Review article

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Development of vaccines to *Mycobacterium avium* subsp. *paratuberculosis* infection

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Johne's disease or paratuberculosis is a chronic debilitating disease in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease causes significant economic losses in livestock industries worldwide. There are no effective control measures to eradicate the disease because there are no appropriate diagnostic methods to detect subclinically infected animals. Therefore, it is very difficult to control the disease using only test and cull strategies. Vaccination against paratuberculosis has been considered as an alternative strategy to control the disease when combined with management interventions. Understanding host-pathogen interactions is extremely important to development of vaccines. It has long been known that Th1-mediated cellular immune responses are play a crucial role in protection against MAP infection. However, recent studies suggested that innate immune responses are more closely related to protective effects than adaptive immunity. Based on this understanding, several attempts have been made to develop vaccines against paratuberculosis. A variety of ideas for designing novel vaccines have emerged, and the tests of the efficacy of these vaccines are conducted constantly. However, no effective vaccines are commercially available. In this study, studies of the development of vaccines for MAP were reviewed and summarized.

Keywords: Vaccines, Immune responses, *Mycobacterium avium* subsp. *paratuberculosis*

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a causative agent of Johne's disease or paratuberculosis, which is a chronic debilitating disease in ruminants that is characterized by incurable enteritis and persistent diarrhea [1]. The disease is distributed worldwide and causes significant economic losses to the livestock industry because of premature culling and production losses [2,3]. In the United States, MAP-positive herds experience economic losses of almost US $100 per cow and a disease cost of US $200 to 250 million annually [4]. At the herd level, it has been estimated that more than 50% of dairy cattle farms were infected with MAP in most major dairy-producing countries [4,5]. Moreover, the most recent herd level prevalence estimates are as high as 90% in the U.S. dairy cattle industry [6]. These findings indicate that an infection rate of MAP is increasing and there is a need to establish an efficient program for control of this pathogen.

Clinical signs of the disease, such as diarrhea, loss of milk production and weight loss, are usually absent until two or more years after initial infection [7]. Stages of the

MAP infection can be divided into four categories according to severity of clinical signs, the potential for shedding organisms, and the possibility of detection using current diagnostic methods [3]. The first stage, silent infection, is generally observed in young animals less than two years of age. These animals have no signs of infection clinically or microbiologically. Furthermore, there are no cost-effective diagnostic methods to detect animals in this stage [8]. The second stage is subclinical infection. Although animals in this stage still have no clinical signs of infection, they may be detected through cost-effective diagnostic tests such as serum enzyme-linked immunosorbent assay (ELISA) and fecal culture [9]. However, many of the animals in this stage are not detected by such tests because the animals shed organisms in an intermittent manner [10], and antibodies against MAP are usually produced when they are close to the next stage of disease (clinical stage) [11]. These undetected subclinical fecal shedders become a source of infection that consistently contaminate the environment. Therefore, many attempts have been made to detect these animals based on immunological knowledge. One of the major attempts is to identify MAP specific antigens that can be used in the interferon γ (IFN-γ) assay to measure Th1-mediated immune response elicited by animals in the early stage of infection [12,13]. Another attempt is to identify biomarkers of the MAP infected animals by analysis of transcriptional changes that show early responses to infection. Accordingly, host transcriptional profiles during the early stage of infection in mouse RAW264.7 cells, MAP infected mouse models, and naturally infected cattle have been analyzed [14-16].

There are three major approaches to reduce or eradicate the Johne's disease, efficient management to decrease transmission, testing and culling, and vaccination [17]. Management using testing and culling practices are used in most countries [18]. Although the incidence of Johne's disease can be reduced by efficient management, eradication can only be accomplished when all the infected animals are detected and culled [19]. Although diagnostic tests for Johne's disease are improving, it is still not possible to detect all infected animals. For these reasons, testing and culling strategies using the present diagnostic methods are ineffective for eradication of the disease except when targeting only-high shedding animals [17,20]. Under these circumstances, vaccination can be the best control strategy unless animals can be detected during early infection. This is because vaccination can reduce the incidence of MAP shedding and manifestation of clinical signs, which is more cost-effective than testing and culling [21]. However, vaccination is probably the least accepted strategy because of several drawbacks, which are discussed in the next section of this review.

MAP can infect a wide range of animals, therefore, it is important to determine if wildlife can act as a maintenance host or act as spillover host because the infection can persist via intraspecies transmission alone in maintenance hosts. In fact, one of the difficulties of eradication of bovine tuberculosis is blocking contacts with livestock from wildlife such as badgers, brush-tailed possums, and white-tail deer [22]. Some species susceptible to paratuberculosis such as farmed deer could maintain the infection when they are in high-density populations [23]. In South Korea, there have been several reports that MAP has infected wild boar, sika deer, and mouflon [24-26]. In this review, the general characteristics of Johne's disease with respect to the pathogenesis and immune response to MAP, as well as recent advances in development of vaccines were briefly examined.

