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Cytokine Profiling of Mice Vaccinated with *Brucella* Polysaccharide

H.S. Bhogal, L.J. McLaws, M.L. Russell, N.D. Barabe, and J.W. Cherwonogrodzky

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In conducting the research described in this report, the investigators adhered to the 'Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.' published by the Canadian Council on Animal Care.

Abstract

Although *Brucella* infection is one of the most common infections acquired in the laboratory, infection could also occur as a result of a deliberate act. The potential for *Brucella* to be weaponized and used as a biological warfare agent is a real concern as it is readily obtained and aerosolized, the infective dose is low, and can lead to a long-lasting disease. Therefore, there is a need for the development of an effective vaccine. A subcellular polysaccharide vaccine derived from *Brucella suis* has been shown to be effective in providing long-term protection from brucellosis in animal studies. The mechanism of action of this subcellular vaccine is unknown. In this study, we report the findings from cytokine profiling of mice injected with subcellular polysaccharide vaccine. A minor increase in IFN-γ and a depressed level of IL-5, coupled with a significant increase in TNF-α, hint at the induction of a Th1 response. Other cytokines such as IL-2, IL-4, IL-10, IL-12, and GM-CSF remain unchanged in response to vaccination with the polysaccharide. Although the polysaccharide may induce a cell-mediated response, this immune response is likely short-lived and, therefore, may not contribute to the long-term protection afforded by this subcellular vaccine.

Résumé

L'infection de la *Brucella* est l'une des infections les plus communes acquises en laboratoire mais elle pourrait aussi résulter d'un acte délibéré. La possibilité que la *Brucella* soit utilisée comme arme et comme agent de guerre biologique est un souci réel puisqu'elle peut facilement s'obtenir et se transformer en aérosol; de plus, la dose infectante est faible et peut résulter en une maladie durable. On a par conséquent besoin de développer un vaccin efficace. Les études animales du vaccin au polysaccharide infracellulaire dérivé de la *Brucella suis* indique que ce dernier s'est démontré efficace à fournir une protection à long-terme contre la brucellose. On ne connaît pas le mécanisme d'action de ce vaccin infracellulaire. Dans cette étude, nous documentons les résultats provenant du profilage de la cytokine de souris injectées avec le vaccin au polysaccharide infracellulaire. Une augmentation mineure en IFN-γ et une diminution du niveau de IL-5, accompagné d'une augmentation importante en TNF-α, suggère l'induction d'une réponse Th1. D'autres cytokines telles qu'IL-2, IL-4, IL-10, IL-12 et GM-CSF ne produisent pas de changement en réponse à la vaccination avec le polysaccharide. Le polysaccharide peut induire une réponse à médiation cellulaire mais cette réponse immunitaire est probablement de courte durée et ne contribue par conséquent pas à la protection à long terme produite par le vaccin infracellulaire.

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Executive summary

Cytokine Profiling of Mice Vaccinated with *Brucella* Polysaccharide

Hardeep S. Bhogal; Lori J. McLaws; Michelle L. Russell; Nicole D. Barabe; John W. Cherwonogrodzky; DRDC Suffield TM 2007-262; Defence R&D Canada – Suffield; December 2007.

Introduction or background: Brucellosis in humans, caused by the *Brucella* bacterium, is a disease that can be transmitted from animals to humans, and approximately 500,000 cases are reported every year globally. The potential for *Brucella* to be weaponized and used as a biological warfare agent is a real concern as it is readily obtained and aerosolized, the infective dose is low, and can lead to a long-lasting disease. Although significant efforts have been made into research for *Brucella* vaccines, no effective vaccine exists for this disease. A polysaccharide vaccine has been developed at DRDC Suffield, but the mechanism of action is unknown. Analysis of immune regulatory proteins may provide clues to help understand the mechanism by which this vaccine elicits protection against infection.

