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Review

Experimental animal modelling for TB vaccine development

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ABSTRACT

Research for a novel vaccine to prevent tuberculosis is an urgent medical need. The current vaccine, BCG, has demonstrated a non-homogenous efficacy in humans, but still is the gold standard to be improved upon. In general, the main indicator for testing the potency of new candidates in animal models is the reduction of the bacillary load in the lungs at the acute phase of the infection. Usually, this reduction is similar to that induced by BCG, although in some cases a weak but significant improvement can be detected, but none of candidates are able to prevent establishment of infection. The main characteristics of several laboratory animals are reviewed, reflecting that none are able to simulate the whole characteristics of human tuberculosis. As, so far, no surrogate of protection has been found, it is important to test new candidates in several models in order to generate convincing evidence of efficacy that might be better than that of BCG in humans. It is also important to investigate the use of "in silico" and "ex vivo" models to better understand experimental data and also to try to replace, or at least reduce and refine experimental models in animals.

Contents

Introduction

Control of tuberculosis (TB) on a global scale requires the urgent development of a vaccine or vaccines which can be applied both prophylactically and post-exposure in order to be deployed as a major preventive intervention. Since 1924 the Bacille of Calmette

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Guerin (BCG) has been available and has been the most widely distributed vaccine in humankind, administered predominantly in neonates, mostly after the Second World War. In 1993, the WHO declared tuberculosis a global emergency[1] because of the re-emergence of the disease, and there was recognition that existing control measures, including the standard antibiotic regimens and BCG vaccination were insufficient to overcome factors such as drug resistance and HIV/AIDs which were driving the TB epidemic. There was a renewed interest to fully ascertain the protective efficacy of BCG, which appeared to be not homogeneous among different populations and to be mainly restricted to preventing severe forms of the disease in children, with a limited ability to prevent lung TB.[2] Since the 1993 WHO declaration there has been progress in halting and even reversing the trend for an increase in cases and deaths, but in order to meet new WHO targets to end TB by 2035,[3] it is absolutely clear that new tools, including vaccines which are better than BCG, will be needed. In order to achieve better efficacy than BCG, either the magnitude (strength and duration) of the BCG-induced immune response could be increased or a broader or even fundamentally different type of immunity might be required.

Do we know what protects against TB?

The search for a new vaccine triggered a resurgence in investigations to identify the immune mechanisms which are associated with protection. Unfortunately, even though immune protection appears to be related to the cellular compartment of the immune response, there is not a clear idea about which particular parameter is correlated with protection. The key role of interferon gamma (IFN-g) in the control of TB infection was soon apparent,[4] but it subsequently became clear that this was not a single and sufficient mediator of protection, as demonstrated by studies which showed that *Mycobacterium tuberculosis* (Mtb) infection itself is able to induce as good an IFN-g response and protection as BCG vaccination[5] and the fact that not all vaccines which induce high IFN-g responses are able to protect against infection. In an attempt to identify an immune mechanism which could define a protective response rather than a response to infection itself, there was a hypothesis that multifunctional T-cells, as demonstrated in *Leishmania* infection,[6] were associated with protection. This turned out not to be the case for TB as a prospective BCG trial showed that these cells are not surrogates of protection.[7] This fact was later confirmed in the phase 2b efficacy trial which evaluated the boosting of BCG with MVA85A in infants.[8] However, recently published studies evaluating immune correlates associated with the risk of developing TB disease have identified activated CD4+ T-cells as being associated with increased risk but reinforced the role of IFN-g in showing that BCG-specific IFN-g secreting T-cells are associated with a decreased risk.[9]

Do we know the Natural History of TB?

