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14. ABSTRACT

This project has 3 hypotheses: 1) Genetically variable BCG vaccine sub-strains produce different amounts of capsular polysaccharides (CP) arabinomannan (AM) and α-D-glucan (DG). 2) Variations in CP affect BCG immunogenicity. 3) Culture media affects CP levels therefore selective modifications of growth media may be a way to enhance BCG immunogenicity. In this reporting period, we found AM does not vary significantly among the 6 BCG vaccine sub-strains studied. However, for the same sub-strain, AM levels decrease when BCG is grown in Sautons compared to 7H9 media. We also found that DG levels were higher in BCG-Moreau compared to the other 5 sub-strains. Like AM, growth in Sautons media also decreases DG although exceptions were noted with BCG-Danish whose DG remain unchanged, and BCG-Pasteur whose DG levels increased. Overall, removal of CP increased phagocytosis of all BCG sub-strains by THP-1 macrophages. However, in the case of BCG sub-strains with intact CPs, BCG-Moreau exhibited significantly reduced phagocytosis likely due to its high production of DG which is a previously documented anti-phagocytosis factor. Although BCG devoid of all CPs were found to be generally less pro-inflammatory, a correlation between CP abundance and capacity to induce pro-inflammatory response in THP-1 macrophages was not observed.

15. SUBJECT TERMS

BCG sub-strains, variations in capsular polysaccharides, BCG-macrophage interactions, innate immune response, cell-mediated immune response

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1. Introduction

The live attenuated *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine has been used for almost 100 years to protect against tuberculosis (TB), an airborne bacterial infection caused by members of the *M. tuberculosis* complex (1). However, the BCG vaccine has a poor record of consistently protecting against the disease (2). A possible reason for this inconsistency may in part be due to the genetic heterogeneity in the 13 extant BCG vaccine sub-strains that are used in different parts of the world (2). Some of these genetic differences likely affect the synthesis of capsular polysaccharides (CP) comprised of arabinomannan (AM) and α-D-glucan (DG) on the surface of mycobacteria like BCG (3). However, it is not known if CP varies among the different BCG sub-strains. Since mycobacterial CP is associated with activation of the immune response, it is not known if variations in CP among BCG sub-strains impact their immunogenicity. Recent studies have also shown that the type of media used to culture BCG influences various aspects of their immunobiology including their protective efficacy. Whether this is mediated through changes in CP is also not known. This project will test 3 main hypotheses:

1) Genetically variable BCG sub-strains produce different amounts of CP and its constituents AM and DG.

2) Variations in CP affect the interaction of BCG with the host immune system including innate and adaptive immunity

3) Alteration of BCG culture media changes CP levels. As such, selective changes to growth media may be a simple way to enhance BCG immunogenicity.

The results of this study could explain why some BCG sub-strains are more immunogenic and protective than others. On a practical level, this work may enable the production of more effective BCG vaccines in the global fight against TB.

2. Keywords

BCG, capsular polysaccharides, BCG-macrophage interactions, innate immune response, cell-mediated immune response

3. Accomplishments

3.1. Major goals of the project.

Aim 1. Examine CP of 6 different BCG sub-strains (BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur).
Major task 1 - Optimize CP extraction and analyses of CP.
Milestone: BCG CP samples prepared and analyzed; Local AREB and ACURO approval to be completed by month 6 – 95% complete.
Major task 2 - LC-MS analyses of CP.
Milestone: BCG CP samples analyzed by LC-MS to be completed by month 5 – 75% complete.

Aim 2. Assess CP-mediated macrophage-BCG interactions.
Milestone: Assessment of CP-mediated macrophage-BCG interactions to be completed by month 12 – 95% complete.

Aim 3. Assess CP-mediated immune-stimulation in mice by different BCG.
Major task 1 - Prep 7H9-grown BCG^{+CP} and BCG^{-CP}, inoculate mice and assess cell-mediated immunity.
Major task 2 - Prep Sautons-grown BCG^{+CP} and BCG^{-CP}, inoculate mice and assess cell-mediated immunity.
Milestone: Assessment of CP-mediated immune-stimulation in mice to be completed by month 13 – in progress.

