

COMPARISON OF THE IMMUNE RESPONSE TO *COXIELLA BURNETII* AND *BURKHOLDERIA MALLEI* IN BALB/C MICE: A DIFFERENTIAL RESPONSE BY TWO DIVERSE BIOLOGICAL AGENTS

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ABSTRACT

We compared the immune response of BALB/c mice to two biological agents, *Coxiella burnetii* and *Burkholderia mallei*. Both *C. burnetii* and *B. mallei* cell preparations induced a proliferative and a mixed Th1- and Th2-like cytokine response in splenocytes from vaccinated mice. *B. mallei* cell preparations induced higher levels of IgG and IgM than did *C. burnetii*. The ratio of the subclasses IgG2a over IgG1 from *C. burnetii* vaccinated mice was higher than 1.0, while the ratio from *B. mallei* was lower than 1.0. The difference in the Th1-like response of the Ig subclasses may be part of the reason why vaccination with the *C. burnetii* can lead to a protective response, while vaccination with *B. mallei* does not.

INTRODUCTION

We compared the immune response in mice to *Burkholderia mallei* and *Coxiella burnetii* to determine the differences in their response that might reveal why one cellular vaccine is efficacious but not the other. *B. mallei* is a gram-negative, rod shaped, facultative intracellular bacterium, which causes glanders primarily in horses¹. Humans can become infected with the organism by coming in contact with infected animals or contaminated products from infected animals. More recently, the highly infectious nature of *B. mallei* was shown in the laboratory, by direct exposure of the organism to the skin or inhalation of an aerosol of the organism^{2,3}. Because of these properties *B. mallei* is considered to be a potential biowarfare agent⁴. Glanders can be presented as an acute or chronic disease, both of which can progress from one state to the other. Death can occur quickly in untreated cases or a chronic disease state can persist for years. Diagnosis of the disease is by isolating the organism from infected exudates or tissues from the suspected animal or human. Identifying the organism is now aided by the discovery of species-specific phages, which can differentiate *B. mallei* from the closely related species *B. pseudomallei*⁵, which causes the more common disease, melioidosis. Currently, there is no vaccine for glanders, but resolution of the disease in humans can occur with long-term treatment with a combination of doxycycline and azithromycin³.

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C. burnetii is an obligate, intracellular bacterium, which causes Q fever. Like *B. mallei*, *C. burnetii* is highly infectious, causes a debilitating disease and is considered to be a potential biowarfare agent⁴. Q fever, is commonly contracted by inhaling infectious particles or aerosols, or by consuming contaminated raw milk in the Middle East⁶. It is less commonly transmitted through the bite of a tick. Q fever can also be presented in an acute or chronic state, with the latter state involving the cardiovascular and reticuloendothelial system. Diagnosis of Q fever is confirmed by indirect fluorescent antibody detection or enzyme-linked immunosorbent assays (ELISA). Unlike for glanders, there is a vaccine for Q fever; Q-Vax is a formalin-killed cellular vaccine, which was demonstrated to prevent Q fever in high-risk individuals of the population⁷. Q fever in humans can be treated with doxycycline and hydroxychloroquine.

This is a report of the results of our study of the immune response to these two diverse organisms in BALB/c mice.

EXPERIMENTAL METHODS

Bacterial Strains. Irradiation-inactivated(IR) and heat-killed(HK) *B. mallei* (ATCC 23344) cellular preparations were grown and prepared as previously described⁷. The phase I Nine Mile strain of *C. burnetii* was propagated and prepared as previously described⁸.

Mice. Female, 6- to 8-week-old, BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). Mice, four to five per group, were vaccinated with 100 µg of either *B. mallei* or *C. burnetii* with 100 µg of Alhydrogel (Superfos Biosector G/S, vedbaek, Denmark). Vaccinations were given subcutaneously, twice, 3 weeks apart. Sera and spleens were collected from mice 3 weeks after the second boost as previously described (Amemiya,2002). All animals used in this research project were cared for and used humanely according to the following policies: the *Public Health Service Policy on Humane Care and Use of Animals*¹⁰; *Guide for the Care and Use of Laboratory Animals*¹¹. All U.S. Army Medical Research Institute of Infectious Diseases animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal use was approved by the Institutional Animal Care and Use Committee and conducted in accordance with federal Animal Welfare Act regulations.