To further complicate the issue, even the natural history of the disease is not clear. There is still not a clear understanding of what mechanism triggers the evolution from infection to disease. The clinical pragmatism probably explains why this question has not had priority for a long time. In particular, strategy against TB has tried to classify persons into three general groups: non-infected, infected and ill. The classification between infected and ill has had some benefit in the respect that a precise treatment for both has been set. Infected persons are those with a tuberculin skin test (TST) positive and no lesion greater than 10 mm in the chest X ray. On the contrary, those who were TST positive and with lesions greater than 10 mm were considered to have TB disease. For the infected persons 6-9 months of INH treatment can be given; for the diseased persons, 2 months with INH, RIF, PZA and ETB plus 4 months with INH and RIF. This treatment strategy has been quite successful for a long time, before the appearance of multidrug resistance, HIV/AIDs and globalization appeared.[10]

Now we are “rediscovering” that TB pathology cannot be only limited to infected or ill people, and that there is an evolution of a spectrum of lesions.[11] In addition, lesions of several ages can be found in the same host, in concordance with the cellular nature of the immune response.[12] We are also rediscovering that large lesions and cavitation are related with exudative responses, i.e. with the neutrophilic attraction to the infectious foci, a fact that is specially favoured at the upper lobes, as it was largely described in the pre-antibiotic era.[13,14]

Therefore, in order to be successful in the discovery and evaluation of vaccination strategies, it is essential that the most appropriate experimental systems are used, taking into account the likely immunological mechanisms which need to be induced and considering the pathological status of the infected host.

The use of Experimental Modelling for Vaccine discovery

In this scenario, the use of experimental models with laboratory animals has had a very clear target: to obtain a better vaccine than BCG. Especially in terms of efficacy, but also in terms of safety, taking into account comorbidities like AIDs that have dramatically increased cases of BCGitis, with a high incidence of fatal outcomes.[15,16] It is not expected to be possible to find one single model which is able to reproduce what is thought to be the situation in humans, not least because there are multiple facets of TB disease in human populations. Every animal model gives us certain information that should be useful for the development of a new vaccine, but it is essential that we discern between prophylactic, boosting and therapeutic vaccines, depending on the particular target population or setting i.e.: in neonates, to boost neonate vaccination or adolescent memory, and in already infected subjects.

Murine models

Mice have been the most extensively used animal for vaccine discovery for several reasons. The most obvious is the economic one, which leads to the mouse model being the most widely used and characterised, such that there is a larger body of data available making it more possible to perform comparisons with humans because both species have been deeply studied.[17–20] In addition, due to the mice being in-bred, it is more feasible to standardise this model between laboratories.

The low dose aerosol

“Popularized” by Ian Orme, this mouse model soon appeared to be a hallmark in the screening of new vaccines.[21] The model was based on the inoculation of about 50 Mtb colony forming units (CFUs) in the lung through an aerosol with droplets of about 2 um diameter to reach the alveolar space, usually through use of a Middlebrook chamber device. Usually, the mouse strain used is the C57Bl/6, and new vaccine candidates must reduce the bacillary load in the lung by at least 0.7 $\log_{10}$CFUs compared with un-vaccinated controls at week 3 post challenge. This is the protection obtained after BCG vaccination and therefore a novel vaccine candidate would be expected to reduce this further.

So far, the majority of the vaccines tested have only weakly increased this protection even if significantly, but none of them have been able to prevent infection.

From the pathology point of view, this infection is characterized by the growth of the bacillary load for the first two weeks, up to a 1×10^5 CFUs in the whole lung without the induction of

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lesions, thus reflecting a "unicellular" phase of the infection.[22] Granulomas appear at week 3 post infection, when the bacillary load is around 1×10^7 CFUs. These granulomas are characterized by a mixture of infected macrophages with rafts of neutrophils. Infection is controlled afterward and the bacillary load decreases to 1×10^6 CFUs at week 6, and the lesions are characterized by the presence of necrosis (depending on the Mtb strain used), epithelioid cells with no presence of acid fast bacilli, surrounded by a ring of lymphocytes and an outermost ring of foamy macrophages, some of them infected, reflecting the "escape" of bacilli from the lesion.[23–25] This has been seen with more or less severity depending on the mouse strain. In particular there is less severity in C57BL/6 and BALB/c, while it is more important in DBA/2 and 129/Sv.[26] The severity is mainly related to the size of the lesions, mainly as a consequence of the increased size of the foamy macrophage ring.