Results: In this study, the levels of a panel of regulatory proteins known as cytokines were observed from mice injected with O-polysaccharide derived from *B. suis*. A small increase in interferon gamma and a significant increase in tumour necrosis factor alpha, hint at the development of a cell-mediated response to the subcellular vaccine, but this response is likely not long-lived and does not provide long-term protection.

Significance: For over a century for several laboratories in several countries, there has been a failure to develop either a killed or an extracted component as a safe vaccine against brucellosis. A paradigm shift, both for the development of the vaccine and its mechanism of action, has successfully led to the development of a highly effective, long-lasting, multi-use polysaccharide candidate at DRDC Suffield. To explain this success, cytokine assessments were conducted to determine if their stimulation had any role to play in host immunity.

Future plans: The mechanism of action of the *B. suis* subcellular polysaccharide vaccine remains enigmatic. A high priority for the CF is the development of one vaccine against several threat agents. Although this has shifted from an impossible to a probable delivery, how this affects cytokines on the soldier's health (adversely or not at all) is critical for the development of the vaccine.

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Cytokine Profiling of Mice Vaccinated with *Brucella* Polysaccharide

Hardeep S. Bhogal; Lori J. McLaws; Michelle L. Russell; Nicole D. Barabe; John W. Cherwonogrodzky; DRDC Suffield TM 2007-262; R & D pour la défense Canada – Suffield; Décembre 2007.

Introduction ou contexte: La Brucellose chez les humains, causée par la bactérie *Brucella,* est une maladie qui peut se transmettre aux humains par les animaux et on documente environ 500 000 cas dans le monde tous les ans. La possibilité que la *Brucella* soit utilisée comme arme et comme agent de guerre biologique est un souci réel puisqu'elle peut facilement s'obtenir et se transformer en aérosol; de plus, la dose infectante est faible et peut résulter en une maladie durable. On a concentré nos efforts sur la recherche des vaccins de la *Brucella* mais il n'existe toujours pas de vaccin efficace contre cette maladie. On a mis au point un vaccin au polysaccharide mais on ne connaît pas son mécanisme d'action. Les analyses des protéines régulatrices immunitaires peuvent donner des indices aidant à mieux comprendre le mécanisme du vaccin qui protège contre l'infection.

Résultats: On a observé, dans cette étude, les niveaux d'un panel de protéines régulatrices appelées cytokines à partir de souris injectées avec du O-polysaccharide dérivé de la *B. suis*. Une faible augmentation en interféron gamma et une augmentation importante dans alpha le facteur de la nécrose de la tumeur suggèrent le développement d'une réponse à médiation cellulaire au vaccin infracellulaire mais cette réponse est probablement de courte durée et n'offre pas une protection à long terme.

Portée des résultats: Depuis plusieurs siècles, dans plusieurs laboratoires de nombreux pays, on a été dans l'incapacité de développer une composante ni morte ni extraite comme vaccin sécuritaire contre la brucellose. Un changement de paradigme, concernant à la fois la mise au point du vaccin et son mécanisme d'action, a réussi à aboutir à la mise au point, à RDDC Suffield, d'un candidat polysaccharide à utilisations multiples, durable et très efficace. Pour expliquer cette réussite, on a conduit des évaluations de cytokines pour déterminer si leur stimulation jouait un rôle dans l'immunité de l'hôte.

Perspectives d'avenir: Le mécanisme d'action du vaccin au polysaccharide infracellulaire *B. suis* demeure une énigme. La grande priorité des FC est de développer un vaccin contre plusieurs agents de menace. Les probabilités de réussite ayant évolué es de l'impossible vers le probable, il est crucial de comprendre comment le vaccin affecte les cytokines et la santé du soldat (effet indésirable ou pas) pour le développement du vaccin.

