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Published in:
Tuberculosis

DOI:
10.1016/j.tube.2017.07.008

Publication date:
2017

Citation for published version (APA):
Pepponi, I., Khatri, B., Tanner, R., Villarreal-Ramos, B., Vordermeier, M., & McShane, H. (2017). A mycobacterial growth inhibition assay (MGIA) for bovine TB vaccine development. *Tuberculosis*, *106*, 118-122. https://doi.org/10.1016/j.tube.2017.07.008

Tuberculosis 106 (2017) 118–122

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

A mycobacterial growth inhibition assay (MGIA) for bovine TB vaccine development

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ARTICLE INFO

Article history:
Received 7 April 2017
Received in revised form
18 July 2017
Accepted 19 July 2017

Keywords:
Bovine tuberculosis
Vaccination
Correlate of protection
Mycobacterial growth inhibition assay

ABSTRACT

Human tuberculosis remains a significant cause of mortality and morbidity throughout the world. The global economic impact of bovine TB is considerable. An effective vaccine would be the most cost-effective way to control both epidemics, particularly in emerging economies. TB vaccine research would benefit from the identification of an immune correlate of protection with which vaccines could be gated at both preclinical and clinical levels. *In-vitro* mycobacterial growth inhibition assays (MGIA) are functional assays that include most aspects of the complex host immune response to mycobacteria, and they may serve as functional immune correlates for vaccine development. We applied to cattle an MGIA that was developed for use with human and murine samples. Several technical difficulties were encountered while transferring it to the cattle model. However, our data demonstrate that the assay was not discriminatory in cattle and further work is needed before using it for bovine TB vaccine development.

1. Introduction

Tuberculosis (TB) remains a major problem for both humans and bovines. In 2015, 1.4 million people died of TB [1] and more than 32,000 animals were slaughtered in Great Britain alone due to the test and slaughter policy used in developed countries to control bovine TB (bTB) in cattle [2]. There is also a considerable economic impact of bTB worldwide including in developing countries where bTB has zoonotic implications since *Mycobacterium bovis* (*M. bovis*) can cause TB in humans, particularly when unpasteurised milk is consumed. The main causative agent of bTB is *M. bovis*, a close relative of *Mycobacterium tuberculosis* (*M.tb*). *M. bovis* is also the source of the only licenced vaccine against TB [3], *M. bovis* bacillus Calmette-Guerin (BCG), which was attenuated from a cattle isolate of *M. bovis*. BCG is widely used in humans throughout the world as it does confer consistent and reliable protection against severe forms of TB in infants, although protection against pulmonary TB in adults is highly variable [4]. BCG vaccination of cattle has shown a comparable degree of protective variability, both at population and individual animal level, as seen in humans [5]. Currently, a leading approach in bovine and human TB vaccine research is the development of a boosting agent that will improve and prolong the immune response induced by BCG [6]. There are many reasons why it is advantageous to address bTB and human TB in parallel. Interdisciplinary collaborations will expand our knowledge with the information available from human, animal and environmental studies. Human and animal health are connected, as demonstrated by the existence of zoonoses.

In the absence of an immune correlate of protection with which to accelerate vaccine design and evaluation, vaccine development is costly and time-consuming. Protection against *M. tb* is likely to be multifactorial. An *in vitro* functional assay that measures the sum of the parts of a host immune response might have utility as a correlate of protection. *In vitro* mycobacterial growth inhibition assays are currently being developed and evaluated as a potential immune correlate for human TB vaccine research [7] and results

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http://dx.doi.org/10.1016/j.tube.2017.07.008

obtained using this assay in the murine model are promising [8,9]. The parallel development, in humans and animals, of an assay such as a mycobacterial growth inhibition assay (MGIA), will help us understand more about the models and the similarities and differences between species and their immune systems.

MGIA could prove useful for the evaluation of vaccine-induced protection and could ultimately reduce or even replace lengthy, costly *M. bovis* or *M. tb* infection experiments in cattle and other animal models, thus fulfilling the 3Rs principle to replace, reduce and refine the use of animals in scientific procedures (https://www.nc3rs.org.uk/).

Therefore, following a translational approach, we selected a BACTEC-MGIT based MGIA, originally developed by Cheon et al. [10] which had already been optimised for mice and humans [7,9]. The assay was adapted and evaluated for the assessment of BCG vaccine efficacy by measuring mycobacterial survival after incubation with bovine whole blood (WB) or PBMC.