Aim 4. Identify exogenous factors that enhance CP production.
Major task 1 - Assess growth in modified Sautons, prep CP and analyze.
Major task 2 - Assess growth in Sautons with serine hydroxamate on BCG CP and cell-mediated immunity in mice.
Milestone: Assessment of effects of exogenous factors on BCG CP and immunogenicity to be completed by month 18 – in progress.

3.2. Accomplishments to date.

Aim 1. Examine CP of 6 different BCG sub-strains (BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur).

Major task 1 - Optimize CP extraction and analyses of CP. The 6 BCG sub-strains were grown in 7H9 and Sautons media without detergent (typically added to prevent clumping) to approximately mid-logarithmic phase of growth. Cells were pelleted by centrifugation, washed once with phosphate buffered saline (PBS) and freeze-dried. 20 mg dry biomass of all BCG sub-strains grown in either Sautons or 7H9 media were weighed out, each resuspended in 1mL PBS with 0.5% of the non-ionic detergent tyloxapol and vigorously agitated in a bead-beater set at maximum strength. Mechanical agitation over several lengths of time were tested for maximal extraction before we settled on 3 cycles of 1 minute each. The mixture was then centrifuged and the supernatant passed through 0.45 micron filters. The filtrates containing extracted CP were each serially diluted 2-fold in PBS. 3 µL each of undiluted, 1/2, 1/4, 1/8 and 1/16 diluted CP extract from all BCG sub-strains grown in either Sautons or 7H9 media were spotted on nitrocellulose membranes before probing with mouse monoclonal antibodies specific for either AM (clone F30.5) or DG (clone IV58B6) (4). Much effort was made to optimize the antibody titers required to detect AM and DG produced by BCG in the dot-blots. Anti-AM antibody at 1:1000 and anti-DG antibody at 1:100 dilution was found to yield the best results. The amounts of AM did not appear to vary dramatically between the 6 BCG sub-strains assessed in this study (Figure 1A). However, AM levels appeared to be reduced 2 to 3-fold in all 6 BCG sub-strains when they were grown in Sautons compared to 7H9 media.

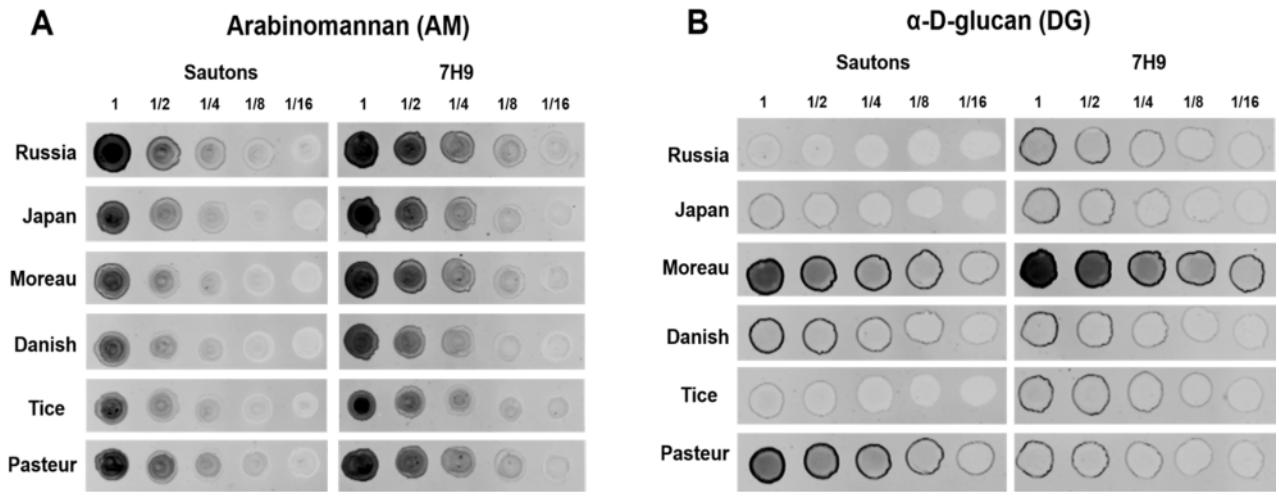

Figure 1. Dot-blots of AM (A) and DG (B) produced by BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur grown in Sautons and 7H9 media. The first undiluted spot is 3 µL of an extract of freeze-dried BCG cells PBS with 0.5% tyloxapol at 20 mg/mL. Subsequent spots are 3 µL of 2-fold serial dilutions. Each dot-blot is representative of 3 independent experiments.

