, which include using fluorescently tagged bacteria or viability stains, but these currently lack the reproducibility and sensitivity of culture on agar. Broth culture using automated detection systems to identify metabolic activity offer an alternative to CFU that might be more sensitive and reliable.

Pathology

Pathological changes that occur in the lungs and extrapulmonary sites of guinea pigs following aerosol infection have been described extensively in the context of previously naïve animals, and these observations have been fundamental to the understanding of experimental TB in guinea pigs (Smith and Harding 1977; Turner

et al. 2003; Basaraba et al. 2006; Basaraba 2008; Palanisamy et al. 2008). Key features of the pathology include human-like granulomas with central necrosis enclosed by lymphocytes, macrophages, and multinucleate giant cells and a fibrotic capsule. Cavitation is observed only rarely—for example, with certain strains of *M. tuberculosis* (Kato-Maeda et al. 2012).

Comparative assessments of pathology have become an accepted means of defining the pathogenicity of strains or evaluating the efficacy of treatments, such as vaccines or drugs (Dascher et al. 2003; Basaraba 2008; Ordway et al. 2008). There is an increasing trend to introduce more quantitative assessments of pathology using a scoring system that is used by a certified pathologist to record the key changes in terms of their severity or quantity (Williams et al. 2005b; Palanisamy et al. 2008). An illustration of the type of data generated when comparing vaccines using such scoring system is shown in Figure 2C.

Immune Response

The guinea pig was frequently used in early studies to determine the effect on the immune system of tuberculosis infection or inoculation with mycobacterial components. Lymphocyte proliferation assays and dermal reactivity were used to characterize and compare the delayed-type hypersensitivity reactions induced, and guinea pigs have continued to be useful for evaluating reagents which are used for diagnostic skin tests (Haslov et al. 1984, 1995; Malaghini et al. 2011). However, compared with other laboratory animal species, there has been limited development of immunological reagents specific for the guinea pig. Despite this, key features of the guinea pig response to *M. tuberculosis* and BCG have been studied by using methodologies such as antibody blocking, bioassays, and relative gene expression, including a boutique microarray covering key cytokines, chemokines, and their receptors/ligands (Tree et al. 2006). These studies have shown the importance of both the innate and adaptive arms of the immune system as summarized by Padilla-Carlin et al. (2008) and the role of CD1-restricted T cells (Hiromatsu et al. 2002) in the interaction

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Figure 2. Examples of measurements of disease used to evaluate the efficacy of vaccines. (A) Bacterial load measured at week 4 postinfection, data from unvaccinated (solid circles) and BCG-vaccinated (open circles) animals are shown; each circle is an individual animal. (B) Kaplan–Meier survival plot showing unvaccinated (solid circles, solid line) and BCG-vaccinated (open circles, dashed line) animals and various test vaccines. (C) Semiquantitative scoring system showing extent of consolidation (mid-gray), caseation/necrosis (dark gray), and calcification (light gray), for unvaccinated animals, BCG-vaccinated animals, and animals vaccinated with test vaccines. (D) Changes in body weight measured over time postchallenge relative to the weight at challenge. Mean percentage weight change ($\pm$ S.E.M.) is shown for each group of animals, which were either unvaccinated (solid circles), BCG-vaccinated (open circles), or given a test vaccine (open triangles).

of *M. tuberculosis* with the host. Ordway et al. (2007) have pioneered flow cytometry techniques for characterizing the changes in the cellular response to *M. tuberculosis* in relevant tissues such as lungs, spleen, and lymph nodes. Using this technique in combination with quantitative polymerase chain reaction (qPCR), Ordway and her colleagues have been able to track changes in lymphocyte populations and have revealed important differences in the response to clinical isolates of *M. tuberculosis* in comparison to laboratory strains, notably the induction of regulatory T cells by highly virulent strains belonging to the W-Beijing genotype (Shang et al. 2011). However, the lack of well-defined, commercially available assays with which to monitor immune responses to vaccines has largely restricted the guinea pig model to efficacy testing, with immunogenicity data derived from mouse studies. This is less than ideal because the differences between mouse and guinea pig immune systems do not enable protection to be correlated with immune response.