The murine scenario reflects poorly the spectrum seen in humans as it is characterized by a mixture of controlled lesions, proliferative ones, without presence of exudative infiltration and cavitation. The other particularity of the mouse model is that lesions progress arithmetically,[24,25] which could reflect a disease infiltration in immune-depressed patients. This sort of progression has been considered as tolerant, as it allows the survival of the host for a long time, while at the same time allowing a large bacillary load with a progressive infiltration of the lung, which is what in the end kills the animal.[27]

Recently, new prospects for the mouse model have emerged with the C3HeB/FeJ mouse. It was long described by the Kramnik team that this mouse strain develops unusually large lesions.[28] Subsequently it was shown that the large lesions had liquefaction, similar to that found in the model of infection reactivation in SCID mice,[29] that was not considered a good "human-like" model to study the evolution from infection to disease.[30] In the C3HeB/FeJ model the neutrophilic infiltration to enlarge lesions and induce liquefaction is paramount, as it is in humans,[14] and consequently several groups are starting to use it to evaluate new vaccine candidates.[31,32]

In addition to the consideration of mimicking more human-like pathology, it is important to consider the nature of the natural challenge. Taking into account the fact that close contacts of an active TB case are those subjects with higher probability to acquire TB, and that receive multiple consecutive infections, it seems logical to explore the influence of new vaccines to control the infection under these conditions.[33]

The murine latent TB model

With the aim to explore the usefulness of a therapeutic vaccine to reduce the chemotherapy treatment of latent tuberculosis infection (LTBI), a new model was set to address the capacity of vaccines to destroy dormant bacilli.[34] Mice are infected with a low dose aerosol and infection progress until week 6, when the antibiotic treatment is started and continued until week 16. At this time point only persistent bacilli are present in the lesions and this is when the therapeutic vaccination is tested. Reduction of bacillary load in the lungs compared to control groups is measured at week 23. This model is the standard for evaluating therapeutic vaccines, and has been widely used so far.[22,34,35]

The intratracheal model

This model is based on the inoculation of a large dose of Mtb intratracheally (1×10^6) in BALB/c mice causing an aspirating pneumonia. Even though this does not appear to follow the natural history of Mtb infection, it has been used to evaluate the capacity of vaccination to reduce the pathology. This was mainly tested with *M. vaccae*, being the basis for considering this vaccine as being responsible for changing the Th2 response to Th1.[36]

Guinea Pig

The guinea pig model has been widely used as a diagnostic tool for detecting Mtb in clinical samples until culture media could demonstrate similar sensitivity. It is usually described as a susceptible model but, unless very high challenge doses are used, infected animals remain clinically well for many weeks post-challenge – a facet which makes this model difficult to use for studies where survival is the main read-out.[39] The main characteristic of this model is the extreme reaction against the bacilli, which has a very important parallelism with the exudative lesions of humans although curiously, this exudative response is very much dominated by eosinophils.[37] In this model secondary lesions occur as a consequence of blood dissemination[38] and a notable feature is the major destruction of pulmonary lymph nodes.[39] The progressive pathology leads to severe disease in the animals, from around 10 weeks post infection (depending on infection dose), as a result of excessive inflammatory response against Mtb.[27] The parallels with human TB features which are found in the guinea pig model have led to it being the most commonly used system to further evaluate vaccine candidates which have performed well in the initial screening in mice.[40] In this model BCG vaccination shows a relatively strong protection compared to that seen in mouse models[41] and it can be difficult to detect a clear effect of vaccine candidates that are better than BCG.[40] Several attempts have been made to simplify the model to obtain equivalent information, looking to detect differences in CFU at week 4, or by using a high dose challenge,[42] although the latter results in a rapid disease progression which could potentially overwhelm vaccine-induced effects.