On the other hand BCG Moreau was found to produce significantly more DG than the other 5 sub-strains (Figure 1B). Furthermore, the DG produced by most BCG sub-strains was reduced when grown in Sautons media compared to 7H9. Notable exceptions were BCG-Danish, whose DG remained unchanged, and BCG-Pasteur whose DG increased significantly when grown in Sautons compared to 7H9 media (Figure 1B). We were able to rule out differences in growth in the two media as the reason for the shifts in AM and DG abundance because the total dry biomass of a given BCG sub-strain was similar regardless of whether it was grown in Sautons or 7H9. Growth measurements by OD600nm of BCG in media with detergent confirmed this finding (Figure 2A and B).

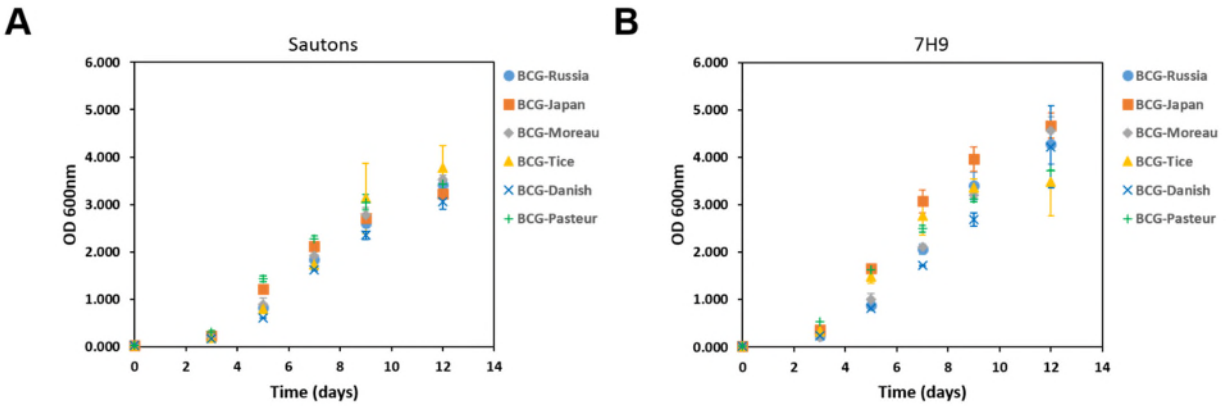

Figure 2. Growth rate measurements of BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur in Sautons (A) and 7H9 (B) media. Each data-point is the mean of 3 independent experiments and error bars represent the standard deviation.

Attempts to quantitatively assess AM and DG production by the 6 BCG sub-strains using sandwich ELISAs has been technically challenging. We suspect this might be due to the presence of relatively high concentration of tyloxapol in the CP extract. Also, due to the limited amounts of monoclonal antibodies against AM and DG available and the lengthy optimization of ELISA conditions required, we opted to concentrate more on improving the dot-blot method. Time permitting, we will revisit the ELISA method to confirm our dot-blot results.

Major task 2 - LC-MS analyses of CP. Extracts prepared as described above were submitted for LC-MS analyses but the results proved to be ambiguous and unclear. We reasoned that the biological complexity of the extract prevents accurate identification and quantitation of AM and DG. Indeed it has been reported that the mycobacterial CP extract contains a multitude other surface molecules such as proteins and glycolipids, and these will likely interfere with MS analysis. Time permitting, we will work further to optimize the preparation of CP extracts as well as the liquid chromatography stage to obtain better resolution.