Spleen Cell Cultures, Proliferation Assays, and Cytokine Assays. Splenocyte cell cultures, proliferation assays, and cytokine expression assays were prepared and performed as previously described⁸.

Antibody Assays. Titers for the major mouse immunoglobulin (Ig) classes and subclasses were determined as described previously⁸. Results were reported as the reciprocal of the highest dilution giving a mean optical density (at 450 nm) of at least $0.1 \pm$ the standard deviation (SD).

RESULTS

Proliferative response by splenocytes. We compared the proliferative response of splenocytes from BALB/c mice vaccinated with either *B. mallei*, or *C. burnetii*. Table 1 shows that in splenocytes from either heat-killed or irradiated *B. mallei* vaccinated mice, there was a small response (1.5 to 1.6X) by the stimulated splenocytes, when compared to the response of splenocytes from mice vaccinated with adjuvant only. This was also true with splenocytes from *C. burnetii* vaccinated mice. In this case, there was a slightly smaller response (1.4X) by the restimulated splenocytes, when compared to the control

splenocytes. All splenocytes, including the cells from the adjuvant-only mice, were stimulated approximately equally by concanavalin A (ConA). Thus, there was a specific proliferative response by stimulated splenocytes from mice vaccinated with either microorganism, although it was a relatively small response.

Table 1. *B. mallei* And *C. burnetii* Both Induce A Low Proliferative Response In Splenocytes From Vaccinated Mice.

Cell Vaccinated	Medium	3H-Thymidine Incorporated (mean cpm±SD)	
		Conditions Antigen	ConA
Heat-Killed (HK) <i>B. mallei</i>	815±235	12,940±1023	174,857±15,026
Irradiated (IR) <i>B. mallei</i>	530±73	10,627±492	172,229±6468
Irradiated (IR) <i>C. burnetii</i>	487±131	3,077±328	165,449±5396
Adjuvant (only)	660±99	7,898±272 (<i>B. mallei</i> HK) 7,312±848 (<i>B. mallei</i> IR) 2,271±277 (<i>C. burnetii</i> IR)	155,325±7251

Table 2. *B. mallei* Induces A Mixed Th1-/Th2-like Cytokine Response In Splenocytes From Vaccinated Mice.

Cytokine	Cell Vaccinated	Amount Cytokine Expressed (pg/ml)		
		Medium	Stimulating Antigen <i>B. mallei</i>	ConA
Th1:IL-2	<i>B. mallei</i> (IR)	40±5	93±19	503±248
	Adjuvant (only)	20±3	42±3	196±23
IFN γ	<i>B. mallei</i> (IR)	9,843±2123	22,967±5,886	21,616±5,693
	Adjuvant (only)	10,808±5018	15,440±7,632	12,738±3,377
Th2: IL-4	<i>B. mallei</i> (IR)	24±2	27±7	45±5
	Adjuvant (only)	19±2	13±4	68±4
IL-10	<i>B. mallei</i> (IR)	165±28	1,210±330	707±71
	Adjuvant (only)	109±40	190±40	862±254

Cytokines Expressed by Splenocytes. We next examined the cytokines expressed after 24 hours by restimulated splenocytes from mice vaccinated with either *B. mallei* or *C. burnetii*. Table 2 shows the representative T cell helper 1 (Th1)-like (IL-2,IFN- γ) and T cell helper 2 (Th2)-like (IL-4,IL-10) cytokines expressed by splenocytes from *B. mallei* (IR)-vaccinated mice. In this case some IL-2, IFN- γ , IL-4, and IL-10 could be detected in restimulated splenocyte cultures from *B. mallei* vaccinated mice. At the same time, we could detect IL-2 and IFN- γ in culture supernatants from Con A-stimulated splenocytes from *B. mallei*-vaccinated mice. Upon examination of restimulated splenocyte cultures prepared from cells from *C. burnetii* vaccinated mice, we found the expression of IL-2, IFN- γ , and IL-10. At the same time in splenocyte cultures stimulated with Con A, we saw slightly higher levels of expression of cytokines in the splenocytes cultures prepared from mice receiving only adjuvant. In summary, essentially the same cytokines (Th1- and Th2-like) were expressed by restimulated splenocytes prepared from mice vaccinated with either *B. mallei* or *C. burnetii*.

