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ORIGINAL RESEARCH PAPER



BCG masking phenomena might depend on the species of *Mycobacterium*

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ABSTRACT

This study investigated BCG masking dependency on the species of *Mycobacterium* through the immune response to the mycobacterial region of deletion 1 (RD-1) associated growth affecting proteins (GEP).

To evaluate the effects of GEP, 8-week old female BALB/c mice were immunized with either the wild type *Mycobacterium bovis* (MBGEP) or the ATCC *Mycobacterium avium* subsp. *avium* (MAGEP) strain and then subjected to further exposure with *Mycobacterium terrae* or *M. avium* subsp. *avium*. Mice immunized with MAGEP and those mice further exposed to *M. avium* subsp. *avium* had increased granulocytes (GRA) and monocytes to lymphocytes rate (MLR) compared to control mice. Immunization of mice with GEP induced an antibody response one month after primary immunization, as observed by cross-reactivity. Our findings suggest that MAGEP is related to a latent hypersensitivity reaction and an increased risk of mycobacterial infection susceptibility. According to the results of the present study, previous sensitization with NTM antigens results in varying immune reactions after contact with different NTM argued that masking phenomena may be dependent on the species of *Mycobacterium*.

KEYWORDS

tuberculosis, BCG vaccine, mycobacterial proteins, region of deletion 1, mice immunization

INTRODUCTION

Globally, an estimated 10 million people in 2018 succumbed to tuberculosis (TB) infection caused by the bacteria *Mycobacterium tuberculosis* [1]. Bacillus Calmette–Guérin (BCG) is currently the only available TB vaccine [2]. However, the BCG vaccine is not fully effective

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due to different BCG strains, the prevalence of environmental mycobacteria, differing host genetics, nutritional factors, and the presence of co-infections such as helminths [3]. Further, there are two specific limitations to the BCG vaccine. First, the mycobacterial RD-1 protein, which is responsible for the bacteria's virulence and pathogenesis, is deleted from the attenuated strain of *Mycobacterium bovis* [4]. Lack of RD-1 in the BCG vaccine hampers mycobacterial defense against the host's immune system, allowing efficient destruction of *M. tuberculosis* [5]. Secondly, a masking phenomenon, possibly due to previous environmental sensitizations to nontuberculous mycobacteria (NTM)-mediated antigens, may confer protection against TB and mask vaccine efficacy [6, 7].

Interestingly, prior exposure to NTM antigens, such as those from the environment, is thought to be a widely accepted explanation as to the BCG vaccine inefficacy [8–13]. Most NTM species, such as *Mycobacterium terrae* or *Mycobacterium avium* are non-pathogenic and do not comprise the RD-1 region, although marginally invasive strains such as *M. avium* do exist [14–16]. These mycobacteria are in direct contrast to *M. bovis*, which are capable of inducing a pathogenic response [17]. More than 140 NTM species have been identified to-date [18], yet little is understood about their specific effect on masking and restricting RD-1 synthesis.

Mycobacteria produce a wide variety of proteins *in vitro* involved in the growth process such as proteins associated with T7S or general secretion pathways [19].

Recently, a study showed that mycobacteria react differently to self-secreted proteins or those provided by closely-related, slow-growing organisms. It was demonstrated that RD-1 associated growth effecting proteins (GEP) secreted by *M. bovis* inhibited *M. bovis* development, while *M. avium* subsp. *avium* was self-stimulated by its proteins. Most GEPs are relevant to the ESAT-6 secretion system-1 (ESX) associated with the RD-1 region. Most GEP proteins interact with signal peptides to carry out metabolic functions essential for mycobacterial growth and are also crucial for homeostasis, iron assimilation, virulence, resistance, and stress response. Interestingly, antibiotic or anticancer activity of these proteins have been reported and these proteins are further involved in the reduction of peroxides, dinitrobenzenes, and detoxification of hydroperoxides *in vitro* [19].

It is necessary to understand the limitations of the current BCG vaccine and consider the variables that influence its efficacy and how they impact on future vaccine design [20]. Understanding the biological mechanisms of mycobacterial proteins and how they control self and cellular activities might provide a better understanding of mycobacteria physiological processes and therefore improve the BCG vaccine.