Aim 2. Assess CP-mediated macrophage-BCG interactions.
Attempts to generate viable BCG lacking CP (BCG^{-CP}) for macrophage infections by vigorous mixing as originally proposed proved unsuccessful. Even sonicating BCG cells over increasing lengths of time (from 30 to 270 seconds in 30 second increments) failed to remove AM or DG (Figure 3A).

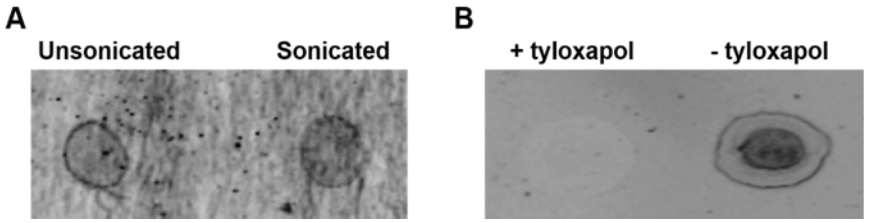

Figure 3. Dot-blot of undiluted AM from extracts of unsonicated or sonicated (A), and tyloxapol treated or untreated (B) cells of BCG-Pasteur. The same results were obtained with DG and was similar with the other 5 BCG sub-strains used in this study. Representative of 3 independent experiments.

We subsequently found that incubating BCG cells in PBS with 0.5% tyloxapol at room temperature for 10 minutes was sufficient to remove CP (AM and DG) without affecting viability (Figure 3B). BCG^{-CP} cells thus obtained were used in all subsequent THP-1 macrophage experiments. BCG^{+CP} cells were obtained by culturing the Sautons or 7H9 media without detergent as described above (Section 3.2, Aim 1).
To assess macrophage uptake, $3x10^5$ adherent THP-1 cells were infected for 4 hours with either BCG^{-CP} or BCG^{+CP} cells at a multiplicity of infection (MOI) of 1. Thereafter, the THP-1 cells were washed to remove unbound and uninternalized BCG and lysed. The lysates were serially diluted and plated out on 7H11 agar plates to enumerate internalized BCG. Consistent with published reports, BCG made devoid of CP by tyloxapol treatment were phagocytosed by THP-1 cells 2 to 3-fold more than BCG with intact CPs (5). However, among untreated BCG sub-

strains with intact CPs, BCG-Moreau appeared to be the least invasive compared to the other 5 sub-strains. Furthermore, when grown in Sautons media most BCG sub-strains appeared to be more phagocytosed by THP-1 cells compared to when they are grown in 7H11 media. However, once the 6 different BCG sub-strains had entered THP-1 cells, we did not observe significant differences in their intracellular replication.
To assess induction of pro-inflammatory responses in THP-1 cells, $3x10^5$ adherent cells were infected for 24 hours with either BCG^{-CP} or BCG^{+CP} cells at a MOI of 10. The levels of pro-inflammatory cytokines IL-1β and TNF-α in THP-1 culture supernatants were quantified by ELISA. We observed that BCG sub-strains with intact CP often