Table 3. *C. burnetii* Induces A Mixed Th1-/Th2-like Cytokine Response In Splenocytes From Vaccinated Mice.

Cytokine	Cell Vaccinated	Amount Cytokine Expressed (pg/ml)		
		Medium	Stimulating Antigen <i>C. burnetii</i>	ConA
Th1: IL-2	<i>C. burnetii</i> (IR)	9 $\pm$ 3	54 $\pm$ 13	5,070 $\pm$ 645
	Adjuvant (only)	8 $\pm$ 1	8 $\pm$ 1	6,242 $\pm$ 3132
IFN γ	<i>C. burnetii</i> (IR)	4,334 $\pm$ 833	123,941 $\pm$ 20,670	99,769 $\pm$ 24,254
	Adjuvant (only)	3,584 $\pm$ 250	81,682 $\pm$ 28,922	131,109 $\pm$ 38,507
Th2: IL-4	<i>C. burnetii</i> (IR)	44 $\pm$ 1	43 $\pm$ 7	732 $\pm$ 287
	Adjuvant (only)	47 $\pm$ 2	42 $\pm$ 2	1,251 $\pm$ 177
IL-10	<i>C. burnetii</i> (IR)	192 $\pm$ 46	678 $\pm$ 326	676 $\pm$ 142
	Adjuvant (only)	153 $\pm$ 8	419 $\pm$ 90	877 $\pm$ 149

Table 4. *B. mallei* Induces a Higher Immunoglobulin Response Than That by *C. burnetii*.

Cell Vaccinated	Immunoglobulin Titer		
	IgM	IgG	IgA
<i>B. mallei</i> (HK)	32,000 $\pm$ 979	512,000 $\pm$ 24,188	1,000 $\pm$ 0
<i>B. mallei</i> (IR)	32,000 $\pm$ 1,866	2,048,000 $\pm$ 1,047,814	1,000 $\pm$ 7
<i>C. burnetii</i> (IR)	12,800 $\pm$ 433	25,000 $\pm$ 1,706	100 $\pm$ 0

Immunoglobulin Class and Subclass Response. Examination of the immunoglobulin (Ig) class response of mice vaccinated with either *B. mallei* or *C. burnetii* showed that the mice vaccinated with *B. mallei* had much higher Ig titers than those vaccinated with *C. burnetii* (Table 4). These results were consistent with all three Ig classes (IgM, IgG, and IgA) examined when tested against the homologous antigen. Further examination of the Ig subclasses induced by either candidate cellular vaccine revealed that the major subclass produced against *B. mallei* was IgG1 (Table 5). On the other hand, the major Ig subclass induced by *C. burnetii* was IgG2a. These results showed that the ratio of IgG2a to IgG1 was consistently less than 1.0 for *B. mallei* (HK or IR) and always more than 1.0 for *C. burnetii*. The major Ig class and subclass titers for the adjuvant-only control mice was less than or equal to 50 for either antigens (data not shown). In summary, the level of the major Ig classes was higher for *B. mallei* than for *C. burnetii*, and the major subclass response to the microorganisms was different for each bacterium.

Table 5. The Major Immunoglobulin Subclass Induced By *B. mallei* And *C. burnetii* Is Different.

Cell Vaccinated	IgG1	IgG2a	Titer IgG2b (X 10 ³)	IgG3	Ratio IgG2a/IgG1
<i>B. mallei</i> (HK)	256±181	40±27	46±55	176±96	0.16
<i>B. mallei</i> (IR)	336±217	54±55	76±60	56±16	0.16
<i>C. burnetii</i> (IR)	14±24	83±55	6±2	38±14	5.9