2. Materials and methods

2.1. Cattle

Holstein-Friesian male cattle (approximately 6 months of age at the beginning of study) were recruited from officially TB-free farms in Great Britain. All animals were housed at the Animal and Plant Health Agency (APHA) at the time of blood sampling, and procedures were conducted under the remit of a United Kingdom Home Office license according to the Animals (Scientific Procedures) Act of 1986 (amended) and were approved by the local animal welfare ethical review board (APHA-AWERB).

2.2. PBMC isolation

PBMC were isolated from heparinised blood by density-gradient centrifugation with Histopaque® (Sigma) according to manufacturer's instructions.

2.3. Bacterial strains

Animals were vaccinated with approximately 1 million CFU of BCG Danish (SSI, Denmark), prepared according to manufacturer's instructions, and challenged *in vivo* with 8000 CFU of *M. bovis* strain AF 2112/97. For the *in vitro* challenge performed within the MGIA, BCG Pasteur grown in-house with 7H9 medium was used (titre: 1.4×10^6 CFU/mL).

2.4. Whole blood and PBMC MGIA

The protocol was adapted from a previously published human MGIA protocol by Fletcher et al. [7]. Briefly, either 300 μL of blood or 1 million PBMC in 300 μL of RPMI medium were added, in a 2 mL tube, with 300 μL of RPMI medium containing 1.167×10^4 CFU/mL of BCG Pasteur. Based on reference monocyte values for 6–12 month old bovines, this dose translated into an approximate MOI of 1:51 ± 17 for WB and 1:6 ± 1 for PBMC MGIA. WB or PBMC were incubated at 37 °C on a rotating platform for 90–96 h and lysed with sterile water. The lysate was transferred to Bactec MGIT supplemented with PANTA antibiotics and acid-albumin-dextrose-catalase (OADC) enrichment broth (all from Becton, Dickinson) and incubated in a Bactec MGIT 960 until detection of growth. The conversion of the TTP into CFU was achieved by means of a standard curve (data not shown). All samples were run in duplicate. Each experiment included duplicate control tubes inoculated with 2.5 μL of BCG stock, equivalent to 3500 CFU/tube, to monitor stock viability. A time to positivity (TTP) of 7 days was chosen as inoculum dose and the average TTP of the control tubes for all experiments was 169.8 h.

2.5. Flow cytometry

For PBMC surface staining, cells were incubated with LIVE-DEAD® Fixable Yellow Dead Cell Stain (YeViD, Invitrogen) and with α-CD3-Alexa488 (Monoclonal Antibody Center), α-bovine δ chain-Alexa405 (Kingfisher Biotech, Inc.), α-CD335-PE (AbD Serotec) and α-CD14-Alexa700 (BIO-RAD). All antibodies were bought unconjugated and labelled as indicated with the appropriate Zenon label (Life Technologies™), according to manufacturer's instructions. Prior to acquisition, cells were washed and fixed.

2.6. Statistical analyses

All statistical analyses were performed using GraphPad Prism (Graph-Pad, USA) with a non-parametric one-way ANOVA (Kruskall-Wallis) followed by Dunn's test of multiple comparison. Correlations were evaluated by means of the Spearman Rank Correlation Test.

3. Results

3.1. Assay optimisation

In order to adapt the assay to bovine samples we performed some optimisation experiments. Factors such as the temperature of blood storage following venesection and prior to processing for WB assays or the processing steps for PBMC were addressed to improve reproducibility. Briefly, to optimise the WB assay, whole blood was incubated at different temperatures (4 °C, RT and 37 °C) for up to 4 h before commencing the assay and the results showed no significant differences related to storage temperature. PBMC MGIA revealed an inconsistency in the number of CFU recovered from replicates, but this was improved by eliminating a centrifugation step which followed cell lysis. The protocol was modified accordingly (Supplementary Fig. SF1 and Supplementary Table ST1).

3.2. MGIA evaluation

To assess the potential of the MGIA to reveal differences in the control of mycobacterial growth between BCG-vaccinated and naïve animals, the assay was evaluated using samples from 24 animals, 12 vaccinated subcutaneously with BCG Danish SSI and 12 naïve controls. Fig. 1 shows the experimental schedule (Panel A) and the result of the MGIA obtained with both WB and PBMC (Panel B and C, respectively).