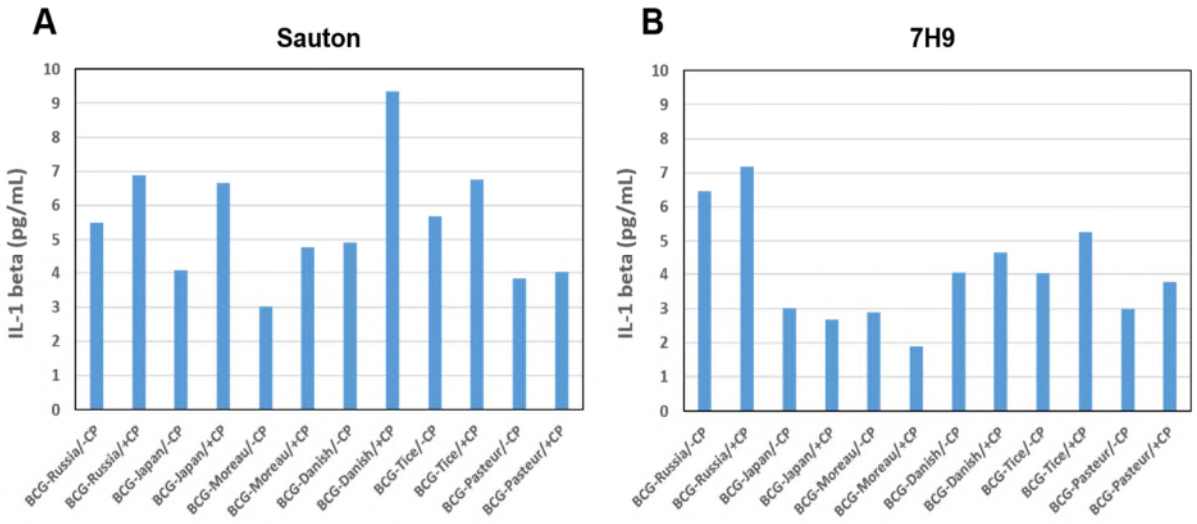

Figure 4. IL-1b production by BCG^{-CP} vs BCG^{+CP} grown in Sautons (A) and 7H9 (B) media. Histogram is the mean of duplicate wells and representative of 2 independent experiments.

induced slightly higher IL-1β production regardless of growth in Sautons or 7H9 media (Figure 4A and B). Notable exceptions were seen with BCG-Japan and Moreau grown in 7H9 media (Figure 4B). We did not see a correlation between AM or DG levels and IL-1β induction by the 6 different BCG sub-strains with intact CPs.

Aim 3. Assess CP-mediated immune-stimulation in mice by different BCG.
Although experimental results from the current reporting period do not show a correlation between AM or DG abundance in the 6 BCG sub-strains and their induction of pro-inflammatory responses in macrophages, a correlation may yet be seen between AM and/or DG abundance with stimulation of cell-mediated immunity. This will be addressed in aim 3. The animal use protocol has been approved by both the institutional animal research ethics board and ACURO. BCG^{-CP} or BCG^{+CP} have been prepared and immunization trials in C57BL/6 mice are ongoing.

Aim 4. Identify exogenous factors that enhance CP production.
Our experimental results from the current reporting period show that BCG grown Sautons tend to produce lower amounts of both AM and DG. A major difference between Sautons and 7H9 media is the presence of significantly higher concentrations of glycerol in the former (6). As such, glycerol which is a carbon source for mycobacteria may negatively regulate CP synthesis. This hypothesis will be tested by culturing BCG in modified Sautons media and assessing their CP levels as described in the proposal and statement of work. In addition, phosphate depletion in culture media triggers the stringent response and upregulates CP production in mycobacteria (4). Serine hydroxamate has also been shown to trigger the stringent response (4). As such, the BCG sub-strains will be grown in culture media containing serine hydroxamate and their levels of CP assessed. The immunogenicity of BCG grown in these modified media will also be assessed in mice as described in the proposal and statement of work.

Main Findings and Conclusions for this Reporting Period: We have found that AM levels among the 6 BCG sub-strains analyzed in this study do not vary significantly when grown in either Sautons or 7H9 media. However, for all 6 sub-strains, AM levels appear to be reduced when they are grown in Sautons compared to 7H9 media. In contrast, DG levels in BCG-Moreau is significantly higher than the other 5 BCG sub-strains when grown in either Sautons or 7H9 media. With the exception of BCG-Danish and Pasteur, DG levels tend to decrease when BCG are

grown in Sautons compared to 7H9 media. These findings support our first hypothesis that genetic variations in existing BCG sub-strains may result in variations in CP. That AM levels from the 6 BCG sub-strains assessed here do not appear to vary significantly may reflect the low sensitivity of the dot-blot assay in detecting quantitative differences. More sensitive assays such as ELISA or LC-MS may better detect bona fide quantitative differences in AM among these 6 sub-strains. That AM and DG levels both decrease for most BCG sub-strains when they are grown in Sautons media partially supports our third hypothesis, although the observation that some sub-strains do not strictly follow this pattern suggests a complex nutrient-mediated regulation of AM and DG exists in mycobacteria.