LTBI model have been also used in the guinea pig, after short term chemotherapy from week 4 post challenge.[39,43]

Non-human primate model

The use of non-human primates (NHP) is very attractive because of the close evolutionary relationship with humans, which makes the immune response very similar to that of humans,[44] and makes possible the use of human reagents but more importantly means that immunological analyses performed in NHP can be directly compared to those generated in clinical studies. This opens the possibility to perform parallel studies in humans and NHPs which have the potential to identify immune correlates of protection or disease, which are then directly applicable to humans. Overall, the natural history of the infection tends toward a constant dissemination, and to the presence of exudative lesions that can evolve to cavitated lesions.[45] Very relevant have been the studies using CT and PET-CT, which allow the evolution of lesions in different evolutionary phases, which leads to a better understanding of the evolution from infection to disease.[43,45]

One of the challenges in using NHPs to test new vaccine candidates for the ability to perform better than BCG is that there can be variable responses after BCG vaccination, depending on which macaque species is used.[45–47] In one study, where very high dose challenge was used, BCG showed almost complete protection in cynomolgus macaques, while rhesus were not protected at all.[47] Further light was shed on this by a study which demonstrated that ultra-low dose aerosol infection of rhesus macaques resulted in a more progressive disease than the same dose delivered to cynomolgus macaques, which showed a reduced disease burden.[48] Thus, the inherent susceptibility of the macaque species is an important factor in determining whether protection may be observed with a vaccine. This diversity in susceptibility can be also seen in the same species of macaque as shown for cynomolgus macaques which vary, depending on the particular origin of the animals.[49] There are other important parameters to consider with

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NHP models such as the challenge dose and route. When evaluating the MVA85A vaccine which was subsequently tested in a clinical trial,[7] Verreck et al. demonstrated that BCG boosting with MVA85A offered a significant protection[50] better than BCG but this result was not correlated with the clinical trial. Many different factors could have accounted for this discrepancy, such as differences in Mtb strains and differences in the environment between an experimental facility and the real world.

However, none of this diminishes the importance of the NHP model for demonstrating the safety and efficacy of vaccines before entering clinical trials, and there are considerable efforts being made to maximise the value of these precious models by harmonising methodology and sharing expertise between the few groups globally who have the capability to perform vaccine evaluations in NHPs. This is imperative to ensure that only the vaccines with the greatest potential to protect in humans are taken forward to clinical trials.

Other small mammals: cotton rat, rat and rabbit

The use of rats as a model for vaccine testing has a quite short history. First of all, it was claimed that rats tend to control TB infection to the extent of being able to sterilize the lesions.[51] Even though this aspect has not been reproduced by other groups, it is apparent that the lesions are better controlled than in mice (around 1×10^4 CFUs in the lungs). The quality of the lesions is similar to those seen in mice, but with much less foamy macrophage infiltration, and BCG vaccination offers a relevant reduction in terms of number of granulomas and bacillary load.[52] This is also the case with the cotton rat, which has been shown to control infection, although this aspect has not been validated. Cotton rats also develop mouse-like lesions and can be protected by BCG vaccination.[53]

The rabbit model has been used for a long time. The major bulk of the information on this model comes from the laboratory of Max Lurie and Arthur M. Dannenberg.[54] The model was very attractive because for several years those authors were able to experiment with two different strains, a susceptible and a resistant one. Interestingly, the resistant one was able to develop exudative lesions and cavitation. The conclusion was that cavitation was the best way to drain bacilli and to protect the host.[55] In this context, the efficacy of vaccines was measured as a decrease in the number of grossly visible primary tubercles.[56] The lack of specific reagents together with the space demanding conditions has made this model very marginal in the vaccine development.

Large mammals: goats, cows and pigs

Large mammals, like humans, have an additional protective mechanism to stop the progression toward active TB. Because of the size of the lung, and the mechanical needs for the breathing process, the parenchyma is not homogeneous but organized in a net of interlobular septae that segments the organ in pieces of around 1 cubic centimetre. This is to allow the transmission of the strength elicited by the diaphragm in order to inflate the lung. Interestingly, these septae are very sensitive to any lesion in the parenchyma and tend to encapsulate it.[14] This was first observed in the mini-pig model, in which it was not possible to reproduce an exudative lesion, and thus large lesions, even after locally inoculating up to 1×10^3 bacilli.[57] In this model only therapeutic vaccination has been tested, showing an increase in the maturation of the lesions, thus limiting the drainage of bacilli from them and also the reinfection process.[57]