Pathogenesis and Immune Responses to MAP

MAP infection is initiated by ingestion of fecal material orally contaminated by MAP (fecal-oral route). Following ingestion, MAP can pass through the M cell, which is specialized for uptake of particles that mainly bind to bacteria and transport them into the submucosal layer. After crossing the epithelial layer, MAP is phagocytosed by submucosal macrophages [27]. Like other mycobacterium, MAP is able to survive and replicate in non-activated macrophages by inhibiting phagosome-lysosome maturation [28,29]. MAP eventually causes the cell death of infected cells, after which liberated MAP can be phagocytosed by freshly accumulated macrophages and dendritic cells that are activated by cytokines such as tumor necrosis factor α and IFN-γ. IFN-γ, which plays an important role in activation of macrophages and T cells, is produced by infected cells, local γδ T cells, and natural killer cells [30,31]. Activated macrophages and dendritic cells produce interleukin (IL) 12 and present antigen to naïve CD4+ T cells through MHC class II molecules. IL-12 triggers the differentiation of naïve CD4+ T cells into T helper 1 (Th1) cells. Th1 cells produce cytokines such as IL-2 and IFN-γ, which play roles in promotion of expansion of antigen specific Th1 cells and maturation of macrophages. These antigen specific responses change from innate immunity to cell mediated adaptive immunity.

In the later stages of infection, increasing antibodies are frequently observed with increasing bacterial shedding [32]. Therefore, it has been accepted that switching from Th1 immune responses to Th2 responses is the cause of disease progression [33-35]. Many researchers investigated the immune regulatory mechanisms of host animals to identify causes of Th1-Th2 switches. An increase of IL-10 production by regulatory T cells (Tregs) or macrophages, which induces the down regulation of Th1 responses and stimulation of antibody production, is considered to cause this switch [36-38]. Along with Tregs, γδ T cells have been known to play a role in immune regulation. The progressive decrease of CD4+ T cell population in local immune response has been shown to be accompanied by increasing γδ T cell population [32]. The cytokine mRNA profiles of γδ T cells demonstrated that subsets of bovine γδ T cells encode IL-10 and transforming growth factor β, suggesting a potential regulatory role of γδ T cells [39,40]. Recently, however, a typical pattern of disease progression that can be explained by the Th1-Th2 switch was observed in only 40% of MAP-infected sheep with other cases showing simultaneous responses of both cellular and humoral immunity or cellular immunity only [41]. A recent study using mathematical modeling suggested that Th1-Th2 switch may be a result of disease progression (increasing of extracellular bacteria) rather than cause [42]. A similar study that used a mathematical model to analyze the correlation between Th1, Th2 expression and bacterial shedding revealed a positive correlation between the amount of bacteria and humoral immune response was observed. However, there was no evidence of competition or synergy between Th1 and Th2 immunity [43]. This study also suggested that MAP-specific cellular immune responses were predicted to increase shedding, whereas in some animals it was predicted to suppress the shedding. As a result, it can be inferred that adaptive immune responses play a limited role in disease protection. However, these mathematical modeling studies have some vulnerable points because they cannot consider all of the parameters. Therefore, these results or hypotheses should be confirmed by both *in vitro* and *in vivo* studies. Another modeling study suggested that long-term subclinical infection may related to innate immunity rather than adaptive immunity [44]. The results using structural models (villus model, granulomatous model) of local infection showed that the long subclinical phase was due to structural organization of the granulomatous lesion. Moreover, the authors predicted that intermittent shedding was due to changes in the recruiting efficiency of macrophages influenced by external factors such as hormonal changes (ex., pregnancy, lactation).