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Acknowledgments

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Introduction

The *Brucella* species, unlike most Gram-negative bacteria, are highly infectious facultative parasites [1]. As these species very rarely kill their host [2] (which can range from marine mammals to wildlife to livestock to humans [3,4,5,6]), are not contagious [7], have a variable incubation time that ranges from a few days to several years [8], and can be treated with antibiotics [9], the Centers for Disease Control and Prevention deem them to be of secondary importance as a bioterrorism threat agent [10]. Nonetheless, they are still of considerable concern as threat agents, as only a few bacteria may be required for infection [11], it is debilitating (with high "undulating" fevers and "night sweats", muscle and joint aches, severe headaches, bone-deformities) [12], it is hardy in the environment [13] and the cell has been described as a "pseudo-spore" (discussion during the 52nd annual Brucellosis Research Conference, Nov. 1999, Chicago), it can infect by the aerosol route [14], and once it becomes established inside the host's cells and bone marrow, it is difficult to eliminate with antibiotics [15].

Perhaps the greatest threat of the *Brucella* species is that these are common infections in third world countries [6]. Indeed, part of the reason these countries are impoverished is the economic impact of brucellosis on the livestock and people [16]. About 500,000 human cases occur each year [17], and acquiring a culture may be as simple as going to a hospital or diagnostic laboratory. Due to its virulence and ease of acquisition, the United States, Japan, and the former Soviet Union have all studied *Brucella* as a putative offensive biological warfare agent [18,19].

The potential use of this bacterium as a biological warfare agent highlights the need for the development of effective prophylactics. In this regard, DRDC Suffield has been most fortunate in the discovery of the first effective subcellular (polysaccharide) vaccine in over a century of investigations around the world [20]. A major impediment to its acceptance as a vaccine is that its mechanism of action is unknown. The accepted effect of antibody stimulation does not appear to play a role and, indeed, investigations reveal that the lower the antibody titer, the better the efficacy of the vaccine (i.e. the best efficacy occurred when the vaccinated mice did not produce detectable titers of anti-*Brucella* antibodies [21]). The induction of cytokine expression (i.e. protein signals that induce cell-mediated responses) by the vaccine that, in turn, enhances host immunity is another possibility. However, depending on the strain of bacterium, state of the host, what antigens are delivered or conditions of the experiment, cytokine expression may be beneficial [22], harmful [23], contradictory [24] or inconsequential [25]. To determine if cytokine expression plays a role for the novel DRDC Suffield polysaccharide vaccine, this study was conducted employing vaccinated and unvaccinated control mice.

Materials and Methods

Polysaccharide Vaccine Production

The preparation of the *B. suis* 145 subcellular (polysaccharide) vaccine is based on previous publications [26]. Briefly the supernatant, containing "exo-polysaccharide" as termed for other bacteria of high virulence [27], and the cells (having lipopolysaccharide or LPS that contain polysaccharide) are treated with a weak solution (3%) of acetic acid and heat (boiling water bath) to cleave the polysaccharide from lipopolysaccharide. The polysaccharide is purified with phenol–water extraction, precipitation and washing of the material with methanol, removal of proteins with trichloroacetic acid, and dialysis against distilled water (which precipitates uncleaved LPS for discard). The polysaccharides from the supernatant and cell are then pooled, freeze-dried and stored at −70°C until required. The polysaccharide was determined to contain less than 1% protein (determined using a protein assay) and no DNA or RNA (determined by measuring absorbance at 260 nm)

Mice Vaccination and Sample Collection

The research was conducted in accordance with the Canadian Council on Animal Care guidelines. At time zero, nine mice were injected intraperitoneally with *B. suis* 145 subcellular vaccine at a dose of 1 µg polysaccharide in 0.1 mL saline. Nine negative control control mice were injected with saline. Six blank control mice did not receive injections. At 6 months after initial vaccination, pre-boost blood samples were collected from all mice (individual mice were not specifically identified at this collection point). The vaccinated and blank control mice were boosted by intraperitoneal injection with *B. suis* polysaccharide at a dose of 1 µg polysaccharide in 0.1 mL saline. The negative control mice were injected with saline in place of antigen. Blood samples were collected from all mice at 6, 24 and 48 hours post boost (individual mice were identified specifically for these collection points). Pre-boost, 6 hour and 24 hour samples were collected via tail bleeds. The 48 hour samples were collected via heart punctures. Blood samples were allowed to clot, centrifuged and serum collected. Serum samples were split into two aliquots and stored at −70°C.