The use of cows as a model of TB vaccination comes from the very beginning of TB vaccine development. In fact, BCG vaccine was tested initially in this model, showing from the very beginning a partial effectiveness.[58] TB infection in cows offers a wide spectrum of the TB disease, resembling very much what is found in humans, from controlled lesions, encapsulated and calcified, to exudative cavitated lesions.[59] This is also the case with goat models, where vaccination has demonstrated a significant reduction on the lesions' size, measured through tomography.[60] In both cases what is significant is the difference in terms of virulence demonstrated after Mtb or *M. bovis* infection, low and high, respectively. In both models it is interesting to note that natural infection can be studied among the herds, which is a very interesting model to test vaccines in "natural" conditions, although always with the difference to test natural infection with *M. bovis*.[61]

A need for replacement, refinement and reduction. The 3R policy

Recently a very interesting review has been published reviewing the use of TB models in the context of the 3R policy.[62] It is important to note the remarkable work to develop ex-vivo models for the detection of bactericidal activity using whole blood[63] in immunized subjects, or by examining the splenocyte bactericidal activity.[64] Another important initiative to be highlighted is the efforts made with mathematical modelling in order to better understand the experimental data from both clinical and experimental modelling resources,[65] from the point of view of understanding the evolution of TB natural history[66] or the design of vaccines.

Conclusions

The discovery of a new TB vaccine able to significantly reduce the TB disease or infection is one of the major challenges for the current scientific community. The lack of a validated surrogate of protection, together with a precise knowledge of the natural history of the infection and its evolution toward active TB, makes this goal even more difficult. Current experimental models using laboratory animals must be correctly interpreted and analysed. Interestingly, the model that was the most used as a pre-screening, the murine one, has traditionally used a tolerant strain (the C57BL/6) that exhibits a limited pathological profile, showing a balanced immune response that does not correspond with the exaggerated inflammatory response, mainly exudative, that usually leads to disease induction in humans. On the contrary, the guinea pig response is always exaggerated, having a close relation with an allergy response (considering its eosinophilic infiltration) that always leads to disease, although BCG vaccination is remarkably efficacious. The rabbit model also displays "human-like" lesions, but has been poorly developed for the evaluation of vaccines. In this regard, the rhesus or cynomolgus models might be the more attractive models, showing the whole spectrum of human lesions, and being the closest species to humans and having the opportunity to conduct parallel human and NHP trials. Additionally, there is also an important opportunity to use developments in bioimaging that will allow a better understanding of TB natural history and to monitor the impact of vaccination on disease progression. There are some limitations to this model such as the variability observed related to the origin of the animals (although this can be taken into consideration), the scarcity of expertise and facilities to conduct these studies, but perhaps of more importance is the anatomy of the lungs, which due to their small size lack the interlobular septae that enable an efficacious encapsulation and control of the pulmonary lesions. In this regard, bigger mammals used as experimental models do have the interlobular septae net that allows the encapsulation of small lesions from the very beginning. Unfortunately, they also have limitations, such as the remarkable difference in the virulence shown by Mtb and *M. bovis* and the potential confusing factor of the complex stomach in the

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ruminant digestive system. Otherwise, both cows and goats show the whole human TB spectrum, and also can be used in the context of natural infection. The model in pigs avoids the complexity of the digestive system, and by using mini-pigs, also avoids the logistic problem of the size of the animals. However this model has only been developed in the context of latent tuberculosis infection. All in all, experimental modelling for TB vaccine discovery and evaluation has some room for improvement both by refining existing models in terms of making them more reproducible and relevant but also by the introduction of new systems, for example by using the C3HeB/FeJ mouse strain, that develops a complete human like pathology spectrum, and also the use of mini-pig to better understanding the protective role of local encapsulation through the interlobular septae. Additionally, the use of "ex-vivo" assays to determine the induction of bacillary killing are very interesting systems which can be used to provide informative vaccine efficacy data whilst reducing or refining animal use. Finally, mathematical modelling validated with experimental data appears to be very relevant for obtaining more value from the available information and to focus and integrate the wide variety of results coming from the different experimental models.

Conflict of Interest

None.

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