MATERIALS AND METHODS

All procedures performed in this study involving animals were conducted in accordance with the ethical standards of the institution at which the studies were conducted. Ethical

approval was obtained from the State Veterinary Service of the Lithuanian Bioethics Committee for the use of laboratory animals, number G2-38. All mice used for this study were provided by the State Research Institute Centre for Innovative Medicine. Nontuberculous mycobacteria ATCC strains of *M. avium* subsp. *avium* (ATCC 15769) and *M. terrae* (ATCC 15755) were used in this study.

The term GEP was chosen based on our previous *in vitro* study. Results showed that these (GEP) proteins are involved in mycobacteria biofilm formation, growth stimulation and inhibitory processes. Protein sequence database from UniProt showed that most of GEP proteins interact with signal peptides to carry out metabolic functions essential for mycobacterial growth. The study was performed according to the *in vitro* results from a previous study [19].

Bacterial strains and GEP preparation

Bacterial cultures were maintained in the Lowenstein-Jensen medium with pyruvic acid (Difco™ & BBL™, Becton, Dickinson and Company). Two different 8- and 16-weeks incubation times cultures were used. For GEP extraction cultures were transferred to Middlebrook 7H9 Broth with Middlebrook ADC Enrichment (Difco™ & BBL™, Becton, Dickinson and Company) and incubated at 37 °C for 8 and 16 weeks. Culture media were prepared according to the manufacturer's recommendations. GEP was extracted from wild type *M. bovis* (MBGEP), and ATCC strains *M. avium* subsp. *avium* (MAGEP). Cultures were centrifuged at 4 °C for 45 min at 4,000 rcf after 8 and 16 weeks of incubation, and the filtrate was passed through a low protein binding 0.2 µm filter (Dismic -13 CP cellulose acetate filters, Advantec, Tokyo, Japan). The Bradford protein assay was used to measure the concentration of total protein in a sample.

Immunization and exposition of mice

A 100 µL emulsion of Freund's adjuvant (Sigma-Aldrich, USA), GEP, and 0.9 % saline solution (Fresenius Kabi, Poland) were injected subcutaneously into 12 mice on day 0 and day 14. Two types of control mice were prepared. The first group of mice was growing in the same conditions as tested mice and did not receive any mixture. The second group, as two-step control, was the mice immunized with GEP. The mixture was prepared according to the guidelines regarding the use of Freund's adjuvant issued by both the University of Pennsylvania Institutional Animal Care and Use Committee and the manufacturer's recommendations. 1 mL of the mixture contained 43.5 µL GEP, 456.5 µL saline solution and 500 µL Freund's adjuvant. After 28 days, mice were exposed to 100 µL of NTM. McFarland standard was prepared using the McFarland (McF) Equivalence Turbidity Standard kit (Remel, USA). A suspension of 1.97×10^6 mycobacteria in saline solution was prepared for *M. avium* subsp. *avium* and *M. terrae*. There were 7 groups of mice, including the control group. Blood samples were taken from two mice from each group and a total of 14 mice were sampled. The different experimental groups, according to immunization and exposure to NTM, are described in Fig. 1.



Blood tests of BALB/c mice

Blood from BALB/c mice was taken from the jugular vein using a 20 µL sterile microcapillary. One month after primary immunization blood was collected from the group of mice immunized with GEP. Three months after primary immunization blood was collected from the GEP immunized and mycobacteria exposed group of mice. Blood analysis was performed using Exigo Eos analyzer (Boule Medical AB, Sweden). Blood serum from selected immunized mice was used for western blotting. After blood was collected in Eppendorf tubes, incubated for 20 min at room temperature, and centrifuged for 10 min at 2,500 rpm they were stored at –20 °C until further use.