WHAT DID WE LEARN FROM THIS MODEL?

Analysis of the subject matter of 550 publications (from 1980s to present) involving tuberculosis and guinea pigs reveals that this animal

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model has contributed widely to our knowledge of the disease, across several disciplines. The distribution of subject categories is shown in Figure 3.

Vaccine Research

A large proportion of the publications describe vaccine evaluations in the guinea pig, because guinea pigs are the preferred small animal model to show the protective efficacy of vaccines. Advantages of guinea pigs over mice include their greater size, enabling more analyses to be performed in the same animal, and the wider range of vaccine types that can be tested compared with mice, which lack some immunological features (e.g., CD1b that are needed to respond to glycolipid antigens). In the absence of positive clinical efficacy data, the predictive value for humans of data obtained in guinea pigs remains unknown but demonstration of efficacy in this model is currently the main “gatekeeper” to progression of a vaccine toward clinical development. Efficacy of the vaccine is measured either by a reduction in the bacterial load in lungs and spleens at 4–5 wk post-infection or by the mean survival time over a prolonged follow-up period, compared with positive (BCG) and negative (unvaccinated)

Figure 3. Pie chart showing distribution of the subject matter of 550 publications from 1982 to the present. The source of the publications was an NCBI (National Center for Biotechnology Information) PubMed search using the term “guinea pig tuberculosis.”

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controls. Evaluations based on bacterial load have increased statistical power to discriminate vaccines and are more rapid and less costly than survival studies, but the latter may be preferred to test the long-term impact of vaccination to prevent severe disease. Subjective or semiquantitative assessment of the pathology at the time of necropsy is also made and, in some studies, improved weight gain over the course of the postchallenge period has been used to define protection (Horwitz et al. 2000). Figure 2 shows examples of these efficacy readouts. Several reviews are recommended for further information, all of which conclude that the guinea pig model has provided essential preclinical efficacy and safety information on vaccine candidates (Orme et al. 2001; McMurray 2003; Orme 2005, 2006; Gupta et al. 2007; Williams et al. 2009).

In addition to its use as a means of proving/confirming efficacy, the guinea pig model has played a key role in identifying antigenic targets for incorporation into vaccines. This was particularly evident in the early days of antigen discovery as described in the previous section of this review. More recently, the key measure of immunogenicity has been the ability to elicit a Th1 response, characterized by the production of interferon γ , which is more readily tested in mice than guinea pigs, and, therefore, mice now tend to be used to test for putative antigenicity.

A less prominent but very important role of the guinea pig in vaccine research has been in preclinical product development. Guinea pigs are used among the battery of tests to show the safety of live attenuated vaccines according to EU regulations and guidelines, and as illustrated in the development of novel live vaccine candidates (Walker et al. 2010; Arbues et al. 2013; Grode et al. 2013). In the future, it would be preferable for such tests to be replaced by in vitro alternatives that would allow, for example, batch-release assays to be conducted without the need for animal testing.

Host–Pathogen Interactions—Identification of Key Pathogenic Mechanisms

Around one-quarter of the publications were related to the use of guinea pigs in understand-

ing host–pathogen interactions, either through the careful study of the disease progression or via screening gene knockout *M. tuberculosis* mutants for their ability to cause disease. This has led to the identification of key pathogenic mechanisms of *M. tuberculosis*. The model has also revealed a number of important, host-related factors that have an impact on the susceptibility of individuals to TB. The effect of malnutrition has been extensively studied and is reviewed by Cegielski and McMurray (2004), who describe the detrimental impact of restricting dietary zinc, vitamin D, and particularly protein on the resistance to *M. tuberculosis* infection. The importance of regulation of iron by both the host and pathogen has been clearly shown in guinea pig studies (Basaraba et al. 2008; Thom et al. 2012). Similarly, studies in the guinea pig showed the strong association of hyperglycemia with exacerbation of disease, which may explain the link between TB disease and diabetes (Podell et al. 2012).