Cytokine Testing

Cytokine testing was performed according to manufacturer instructions using components contained in the Bio-Plex Cytokine Reagent Kit, Bio-Plex Mouse Serum Diluent Kit and Bio-Plex Mouse Cytokine Th1/Th2 Panel kit (Bio-Rad, Mississauga, ON). Buffers and diluents were adjusted to room temperature prior to use. Prior to performing the Bio-Plex Cytokine assay (Bio-Rad), a vacuum apparatus (Millipore, Etobicoke, ON) was calibrated and set to 1-2 mm Hg. Standards, unknown samples and blanks were tested in duplicate in a 96-well filter plate (Bio-Rad). All plate mixing and incubations were carried out in light free conditions at room temperature.

Standard Dilution

One vial of lyophilized cytokine standard (Bio-Rad) was reconstituted with 500 µL Mouse Serum Standard Diluent (Bio-Rad) resulting in a master multiplex cytokine standard stock solution of 50,000 pg/mL. The vial was mixed gently and incubated on ice for 30 minutes. The standard

stock was further diluted in Mouse Serum Standard Diluent to produce a working standard solution of 32,000 pg/mL. Seven additional 1:4 serial dilutions of the working standard solution were prepared. The eight working standard solutions (1.95 to 32,000 pg/mL) were maintained on ice until ready for use.

Sample Dilution

Three representatives from each of three treatment groups were selected for testing. An aliquot of serum from the four collection points for all representatives was removed from −70 °C storage and allowed to thaw on ice. Serum samples were diluted 1:4 in Mouse Serum Sample Diluent (Bio-Rad) and maintained on ice until tested.

Reagent Preparation

Working solutions were prepared from stock anti-cytokine beads, detection antibody and streptavidin-PE (Bio-Rad) in sufficient volume to accommodate the total number of wells to be tested. Stock anti-cytokine beads (25X) were diluted 1:25 in Bio-Plex Assay Buffer (Bio-Rad), detection antibody (50X) was diluted 1:50 in Bio-Plex Detection Antibody Diluent (Bio-Rad), and streptavidin-PE (100X, Bio-Rad) was diluted 1:100 in Bio-Plex Assay Buffer. Tubes were maintained in light free conditions. Anti-cytokine beads were kept on ice, while detection antibody and streptavidin-PE were kept at room temperature until ready for use.

Assay Procedure

The desired numbers of wells on the 96-well filter plate were pre-wet with 150 µL Bio-Plex Assay Buffer. Unused wells were covered with sealing tape and the buffer was removed by vacuum filtration. Working anti-cytokine bead solution was mixed by vortex, and then 50 µL was deposited in each well. Liquid was removed by vacuum filtration. The beads were filter-washed two times with 100 µL Bio-Plex Wash Buffer (Bio-Rad). Each diluted standard and sample was mixed gently. Fifty µL of diluted standard or sample was deposited in each of two wells on the 96-well filter plate. Mouse Serum Sample Diluent was deposited in each of the blank control wells. The plate was covered with sealing tape and shaken on a microplate shaker (Wallac PerkinElmer, Woodbridge, ON) at 1,100 rpm for 30 seconds then 300 rpm for 30 minutes. Sealing tape was removed from test wells and liquid removed by vacuum filtration. Contents of wells were washed three times with 100 µL Bio-Plex Wash Buffer, removing the buffer by vacuum filtration after each wash. Detection antibody working solution was mixed gently and 25 µL was added to all wells. The shaking/incubation and wash procedures were repeated. Streptavidin-PE working solution was mixed gently and 50 µL was added to all wells. The shaking/incubation and wash procedures were repeated, with the final shaking/incubation reduced to 10 minutes. The beads were resuspended in 125 µL Bio-Plex Assay Buffer. The plate was sealed and shaken at 1,100 rpm for 30 seconds. Sealing tape was removed from test wells, and fluorescence was read immediately using the Bio-Plex system (Bio-Plex Manager Software, Bio-Rad).