Western blotting

Western blotting was performed to identify the immune response towards GEP. Protein electrophoresis in 12% SDS-polyacrylamide gel under reducing conditions was performed as described elsewhere. Samples were prepared in a 1: 1.4 ratio of protein sample to sample loading buffer (pH 6.8: 1.5 M Tri-HCl, 10% 2-ME, 20% glycerol, 4% SDS, and 0.02% bromophenol blue). Samples were heated at 95 °C for 5 min prior to being loaded into the wells alongside the molecular weight ladder for electrophoresis separation (Super Signal Molecular Weight Protein Ladder, USA). Following electrophoresis, proteins were semi-dry transferred to an Immuno-Blot PVDF membrane (Bio-Rad, JAV)

with a pore size of 0.2 µm, using a MilliBlot – Graphite Electroblotter I apparatus (Millipore, USA). The transfer was performed for 30 min at 250 mA. After the transfer process was completed, the membrane was blocked with 0.05% Tween-20 buffer containing 1% polyvinylpyrrolidone in PBS (Sigma, USA) at 4 °C overnight. After blocking, the membrane was washed three times for 5 min with 0.05% Tween-20 in PBS (washing buffer) on a shaker and then incubated with the hyperimmune serum of mice (1: 50,000 for samples from MBGEP immunization and 1: 10,000 from samples with MAGEP immunized) for 2 hours shaking at room temperature. After incubation, the membrane was rewashed using a wash buffer three times, each time for 5 min. The membrane was then incubated with conjugate 1:10 00 goat-anti-mouse IgG (H&L) for 1 h at room temperature followed by washing. In preparation for exposure and imaging, ECL detection reagent (ECL Western Blotting Substrate Thermo Scientific, USA) was added to the membrane according to the manufacturer's instructions and then imaged (Chemi Doc MP, Bio-Rad, USA). All western blot data were processed with Image Lab 5 imaging software (Bio-Rad, USA).

RESULTS

The MBGEP immunized mice had higher white blood cells value (WBC) ($12.35 \times 10^3 \mu\text{L}$) compared to the mice that

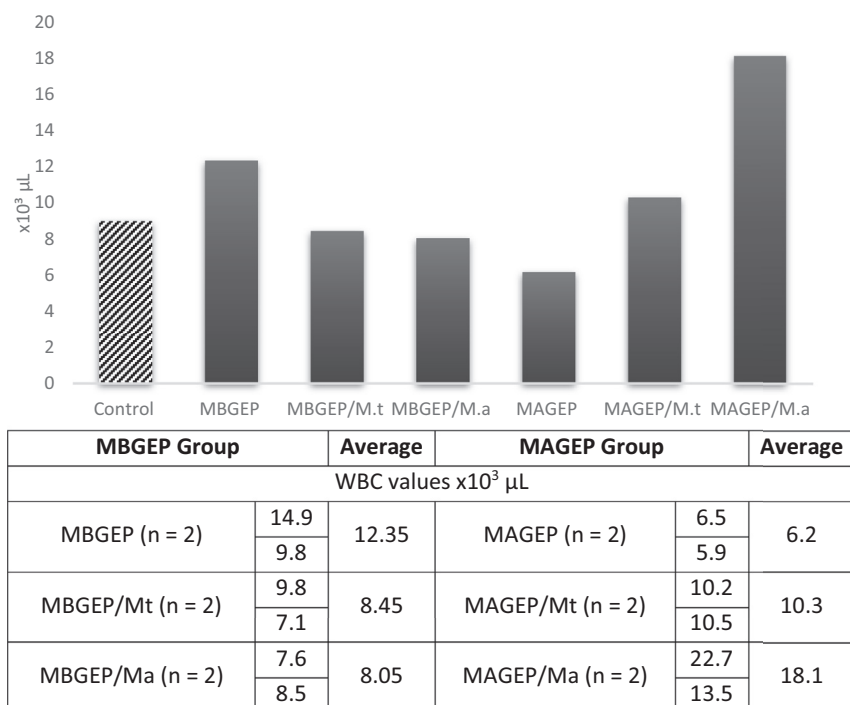


Fig. 1. WBC of mice and GEP groups. Histograms (top) shows different mouse treatment groups. Control mice did not receive GEP and were not exposed to NTM. The following groups were exposed to GEP or GEP and NTM. MBGEP only mice were immunized with GEP extracted from *M. bovis* and MAGEP only mice were immunized with GEP obtained from *M. avium* subsp. *avium*. MBGEP/M.t mice were immunized with MBGEP and exposed to *M. terrae*; MBGEP/M.a mice were immunized with MBGEP and exposed to *M. avium* subsp. *avium*; MAGEP/M.t mice were immunized with MAGEP and exposed with *M. terrae*; MAGEP/M.a mice were immunized with MAGEP and exposed to *M. avium* subsp. *avium*. The table (bottom) summarizes the findings presented in the histogram