Blood samples were taken at 2, 6, 9, 12 and 14 weeks after BCG vaccination and employed in the WB-MGIA. At the same time, PBMC were isolated and stored. When all samples had been collected, PBMC from all time points were thawed and employed in a single experiment. In Fig. 1B and C, each symbol corresponds to a single animal. No significant difference in mycobacterial growth was detectable between BCG-vaccinated and naïve animals, using either WB or PBMC at any time-point assessed. WB-MGIA resulted in similar CFU numbers between the two groups for all time points analysed. In the case of PBMC-MGIA, CFU recovery decreased for BCG-vaccinated animals at weeks 9 and 12, but this trend towards higher growth inhibition in vaccinated calves compared to BCG-naïve animals was statistically not significant. Interestingly, in WB, a significant decrease in mycobacterial growth inhibition, irrespective of whether the animals were vaccinated or not, between early sampling times (week 2) and later tests (weeks 9, 12, 14) was found (Fig. 1B). This was not observed in PBMC (Fig. 1C).

3.3. Correlation of MGIA with cellular subpopulations

PBMC samples used in the assay were also analysed by flow cytometry in order to identify potential cell populations likely to be involved in mycobacterial growth inhibition. Antibody panels used contained markers for T lymphocytes (CD3), monocytes (CD14), gamma delta (γδ) T cells and natural killer (NK) cells (gating strategy described in Supplementary Fig. SF2).

Individual cell percentages were analysed against the result of the MGIA. Fig. 2 shows the relationships between the PBMC-MGIA and the cell subsets characterised at weeks 6 and 9 combined. These time-points were selected in order to focus on the immune response pre-challenge (for technical reasons not all data for week 2 were available). The results for monocytes, γδ T cells, NK cells and NK T-like cells are shown. A significant correlation was found only between the proportions of NK T-like cells ($CD3^{+}CD335+$) and the extent of growth inhibition *in vitro*, i.e. the higher the proportion of NK T-like cells present in the sample, the less CFU were recovered in the MGIA (Fig. 2).

4. Discussion

In this study, we applied a mycobacterial growth inhibition assay to bovine samples from BCG-naïve and BCG-immunised calves to assess the discrimination potential it could afford in this animal model.

The literature describes several attempts to develop an *in vitro* growth inhibition assay to estimate immunity against mycobacteria. Silver et al. had developed a cell-based assay where *M. tb* growth inhibition was achieved in infected monocytes incubated with autologous non-adherent cells from peripheral blood of both PPD-positive and PPD-negative healthy subjects [11]. A similar approach, although based on whole blood, showed that tuberculin-positive individuals were better at controlling growth of intracellular BCG *lux* than tuberculin-negative counterparts [12]. Wallis et al. employed the WB assay to measure the bactericidal potential of drugs combined with immune mechanisms of TB-naïve healthy subjects [13]. The innovation of his work included the use of the Bactec MGIT detection system based on liquid cultures instead of standard agar plates. Our work was based on the same detection system while the protocol had to be adjusted to suit our purpose.

Optimisation experiments were carried out to minimize the variability seen when using PBMC instead of WB in the MGIA. Assay variability was observed also by Fletcher et al. [7] who found that, for a human MGIA, variability was higher when using WB compared to PBMC. However, the variability in the human MGIA study referred to repeated sampling over time. One of the reasons suggested to explain this variability was the processing of WB in different experiments, while cryopreserved PBMC could be processed simultaneously. The variability between replicates in our PBMC assay could be improved by modifying the test protocol. In WB, we observed differences in MGIA results between different bleed time points rather than between replicates. This cannot be accounted for by assay variability as the positive control tubes used in every assay were giving highly consistent results between different experiments (Fig. 1, panel B, black squares and line). The WB-MGIA variability between sampling time points manifested itself as higher growth inhibition at early bleeds, which was reduced at later bleeds (week 9 and after, Fig. 1B). This could be due to different levels of innate immune cell numbers or functionality in these animals at earlier time points when the immune system of these young calves is still being re-modelled. This intensive innate immune system re-modelling in the first 6 months of age is a common observation in ruminants, particularly in the number of γδ T cells and NK cells [14]. This may have less of an impact using the more standardised approach of PBMC-MGIA. Unfortunately, we were unable to detect a significant difference in growth inhibition between BCG-immunised and BCG-naïve animals using either the WB- or PBMC-MGIA.