Statistical Analysis

Statistical analysis of data using the t-test was performed with SigmaStat 3.0 (Systat Software Inc., San Jose, CA). Statistical analysis of non-normally distributed data sets was performed with SigmaStat using the Mann-Whitney U test [28].

Results

Groups of mice were vaccinated with the *B. suis*-derived polysaccharide 145 subcellular vaccine. Cytokine profiling was performed on serum samples which were acquired prior to and at various times after a booster injection of polysaccharide either via tail-bleed or heart puncture. Using the Luminex fluorescent bead-based xMAP technology, samples were tested for the presence of a panel of 8 different cytokines. These included the Th1 cytokines IL-2, IL-12(p70), IFN-γ, TNF-α, and GM-CSF, and the Th2 cytokines IL-4, IL-5, and IL-10.

Prior to the booster injection, baseline cytokine levels were assessed (Fig. 1). Only IL-5, IL-10, and TNF-α were measurable above 1 pg/mL. The mice injected with saline showed slightly higher levels of TNF-α and IL-5 compared to their vaccinated and non-treated counterparts.

Six hours after a booster injection of *B. suis* polysaccharide, IFN-γ levels became slightly elevated and IL-5 levels were depressed relative to both groups of control mice (Fig. 2). In addition, levels of each of GM-CSF and IL-12(p70) were elevated only in the non-treated mice and saline-injected mice, respectively.

After 24 hours, GM-CSF and IFN-γ levels dropped below measurable levels in all groups of animals (Fig. 3). Although IL-5 levels were slightly lower in the boosted animals compared to the saline-injected animals, there was no measurable IL-5 in the non-treated mice.

Interestingly, 48 hours after post-injection, TNF-α levels increased significantly in the boosted animals relative to the saline control group ($p=0.032$) (Fig 4).

Figure 5 provides an overview of the cytokine levels across the different groups of animals over the 4 different time points. Taken together, these results suggest that there may be a Th1-type bias in the cytokine profile, after injection with polysaccharide.

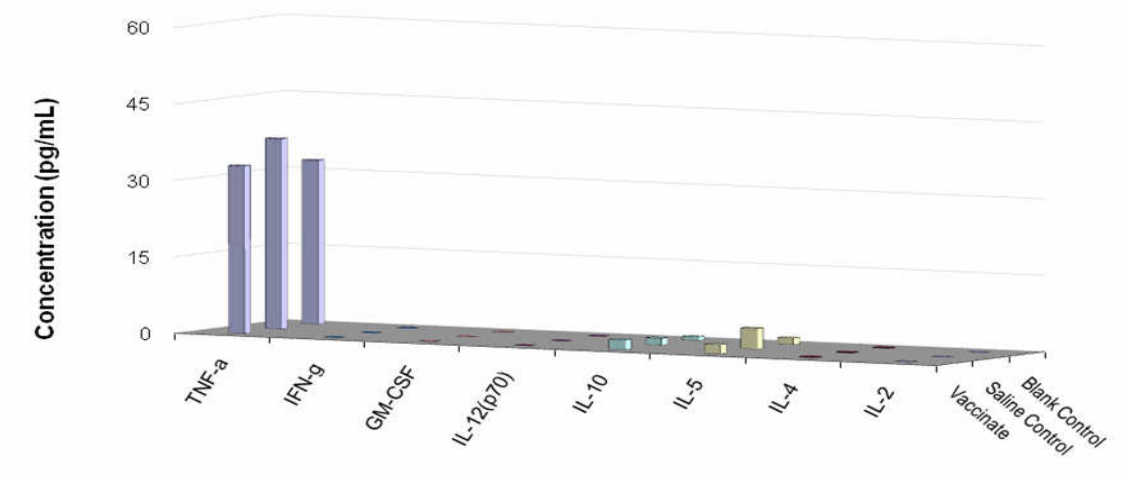

Figure 1: Pre-boost serum samples.