were exposed to both MBGEP and NTM (8.45 observed in MBGEP/M.t and 8.05 in MBGEP/M.a). The MAGEP immunized mice had a lower WBC value (6.2) in comparison to the MAGEP immunized and exposed to NTM (10.3 observed in MAGEP/M.t and 18.1 in MAGEP/M.a). Amongst the mice vaccinated with MAGEP, those exposed to *M. avium* subsp. *avium* had a WBC value 2.9 times higher than MAGEP alone (Fig. 1).

Considering the values of obtained WBC, we further sort to clarify changes to granulocytes (GRA) in MAGEP immunized mice. As shown in Fig. 2, the GRA value of mice immunized with MAGEP only (1.5) or MAGEP/M.t (2.1) was lower than the control group (2.3). Interestingly, mice in the MAGEP/M.a experimental group presented with a GRA value 2.6 times higher (6.1) than the control group (Fig. 2).

Reduced or elevated ratios of MLR are associated with enhanced lethality of mycobacterial infection or increased risk of TB susceptibility, respectively [21, 22]. The highest MLR value observed was 0.13 and 0.12 in mice immunized with either MBGEP or MAGEP, respectively (Fig. 3). The MLR was not significantly higher in mice immunized and exposed to NTM, except MLR values for MAGEP/M.a exposed mice (0.13), which was similar to MBGEP or MAGEP only treatment groups.

Mice immunized with either MAGEP or MBGEP developed an immune response observed due to the presence of an antibody response one month after primary immunization (Fig. 4). Two antigen proteins were detected at

50 kDa and 80 kDa. An additional protein was detected at 45 kDa in mice immunized with MAGEP.

DISCUSSION

The efficacy of the BCG vaccine in adults is region-dependent; for example, vaccine efficacy is particularly poor in tropical and subtropical climates. The reduced vaccine outcome may be due to a vast number of environmental mycobacteria species that are found throughout the world. Over time, adults increase their physical contact with mycobacteria as well as environmental bacteria, which is not observed in newborns or infants. Most studies regarding the masking hypothesis are investigated on a few environmental mycobacteria and mostly with organisms vaccinated with BCG [9–13]. We found *in vitro* and *in vivo* evidence that suggests mycobacteria react differently towards their own species and to species-related, slow-growing organisms by secreting proteins that have an impact on the immune response [19].

Within several days after immunization, increased WBC value is a normal response to vaccination [23]. In immunized mice, a higher WBC value compared to the control group was observed after initial immunization with MBGEP vaccine. The WBC value in MAGEP immunized mice remained unchanged, which suggests that the increased WBC value in MBGEP immunized mice is influenced by the adjuvant's interaction with MBGEP proteins. There are