This is consistent with the findings of Carpenter et al. who used a cellular MGIA involving the addition of autologous lymphocytes to BCG-infected macrophages, and found no difference in the ability to inhibit mycobacterial growth between naïve and BCG-vaccinated cattle [15]. However, it is in contrast to previous

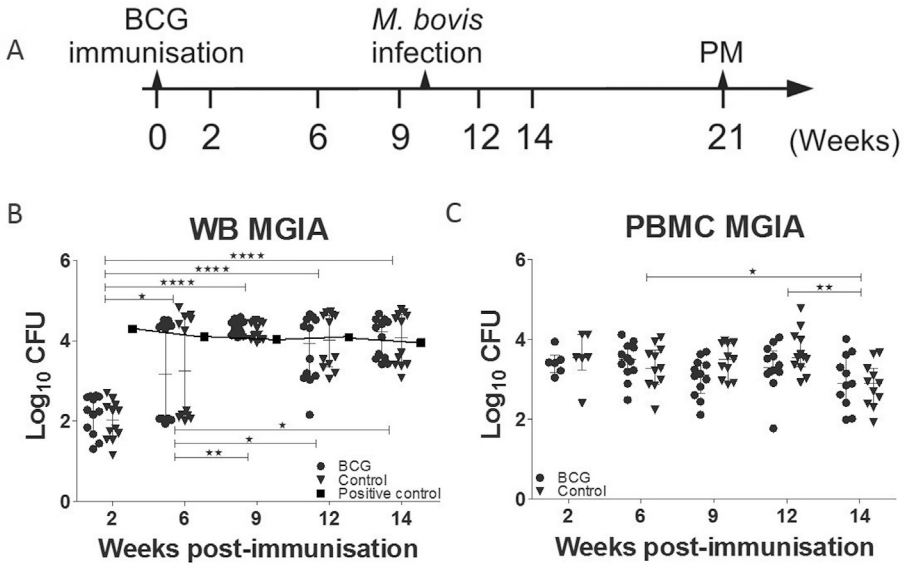

Fig. 1. Evaluation of the vaccine-effect detection level afforded by the assay. Panel A: time line for animal immunisation and blood withdrawal. Panel B: WB- and PBMC-MGIA at consecutive time points. Symbols represent single animals and bars show the mean and standard deviation. Statistical significance between time points was calculated considering all 24 animals as one group. Black squares on panel B, WB-MGIA, represent the assay internal positive control. Due to technical issues, the results for PBMC-MGIA at week 2 post-immunisation is based on 12 animals only, 6 BCG-vaccinated and 6 controls. Statistical analysis: non-parametric 1 Way ANOVA followed by Dunn's multiple comparison test. *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$.

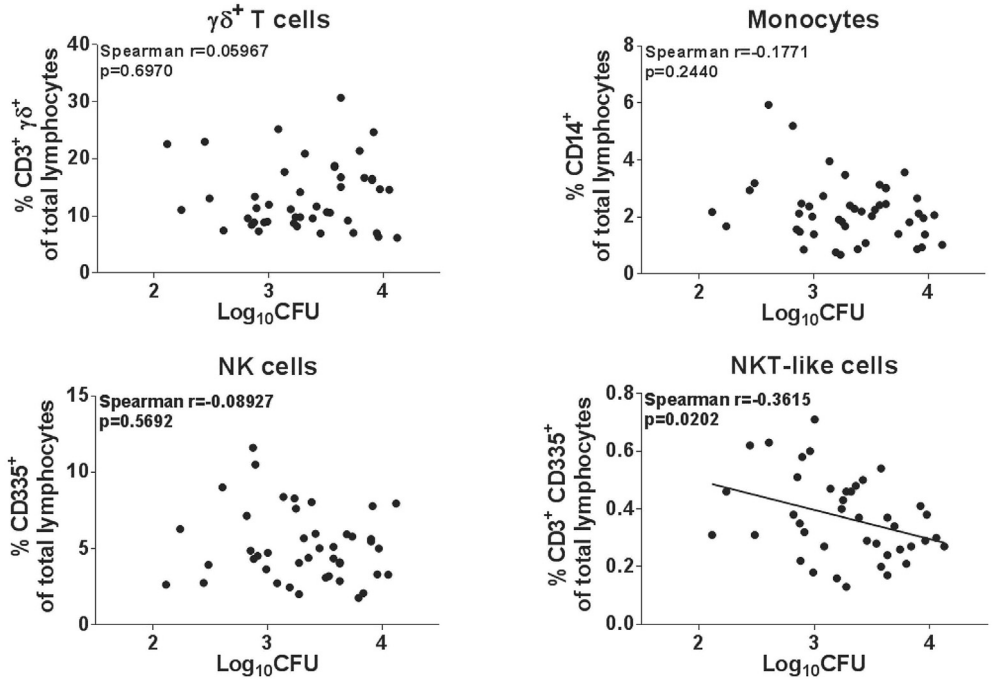

Fig. 2. Correlation at week 6 and 9 post-BCG immunisation, of PBMC-MGIA with characterised immune cell populations. PBMC-MGIA (Fig. 1) and cell frequencies were correlated to highlight which cell population might have a stronger impact on mycobacterial growth inhibition. Correlation strength was evaluated by means of the Spearman rank correlation coefficient (Spearman r).

studies using similar assays in humans and mice [7,8,11,12] and the findings of Denis et al. using an expanded PBMC-MGIA [16], which all showed a significant enhancement of growth inhibition following BCG vaccination.