DISCUSSION

We previously examined the efficacy of *B. mallei* cellular candidate vaccines in BALB/c mice and found that neither vaccination with neither the heat-killed, irradiation-inactivated, nor the capsule-negative mutant of *B. mallei* protected BALB/c mice from a live challenge⁸. This is in contrast to the *C. burnetii* Q-Vax vaccine, which is a formalin-killed cellular vaccine, that can protect both mice and guinea pigs from an aerosol challenge¹². The phase I Nine Mile strain of *C. burnetii* used in our study is closely related to the strain used in the preparation of the Q-Vax vaccine. It has been shown to increase non-specific resistance to infection and stimulates lymphokine production in mice⁹. In our previous study, we found that the *B. mallei* cell preparations (HK, IR, and IR-capsule negative) induced similar cellular immune responses. They induced splenocyte proliferation in restimulated splenocyte cultures. In addition, all three *B. mallei* preparations induced a mixed Th1-/Th2-like cytokine response (IL-2, INF- γ , IL-4, IL-5, and IL-10) in splenocyte cultures under similar conditions. The immunoglobulin subclass response to all three *B. mallei* preparations also were identical, that is IgG1 was the predominate immunoglobulin subclass induced by the cellular preparations. IgG1 is considered to be a Th2-like immunoglobulin subclass response¹³.

In our present study, we chose to examine the immune response to *C. burnetii* at the same time as *B. mallei* because cellular vaccines from the former organism had been shown to be efficacious in animals and humans^{14,15,16}. We found a similar immune response for both heat-killed or irradiation-inactivated *B. mallei* and *C. burnetii* cellular preparations, when we examined the proliferative response and induction of cytokine expression by splenocytes from vaccinated mice. We were able to detect a proliferative

response in splenocytes from *C. burnetii* vaccinated mice, although at a slightly lower (1.4X) amount than that seen in splenocytes from *B. mallei* vaccinated mice (1.5-1.6X). It has been reported that *C. burnetii* usually suppresses the lymphocytic proliferative response in mice vaccinated with phase I *C. burnetii*.^{17,18,19,20} This difference in our study and those previously reported may be attributed to the use of BALB/c mice in the present study rather than C57BL/10ScN mice, which are endotoxin-nonresponder mice. Another difference may be that we vaccinated our mice subcutaneously with the phase I *C. burnetii* cells mixed with alhydrogel compared to the intraperitoneal injection of the Q-Vax or *C. burnetii* cellular vaccine without any adjuvant. The cytokine response was also similar in splenocytes from *B. mallei* and *C. burnetii* vaccinated mice. Both Th1- and Th2-like cytokines were expressed in these splenocytes. Interestingly, we did see some suppression of cytokine expression in concanavalin A stimulated splenocytes from *C. burnetii* vaccinated, when compared to the splenocytes from mice receiving only adjuvant. These results were observed more often than not at the same time, when we were able to detect expression of cytokines in splenocytes from the same mice. We also saw this seemingly contradictory result occasionally in splenocytes from *B. mallei* vaccinated mice. However, stimulation by ConA or by the organism are most likely through different mechanisms (receptors?), and measuring different parameters may lead to divergent results.

The differences we observed between the two organisms were the specific immunoglobulin class titer and the major immunoglobulin subclass to the cellular vaccines. It is not clear if the different response to *C. burnetii* was the result of the immunosuppression reported associated with the *C. burnetii* vaccines¹⁷⁻²⁰, and/or the result of how *C. burnetii* may be taken up by host cells, processed, and presented to the immune system. *C. burnetii*, either alive or dead, can be taken up internally²¹, but once in the cell (dendritic or macrophage?) nonviable cells may be processed and presented through a Th1-like cellular immune pathway. Although *B. mallei*, is a facultative intracellular bacteria, it must be processed differently. The capsule is considered a virulence factor²², which may protect the bacteria from being actively processed by a dendritic or macrophage cell. However, even the nonviable, capsule-negative mutant of *B. mallei* is not processed through a Th1-like pathway, as the results of our studies suggest, and consequently does not lead to a protective response⁸. Further studies are underway to modulate the murine immune system so that the *B. mallei* cellular vaccine can be processed so that the protective response is enhanced.

CONCLUSION

We found differences in the way the immune system in BALB/c mice responds to the cellular vaccines of *C. burnetii* and *B. mallei*. A better understanding of the interaction of the immune system with different vaccines, such as one that is efficacious and one that is not, will help us in the future to decide on how to approach the development or the modification of new and current vaccines. With the use of different classes of immunomodulators, such as cytokines, immunostimulatory oligonucleotides, adjuvants, or immunogenic proteins, the interaction of the immune system with the candidate vaccines can be modified, which may lead to a more favorable immune response.

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