Paratuberculosis Vaccines

Live attenuated vaccines

Recently, many researchers have been interested in development of live attenuated vaccines against MAP. These types of vaccines can elicit protective mucosal and systemic immune responses because the diverse antigens included in this vaccine can stimulate both innate and adaptive immunity [45,46]. Another advantage of this vaccine is that manufacture of live attenuated vaccine is cost effective and easies than that of other vaccines such as subunit vaccines [47]. Many vaccine candidates have been produced by mutagenesis to attenuate the virulence of MAP. Mutants of MAP have been made by phage-mediated techniques, transposon mutagenesis and allelic exchange mutagenesis [48-50]. Many transposon mutant libraries have been created to identify virulence mechanisms thereby finding vaccine candidates [49,51,52]. Direct mutagenesis using allelic exchange techniques has also been tried by deletion of genes already known to be pathogenic or essential for intracellular survival in *M. tuberculosis* or *M. bovis* [46,47,50,51,53]. The ΔrelA, Δlsr2, and ΔpknG mutants were generated by Park et al. [50] and each gene was known to be related to virulence factors in *M. tuberculosis* and *M. bovis* [54-56]. Two of these candidates, ΔrelA and ΔpknG were evaluated for virulence attenuation and efficacy as vaccine candidates using macrophages and ileal cannulation models of natural hosts (cattle) and goats [57]. The result showed that ΔrelA mutant was a better vaccine candidate than ΔpknG mutant based on virulence attenuation and inhibition of MAP challenge in baby goats. The WAg906 (ΔMAP1566), WAg913, and WAg915 (ΔppiA) mutants were evaluated by Scandurra et al. [51]. WAg906 and WAg913 were made by transposon mutagenesis using MAP 989 strain, and WAg915 was made by allelic exchange of the ppiA gene [51,58]. These live attenuated vaccine candidates were evaluated using monocyte derived macrophages (MDM) apoptosis, IL-10 production and animal models (mouse and goat) [51]. The results revealed that WAg906 mutant was the most attenuated strain. Mouse vaccinated with WAg915 mutant reduced bacterial loads in the spleen and liver after challenge with MAP. The ΔleuD, Δmpt64, and ΔsecA2 mutants were constructed by allelic exchange of these three genes to develop effective live attenuated vaccine [53]. These mutant candidates were selected based

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on previous studies. The auxotroph *leu*D mutant of *M. bovis*-BCG strain showed lower survival rates [59,60], and *M. bovis leu*D mutant induced significant protective immune responses against a virulent *M. bovis* strain in cattle [61]. The *mpt*64 gene is related to apoptosis of multinucleated giant cells [62, 63], while the *sec*A2 mutant of *M. tuberculosis* enhanced apoptosis of infected macrophages [64]. Testing using the mouse model revealed that the most obviously attenuated mutant was ΔleuD mutant strain. In addition, ΔleuD mutant induced a significant reduction of inflammation and bacterial load compared with the non-vaccinated group. The ΔsigL and ΔsigH mutants that are knocked out of the sigma factor gene were selected as live attenuated vaccine candidates because the sigma factors involved in part of the global virulence regulation provide resistance to the host bactericidal activities [46,65]. The ΔsigL and ΔsigH mutants showed attenuated virulence in mice, and these mutants elicited significant protective immune responses against MAP infection in mouse models [46,47].

Recently, a three phase vaccine candidate evaluation strategy was established by Johne's Disease Integrated Program (JDIP) research consortium to improve the efficiency of the efficacy test on the MAP live attenuated vaccines [66]. Phase I is a screening test using the MDM model, phase II is a challenge test using the mouse model, and phase III is an evaluation of protective effects using a goat model. The phase I test was conducted by Lamont et al. [67] to evaluate many live attenuated vaccine candidates constructed until 2014.

Subunit vaccines

Subunit vaccines have been developed to overcome the drawbacks of whole-cell based vaccines. Whole-cell based vaccines interfere with the diagnosis of both tuberculosis and paratuberculosis in vaccinated animals. However, subunit vaccines using well defined recombinant MAP proteins or DNA encoding immunogenic antigens can overcome the interference issues [68]. Many attempts have been made to identify MAP antigens to develop subunit vaccines using genomic or proteomic analysis. Because the production of IFN-γ induced by Th1-mediated immune responses is crucial to reducing the number of bacteria in the early stages of MAP infection, identifying antigens that induce strong Th1 responses is essential to the development of subunit vaccines [68]. Finding an antigen is also related to development of immunodiagnostic method as well as development of subunit vaccines. Several proteins have been identified as vaccine candidates. Several antigens were tested for their potential for use as a vaccine candidates: heat shock protein 70 (Hsp70) [69], antigen 85 complex proteins (Ag85A, Ag85B, and Ag85C) [70], lipoproteins (LprG and MAP0261c) [71,72], PPE family proteins (MAP1518 and MAP3184) [73], superoxide dismutase [70], and alkyl hydroperoxide reductases (AhpC, AhpD) [74]. Among many antigens, the protein Hsp70 has been widely studied as a subunit vaccine candidate. Cattle vaccinated with Hsp70 containing an adjuvant, dimethyl dodecyl ammonium bromide, showed reduced bacterial load compared with a non-vaccinated group in animals experimentally challenged with MAP [75]. Furthermore, the cross-reactivity with serologic test of paratuberculosis was not observed when a preabsorption step with Hsp70 was included, and Hsp70 vaccination did not interfere with the skin test of tuberculosis, despite Hsp70 being a major component of mycobacterial tuberculin [76,77]. However, recent study suggested that the protective effects of Hsp70 protein are due to B-cell activation and therefore the production of Hsp70-specific IgG1, instead of Th1-mediated immune response producing IFN-γ [78]. To date, researchers have only focused on cell-mediated immune responses to identify candidate vaccines. However, further studies are needed to understand protective mechanisms to MAP of host animals in greater detail, including humoral immune responses in the early stages of infection.