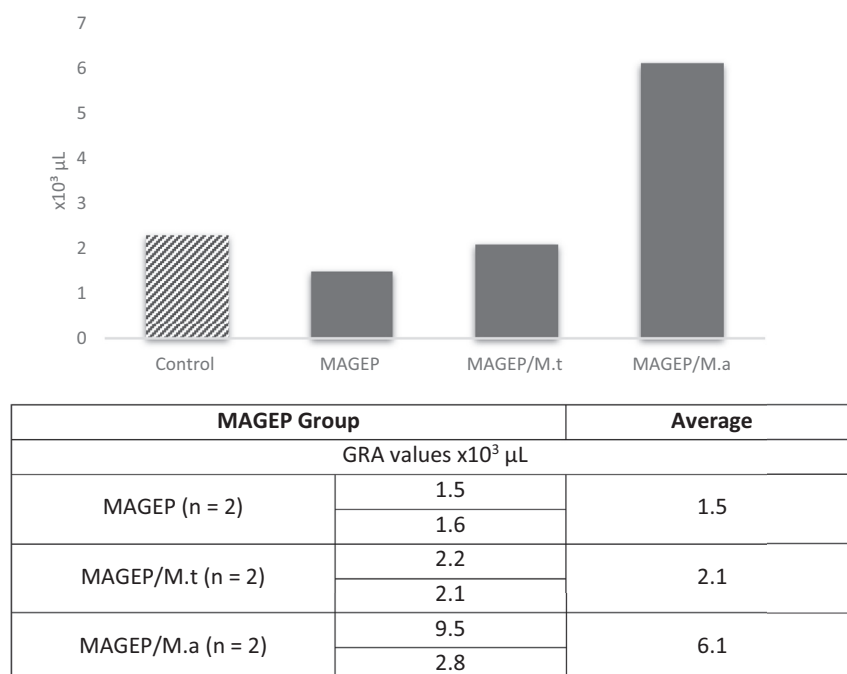


Fig. 2. GRA of mice and GEP groups. Histograms (top) shows different mouse treatment groups. Control mice did not receive GEP and were not exposed to NTM. The following groups were exposed to GEP or GEP and NTM. MAGEP only mice were immunized with GEP obtained from *M. avium* subsp. *avium*. MAGEP/M.t mice were immunized with MAGEP and exposed with *M. terrae*; MAGEP/M.a mice were immunized with MAGEP and exposed to *M. avium* subsp. *avium*. The table (bottom) summarizes the findings presented in the histogram

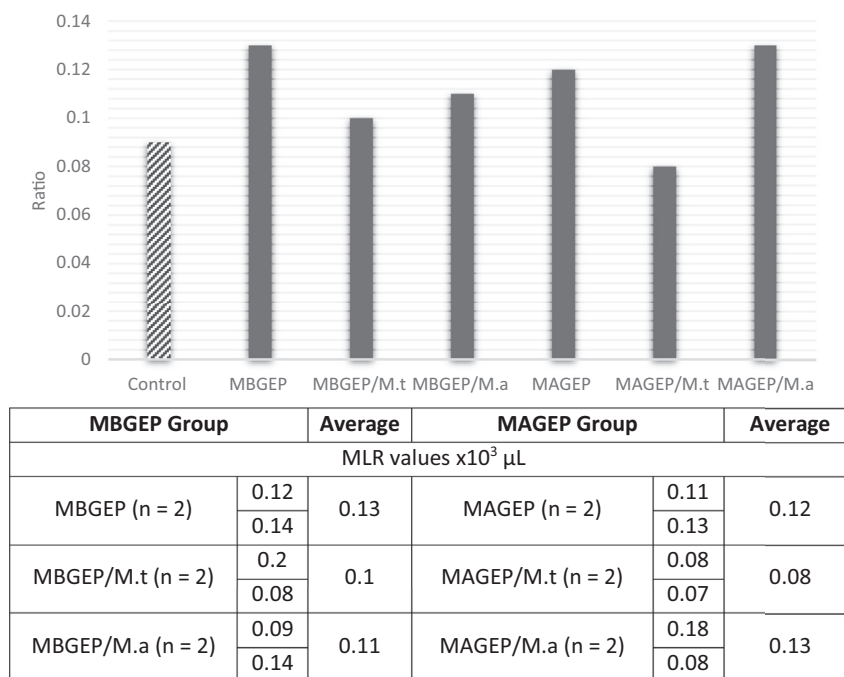


Fig. 3. MLR of mice and GEP groups. Histograms (top) shows different mouse treatment groups. Control mice did not receive GEP and were not exposed to NTM. The following groups were exposed to GEP or GEP and NTM. MBGEP only mice were immunized with GEP extracted from *M. bovis* and MAGEP only mice were immunized with GEP obtained from *M. avium* subsp. *avium*. MBGEP/M.t mice were immunized with MBGEP and exposed to *M. terrae*; MBGEP/M.a mice were immunized with MBGEP and exposed to *M. avium* subsp. *avium*; MAGEP/M.t mice were immunized with MAGEP and exposed with *M. terrae*; MAGEP/M.a mice were immunized with MAGEP and exposed to *M. avium* subsp. *avium*. The table (bottom) summarizes the findings presented in the histogram