Drug Development

Although mice are commonly used to screen compounds for their potency to kill *M. tuberculosis* in vivo, testing of drug efficacy in guinea pigs is considered important because a wider range of pathological lesions develop and these are not only more reflective of human pathology but also represent a more stringent target for chemotherapy (Basaraba 2008). The necrotic granulomas that are a feature of guinea pig lung pathology are proposed to harbor bacteria that are refractory to standard chemotherapy regimens. Lenaerts et al. (2007) clearly showed the presence of extracellular, biofilm-like clusters of acid-fast bacilli in granulomas of guinea pigs, and this was associated with the failure of standard chemotherapy to sterilize the lungs. Therefore, guinea pigs are a highly relevant species to test novel drugs or regimens to show activity against persisting bacilli.

Studies of *Mycobacterium bovis* Infection

Despite the opportunities to study infection caused by *Mycobacterium bovis* in the natural

hosts (e.g., cattle and deer), guinea pigs have proven useful as an experimental tool to develop diagnostic skin test reagents (van Pinxteren et al. 2000), screen mutants (Collins 2001), or perform early-stage down-selection of vaccines intended for use in cattle or wildlife (De Lisle et al. 1999; Chambers et al. 2002; Clark et al. 2008).

FUTURE PERSPECTIVES FOR THE MODEL

Vaccine Evaluation and Natural Transmission Studies

It is likely that the guinea pig model will remain an important in vivo system to test the potency of interventions to reduce disease burden, at least until a reliable marker of protective immunity is defined. Researchers with expertise in the model are continually seeking ways to improve the model and advances in methods to quantify disease burden, such as advanced imaging and the use of bacterial reporter strains, are on the horizon. Developments that allow disease progression to be monitored in real time in the same animal will help to clarify which are the optimal readouts to define protective efficacy. There is a renewed interest in understanding natural transmission of TB with a view to improving control measures. In the original natural transmission studies performed by Riley in the 1950s (Riley 1961), guinea pigs were used to sensitively “capture” tubercle bacilli, which were shed from patients with TB disease, and similar experimental systems are being developed (Dharmadhikari et al. 2011) with funding initiatives such as the Bill and Melinda Gates Foundation, TB Vaccine Accelerator grants. The reliance on the guinea pig model has sparked several new initiatives to develop immunological reagents and assays to monitor responses to exposure/infection. These new tools will substantially improve vaccine evaluation studies by allowing vaccination schedules to be optimized before performing lengthy and costly challenge studies and will additionally enable correlates of protection and immune markers of disease progression to be identified.

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Postexposure Vaccination and Clinically Relevant Scenarios

Although mice are the more commonly used species to test therapeutic or postexposure vaccines, guinea pigs might be considered to measure both efficacy (to prevent reactivation of persisting organisms) and the safety of vaccines or immunotherapies because the characteristic necrotic granulomas, which develop in guinea pigs, are an environment that has been associated with persistence of bacteria and the expression of antigens believed to be important in latent *M. tuberculosis* infection.

Guinea pigs have been shown to be useful to study TB in the context of other underlying factors such as diabetes and malnutrition. The development of immunological tools should make possible the manipulation of host factors to determine their specific interactions with *M. tuberculosis*.

CONCLUDING REMARKS

The guinea pig has been the faithful servant of the TB researcher for more than 200 years, providing information to understand disease processes and identify virulence determinants of *M. tuberculosis*. It has enabled preclinical safety and efficacy screening of new drugs and vaccines, which are urgently needed. Although overshadowed for many years by mouse models, which have allowed identification and study of the immune responses to *M. tuberculosis*, guinea pigs have generally been regarded as the more relevant species to replicate human disease. New immunological tools are being developed, sparked by the use of guinea pigs as recipients of naturally transmitted *M. tuberculosis*; such tools will further increase the usefulness of the guinea pig in the future.

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