Blood samples were collected prior to boosting with B. suis 145 polysaccharide from three mice in each of the Brucella vaccinated, saline injection and blank control groups. Serums were tested for a panel of eight Th1/Th2 cytokines. The results are displayed as an average of the three samples in each group.

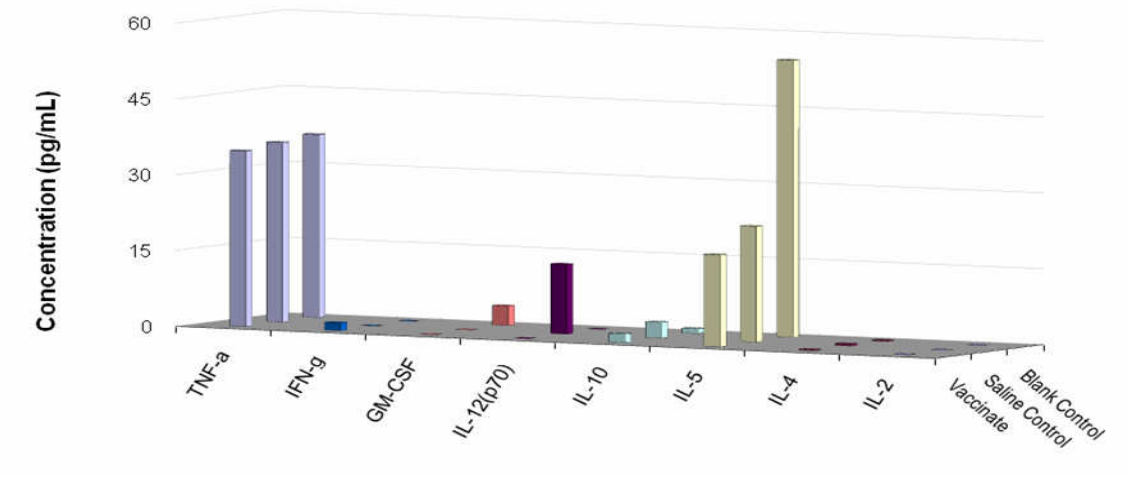

Figure 2: 6 hour post-boost serum samples

Blood samples were collected 6 hours post B. suis 145 polysaccharide boost from three mice in each of the Brucella vaccinated, saline injection and blank control groups. Serums were tested for a panel of eight Th1/Th2 cytokines. The results are displayed as an average of the three samples in each group.