DNA vaccination against mycobacteria showed very effective protective immune responses in small rodents [79,80]. Moreover, DNA vaccines have advantages of storage and delivery because they are very stable. Several candidates were evaluated for their ability to induce protective immune responses; however, they were only evaluated in mouse models. Recently, combination of MAP-specific antigens and viral vectors was attempted to increase the ability of antigenic effects of DNA vaccines [81,82]. The advantage of viral vectored vaccines is to provide high delivery of antigens to antigen presenting cells, thereby increasing antigen specific CD4+ and CD8+ immune responses [83-85].

Commercially available vaccines

The first MAP vaccine, which was developed in 1926 by Vallee and Rinjard, consisted of a live non-virulent MAP and oil based adjuvants. Since then, a number of whole-cell based vaccines, live attenuated vaccines and inactivated vaccines were developed to prevent bovine and ovine Johne's disease. Currently, three commercial vaccines are all based on inactivated whole bacteria, Mycopar, Gudair, and Silirum, of which

only Mycopar is approved for use in the United States [17]. Mycopar is manufactured by Boehringer Ingelheim Vetmedica Inc. using MAP strain 18 for use in cattle. Interestingly, strain 18 is not a MAP, although it is a member of the family of *Mycobacterium avium* species [86]. Gudair is manufactured by CZ Veterinaria in Spain for use in sheep and goats using heat inactivated MAP F316 strain adjuvanted with mineral oil. Gudair vaccination is encouraged in Australia for controlling ovine Johne's disease [87]. An Australian study revealed that vaccination could reduce the prevalence of MAP shedding with their longitudinal study [88]. However, another cross-sectional study reported that shedding of MAP persisted in the majority of flocks, despite vaccination of lambs [89]. Silirum consists of MAP F316, similar to Gudair. This vaccine is manufactured by Zoetis to prevent bovine Johne's disease. An efficacy test with a randomized control of Johne's disease in young farmed deer in New Zealand revealed that vaccination of Silirum reduced the prevalence of clinical disease [90]. Meta-analysis of the efficacy of MAP vaccination, especially its production, epidemiological effects, and pathogenic effects, was conducted by Bastida and Juste in 2011 [17] using previously published papers. From this meta-analysis, it was concluded that vaccination against MAP is a useful strategy for reducing contamination by this pathogen, production losses and pathologic effects. Despite the many advantages of vaccination, it has not been encouraged in cattle in most of countries because of several drawbacks mentioned in the introduction section. One major drawback of whole-cell based vaccination is interference with diagnostic tests currently used in bovine tuberculosis and paratuberculosis [91,92]. These vaccines have the potential to produce false positive animals in serological tests for paratuberculosis such as ELISA because the commercial ELISA kit consisted of crude MAP antigens, which hinder differentiation of infected animals from vaccinated animals [93]. The caudal fold skin test using *M. bovis* purified protein derivatives (PPD-B) is most widely used field screening tool for diagnosis of bovine tuberculosis [94]. However, in the IFN-γ assay, stimulation with PPD-B produced robust responses similar to PPD-J (MAP purified protein derivatives) in MAP vaccinated animals [92,95]. Because of this cross-reaction with other mycobacteria such as *M. avium* subspecies, comparative cervical test has been used as a complementary test to discriminate *M. bovis* infection from other mycobacterial infections by comparing the reactivity of each antigen using PPD-B and PPD-A (*M. avium* purified protein derivatives). However, this strategy may also cause problems with diagnostic sensitivity owing to the higher PPD-A reactivity because MAP vaccination can reduce the differences between PPD-B and PPD-A in *M. bovis* infected animals [93]. Therefore, many countries that are running *M. bovis* eradication programs do not use vaccination policies. However, these problems can be overcome by development of new diagnostic methods or vaccines. Emerging serologic tests using *M. bovis* specific antigens such as ESAT-6, CFP-10, and MPB83 did not produce positive results in MAP vaccinated animals [95]. Another drawback of whole cell based vaccines is the substantial tissue damage at the injection site and accidental self-inoculation, which may cause serious side-effects [96]. However, there is a vaccine adjuvanted with highly refined mineral oils such as Silirum to reduce the formation of granuloma at the site of injection [68].

Conclusion

Vaccines against paratuberculosis have been developed by diverse approaches. The most important factors to consider in vaccine studies are the mechanisms related to the host-pathogen interaction. Much more efforts are needed to understand exactly how bacteria can evade the host defense system, and these should focus on not only an adaptive immune system, but also innate immunity. Vaccines that can induce both immune responses may have improved protective effects. Despite some limitations, vaccines might still be an effective strategy to reduce or eradicate Johne's disease in livestock industries.

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