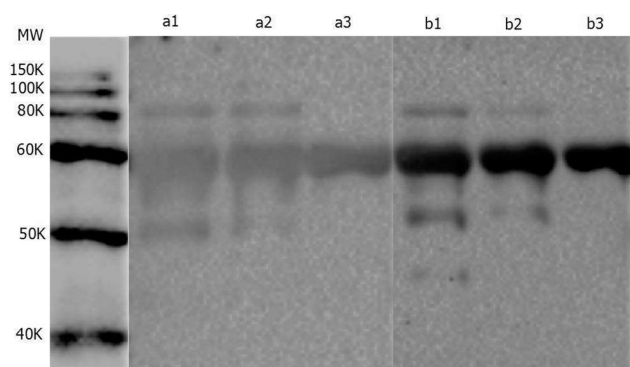


Fig. 4. Western blots of GEP's with specific anti-GEP antibodies. Columns marked with "a" represent hyperimmune serum of mice immunized with MBGEP; columns marked with "b" represent hyperimmune serum of mice immunized with MAGEP. Each column number refers to the following groups: number 1, MAGEP Ag; number 2, MBGEP Ag; number 3 is the control (Middlebrook 7H9 medium). MW represents molecular mass in kilo Daltons (kDa) of known protein sizes

many immunogenic proteins present in MBGEP, for example, mycobacterial virulence factors such as ESAT-6 and other ESX substrates. Higher WBC values seen in the group immunized and exposed to NTM in comparison with MAGEP immunized mice may have resulted from components of MAGEP interacting with NTM, and not through NTM alone. Further, WBC values of *M. terrae* infected mice

remained within control limits, and MAGEP stimulated *M. avium* subsp. *avium* improved *in vitro* growth [19].

GRA value of immunized and exposed to *M. avium* subsp. *avium* exceeded the control range (6.1). It could be assumed that increased GRA value can be the result of latent hypersensitivity reaction to antigens. GRA value is an essential participant of inflammation and plays a vital role in the etiology of allergic and hypersensitivity reactions [24, 25].

The high MLR value observed in the (MAGEP and MBGEP) group of immunized mice may be due to the interaction between GEP and Freund's adjuvant. This observation is supported by the increase in MLR values seen in both MAGEP and MBGEP immunized mice compared to the control group. MLR was similar to the control MBGEP group and those further exposed to NTM (0.1 and 0.11). Therefore, the increase in MLR values due to MBGEP immunization may not be related to the increased risk of mycobacterial infection susceptibility. For MAGEP immunized and mycobacteria exposed mice, MLR values were similar to the control group and in the group further exposed to *M. terrae* (0.08). In contrast, the MLR value increased in *M. avium* subsp. *avium* exposed mice (0.13). The cross-reactivity observed in the study is not surprising [26] considering the high antigenic and genetic homology between GEP and mycobacteria. The induced cross-reactivity against mycobacterial antigens supports GEP as a vaccine candidate.

CONCLUSIONS

According to the results of the present study, previous sensitization with NTM antigens results in varying immune reactions after contact with different NTM. In conclusion, the immune response depends on sensitization, and masking may be dependent on the species of *Mycobacterium* rather than the dependency of the RD-1 region, as bacteria used in our study do not have this region.

Our findings suggest that immunization with MAGEP might reduce the risk of susceptibility to mycobacterial infection after exposition with *M. avium* subsp. *avium*. MAGEP showed an increased GRA value, possibly due to the latent hypersensitivity reaction to antigens present in MAGEP and an elevated susceptibility risk to mycobacterial infection. The increased GRA value may occur due to an increased MLR following the exposition with *M. avium* subsp. *avium*. We found that GEP proteins affect the immune response by detection of a humoral cross-reactive reaction against *M. bovis* and *M. avium* subsp. *avium* antigens.

Our results further confirm that previous sensitization confers some level of protection against TB that masks vaccine efficacy, and a larger sample study will help to validate the findings in this report.

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