In terms of BCG-macrophage interactions, BCG devoid of CP after tyloxapol treatment invaded THP-1 cells more than BCG with intact CPs regardless of culture media used and is consistent with published reports (5, 7). Strikingly, untreated BCG-Moreau which produces high amounts of DG was found to be phagocytosed less than other 5 BCG sub-strains. This is consistent with studies that have reported DG to be anti-phagocytic (3, 7). Likewise when the BCG sub-strains are grown in Sautons media which generally reduces both AM and DG levels, they appeared to be more easily phagocytosed by THP-1 macrophage cells. With respect to the induction of pro-inflammatory responses in macrophages however, BCG with intact CPs tended to induce higher responses but notable exceptions with BCG-Japan and Moreau grown in 7H9 media were observed. The reasons for this are presently unknown and is the focus of ongoing experiments.

3.3. Opportunities for training.

Nothing to report.

3.4. Dissemination of results to communities of interest.

Nothing to report.

3.5. Plans during next reporting period to accomplish goals.

As outlined in the proposal and statement of work, experiments to assess cell-mediated immunity in BCG^{-CP} and BCG^{+CP}-immunized mice will be completed. In parallel, the effect of modifying culture media on CP and cell-mediated immunity in mice will also examined as described in the proposal and statement of work. Further efforts will also be made to continue optimizing ELISAs and LC-MS approaches to quantify AM and DG produced by the 6 different BCG sub-strains under investigation in this project.

4. Impact

4.1. Impact on the development of the principal discipline of the project.

Nothing to report.

4.2. Impact on other disciplines.

Nothing to report.

4.3. Impact on technology transfer.

Nothing to report.

4.4. Impact on society beyond science and technology.

Nothing to report.

5. Changes/Problems

5.1. Changes in approach and reasons for change.

As explained in section 3.2, aim 2, our initial plan to produce BCG^{-CP} by mechanical agitation including sonication was unsuccessful. Subsequent testing revealed that incubating BCG in PBS with 0.5% tyloxapol effectively removes CP without causing loss in viability. Going forward, all BCG^{-CP} will be prepared in this way.

5.2. Actual or anticipated problems or delays and actions or plans to resolve them.

Due to the lengthy process of grant contract execution and setting up of expense accounts, some equipment and reagents required for this project did not arrive till late October 2018. As a result, work for this project did not begin

till 1 November 2018. Despite these initial set-backs, we dedicated more working hours to the project and have managed to keep it on track with statement of work time-lines.

5.3. Changes that had a significant impact on expenditures.
Nothing to report.

5.4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Not applicable and nothing to report.

5.5. Significant changes in use or care of human subjects.
Not applicable

5.6. Significant changes in use or care of vertebrate animals.
Nothing to report.

5.7. Significant changes in use biohazards and/or select agents.
Nothing to report.

6. Products

6.1. Publications, conference papers, and presentations.
Nothing to report.

6.2. Website(s) or other Internet site(s).
Nothing to report.

6.3. Technologies or techniques.
Nothing to report.

6.4. Inventions, patent applications, and/or licenses.
Nothing to report.

6.5. Other Products.
Nothing to report.

7. Participants & Other Collaborating Organizations

7.1. What individuals have worked on the project?

Name:	*Jeffrey Chen Ph.D.*
Project Role:	*Principal investigator*
Researcher Identifier:	***http://orcid.org/0000-0001-8431-3802***
Nearest person month worked:	*10*
Contribution to Project:	*Conceptualized, planned, helped execute experiments, collected and analyzed data*
Funding Support:	*VIDO-InterVac, University of Saskatchewan*

Name:	*Ze Lim M.Sc.*
Project Role:	*Research technician*
Researcher Identifier:	*None*
Nearest person month worked:	*12*
Contribution to Project:	*Mr. Lim executed experiments, collected and analyzed data*

Funding Support:	

7.2. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

7.3. What other organizations were involved as partners?
Nothing to report.

8. Special Reporting Requirements

8.1. Collaborative Awards.
Not applicable.

8.2. Quad Charts.
Not applicable.

9. Appendices

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