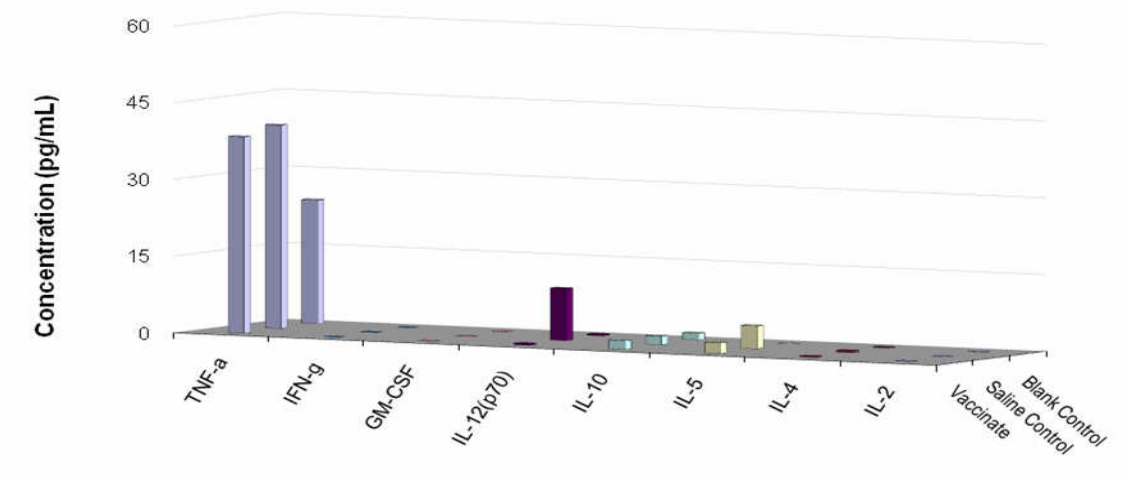

Figure 3: 24 hour post-boost serum samples

Blood samples were collected 24 hours post B. suis 145 polysaccharide boost from three mice in each of the Brucella vaccinated, saline injection and blank control groups. Serums were tested for a panel of eight Th1/Th2 cytokines. The results are displayed as an average of the three samples in each group.

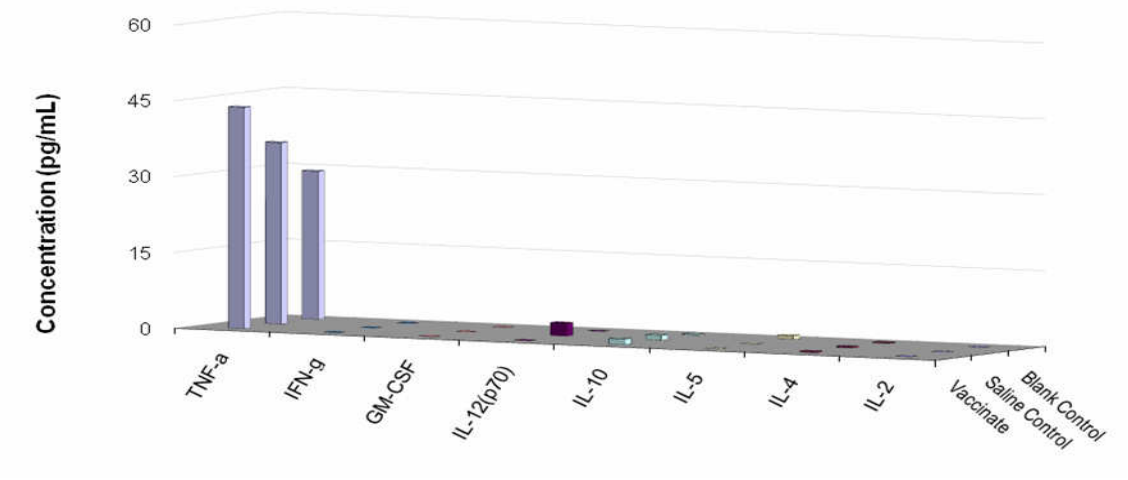

Figure 4: 48 hour post-boost serum samples

Blood samples were collected 48 hours post B. suis 145 polysaccharide boost from three mice in each of the Brucella vaccinated, saline injection and blank control groups. Serums were tested for a panel of eight Th1/Th2 cytokines. The results are displayed as an average of the three samples in each group.