The demonstration of a known BCG vaccine effect using this assay would biologically validate the assay. The lack of detection of a vaccine effect in the study reported might indicate that unidentified environmental parameters such as co-infection with environmental mycobacteria or *Mycobacterium avium* sub. *paratuberculosis* (MAP) could have a stronger effect on mycobacterial growth inhibition than BCG vaccination. Cattle, in contrast to specific-pathogen-free mice, are more likely to face constant challenge by environmental mycobacteria or being infected with MAP, and our results could therefore be a reflection of this scenario inherent to non-laboratory animal species. Responses to avian tuberculin purified protein derivative (PPD) were found in the control animals (Supplementary Fig. SF3), which is an indicator of exposure to environmental bacteria such as MAP. It has been shown that sensitisation with MAP can impart a degree of protection against *M. bovis* challenge [17] and this could be reflected in the lack of differences between MGIA responses in vaccinated and naïve calves. The significant inhibition seen in the human assays cited [7,11,12] could also potentially be explained considering that the samples tested were all from healthy subjects from developed countries. It is very unlikely that throughout their lives, those subjects were constantly challenged by environmental mycobacteria as could happen in non-developed countries or to bovines. It is also possible that reproducibility in mice is higher due to genetic identity and the controlled environment where they are kept. However, BCG is known to confer partial protection in cattle against *in vivo M. bovis* challenge [18].

As we did not observe a vaccine-associated effect using the MGIA, and it is likely that innate immune responses have an impact on *in vitro* mycobacterial growth inhibition, we analysed four innate immune cell populations (monocytes, γδ T cells, NK cells and NK T-like cells) in order to investigate a possible association between the proportions of these cells in PBMC and MGIA reactivity and we detected a statistically significant association between MGIA responses and the proportion of NK T-like cells ($CD3^{+}CD335+$) suggesting a role for these cells in the restriction of mycobacterial growth.

An immune correlate of protection in both the human and bovine field would be extremely useful. As the response to tuberculosis involves several features of the immune system, a functional assay that measures the sum of the parts could potentially be game-changing. Considering the synergies between the human and bovine field, the development of TB vaccines according to a "One Health" approach could bring many advantages for both. However, such an immune correlate would need to be sufficiently discriminatory to detect a vaccine effect that correlates with the protection observed after *in vivo* challenge, otherwise its benefit would be limited. We present here our efforts to transfer to the bovine field, a functional assay that has proven promising for mice and humans. We found preliminary evidence that NK T-like cells have a role in *in vitro* mycobacterial growth inhibition. Unfortunately, we did not find a significant difference in growth inhibition between naïve and BCG-vaccinated animals. We also had different reproducibility issues compared to those found in humans. These findings suggest that the transferability of this assay is not as straight forward as would be anticipated considering the results obtained in mice and humans. External factors like pre-sensitisation to environmental mycobacteria or infection from other pathogens may be confounding MGIA results.

5. Conclusion

MGIAs have potential as a tool for the evaluation of TB vaccine candidates at a preclinical and clinical level, by minimising the number of *M. tb* challenge experiments needed and by reducing the size of TB vaccine clinical trials. Further research is needed to

elucidate the effector mechanisms involved and other factors that might influence the MGIA in cattle. The establishment of specific cell populations, which have a role in mycobacterial growth inhibition, together with their mode of action will provide valuable information for the TB vaccines and biomarkers field.

Funding

This work was jointly funded by intra mural grants from The Jenner Institute, University of Oxford, and the Department for the Environment, Food and Rural Affairs (DEFRA), Welsh and Scottish governments (project AP0064).

Conflict of interest

The authors declare no conflict of interest. HMcS is a Wellcome Trust Senior Clinical research fellow, HMcS and MV are Jenner Institute investigators.

Acknowledgements

We thank the staff of the APHA animal care unit for their dedication to animal welfare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2017.07.008.

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