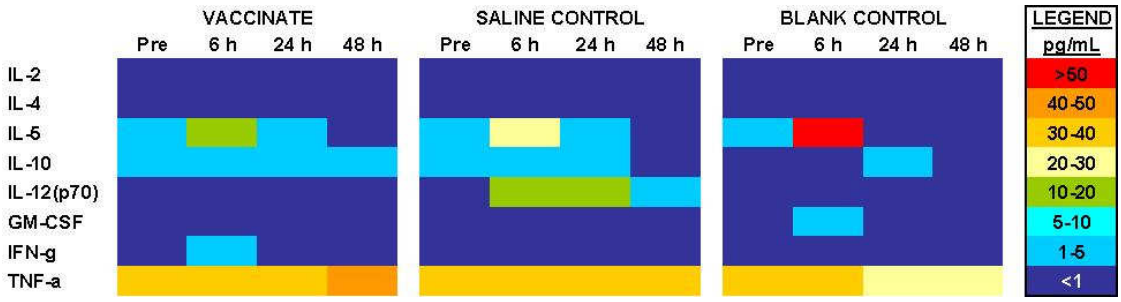

Figure 5: Th1/Th2 Cytokine Array

Blood samples were collected prior to B. suis 145 polysaccharide boost and 6, 24 and 48 hours post B. suis OPS boost from three mice in each of the Brucella vaccinated, saline injection and blank control groups. Serums were tested with a panel of eight Th1/Th2 cytokines using the Bioplex system. The results are displayed in an array showing individual cytokines for each collection time point and represent an average of the three samples in each group.

Discussion

In this study, cytokine profiling experiments have been performed on serum samples isolated from mice that have been vaccinated and boosted with *B. suis*-derived polysaccharide, to determine whether this polysaccharide induces secretion of specific cytokines related to Th1 or Th2-type immune responses. This has been performed using xMAP-based protein suspension array technology which allows for the simultaneous detection of a number of different cytokines from a relatively small sample volume.

After a booster injection of the *B. suis* polysaccharide antigen, a small increase in IFN-γ and slightly depressed IL-5 levels, a Th2 cytokine, were observed at 6 hours post-injection. More importantly, the observation of a statistically significant increase in TNF-α at 48 hours post-booster injection suggests a bias towards a Th1-type response. This Th1 bias, however, in the absence of increases in the other Th1-type cytokines such as IL-2 and IL-12, may not be conclusive and further study will be required to determine if specific Th1 or Th2 cytokine patterns are elicited by the bacterial polysaccharide.

The polysaccharide has been demonstrated previously to provide long-term protection against *Brucella* infection. Although the mechanism of protection has not been elucidated (antibodies do not appear to play a role in providing long-term protection), the extent to which cytokines may play a role in immunity will require further study. Based on results presented here, it is likely that the observed cytokines are elicited in response to *Brucella*-derived polysaccharides, but may not contribute to significant long term protection.

Further research is required to resolve if cytokine responses elicited by the *B. suis* polysaccharide do or do not play a role in the response and hence immunity. Parameters to be studied include the purity of the polysaccharide antigen preparation, the dose of antigen, the route of administration, and the half-life of the antigen *in vivo*. Since no subcellular vaccine has been developed by conventional methods, the existing DRDC Suffield vaccine may well have unconventional influences on an effective immune response.

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List of symbols/abbreviations/acronyms/initialisms

CDC	Centers for Disease Control
CF	Canadian Forces
DRDC	Defence Research & Development Canada
GM-CSF	granulocyte macrophage-colony stimulating factor
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
PE	phycoerythrin
Th1	T helper type-1
Th2	T helper type-2
TNF	tumour necrosis factor

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Although *Brucella* infection is one of the most common infections acquired in the laboratory, infection could also occur as a result of a deliberate act. The potential for *Brucella* to be weaponized and used as a biological warfare agent is a real concern as it is readily obtained and aerosolized, the infective dose is low, and can lead to a long-lasting disease. Therefore, there is a need for the development of an effective vaccine. A subcellular polysaccharide vaccine derived from *Brucella suis* has been shown to be effective in providing long-term protection from brucellosis in animal studies. The mechanism of action of this subcellular vaccine is unknown. In this study, we report the findings from cytokine profiling of mice injected with subcellular polysaccharide vaccine. A minor increase in IFN-γ and a depressed level of IL-5, coupled with a significant increase in TNF-α, hint at the induction of a Th1 response. Other cytokines such as IL-2, IL-4, IL-10, IL-12, and GM-CSF remain unchanged in response to vaccination with the polysaccharide. Although the polysaccharide may induce a cell-mediated response, this immune response is likely short-lived and, therefore, may not contribute to the long-term protection afforded by this subcellular vaccine.

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Brucella; brucellosis; cytokine; suspension array; vaccine; polysaccharide

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