pISSN 1229-1153 / eISSN 2465-9223 *J. Food Hyg. Saf.*
Vol. 33, No. 4, pp. 221~228 (2018)
https://doi.org/10.13103/JFHS.2018.33.4.221

Journal of Food Hygiene and Safety
Available online at http://www.foodhygiene.or.kr

Review

Progress Towards Control of a Mycobacterial Pathogen, *Mycobacterium aviumsubsp. paratuberculosis*, the Causative Agent of Johne's Disease in Cattle and Humans

William C. Davis[1] and Kun Taek Park[2]*

[1]*Department of Veterinary Microbiology and Pathology, Washington State University, Pullman WA, USA*
[2]*Department of Biotechnology, Inje University, Injero 197, Kimhae-si, Gyeongsangnam-do, Republic of Korea*
(Received June 5, 2018/Revised June 9, 2018/Accepted June 12, 2018)

ABSTRACT - Since the discovery that *Mycobacterium avium*subsp. *paratuberculosis* (*Map*) is the causative agent of Johne's disease (JD) in cattle at the end of the nineteenth century, movement of livestock latently infected with *Map* has led to the spread of JD throughout the world. A new form of enteritis with clinical features of JD in cattle appeared in humans concurrent with the appearance of *Map* as a disease problem in livestock. The demonstration that *Map* is a zoonotic pathogen and the causative agent of the new form of enteritis in humans, however, wasn't recognized until late in the twentieth century when methods were developed to detect the presence *Map* in tissues from patients with the new form of clinical enteritis. The objective of this short review is to provide a brief history explaining how *Map* has become a major disease problem in livestock and humans and then provide a review of the progress that has been made in treating patients with an enteritis caused by *Map* and the strategies underway to develop a vaccine to control infection in livestock.

Key words : Johne's disease, Crohn's disease, *Mycobacterium paratuberculosis*, Vaccine, Zoonosis

*Mycobacterium avium*subsp. *paratuberculosis* is a zoonotic pathogen of worldwide importance with a broad host range that includes humans. It has been difficult to control because of a lack of understanding the immune response to *Map* and the lack of a vaccine that elicits a protective immune response. It remains unclear as to when and where *Map* was first introduced into livestock. A debilitating intestinal enteritis of unknown etiology was recognized in cattle well before methods were developed to identify the causative agent. Similar to another zoonotic mycobacterial pathogen, *M. tuberculosis* (*Mtb*), infection leads to development of a latent infection under immune control. When the immune responseis compromised by the pathogen and other poorly understood factors, infection becomes clinical in cattle and other ruminant species. Although it causes disease in other species data are limited (reviewed in[1)]). The intestine is the primary target tissue affected by *Map*. Infection leads to erosion of the absorptive epithelium, persistent diarrhea and death by starvation. Infection is undetectable during the initial stages of infection. This has led to the inadvertent spread of *Map* into livestock in many countries through introduction of latently infected animals. A concurrent increase in a new form of enteritis emerged with the spread of *Map*. Because of the lack of understanding of the enteritis appearing in livestock, the association with appearance of the new form of enteritis in humans went unnoticed until the end of the 19th century when a veterinarian in Germany, H. A. Johne, in collaboration with a visiting investigator from the United States, L. Frothingham, identified an acid fast bacterium in tissues from a malnourished emaciated cow with persistent diarrhea[2)]. It was differentiated from *M. bovis* (*Mbv*) by morphology and inability to cause tuberculosis in guinea pigs. As reviewed in a summary of the history of *Map*, maintained by the University of Wisconsin https://johnes.org/history/index.html, *Map* was later successfully cultured independently by two investigators, F. W. Twort and H. Holth during the beginning of the twentieth century and referred to as pseudotuberculosis, paratuberculosis and Johne's disease (JD). It was during this same time frame that paratuberculosis or JD was first recognized as an important emerging disease problem in the US by aveterinarian, L. Pearson, in 1908 https://johnes.org/history/index.html. The suggestion that *Map* might also be an important disease problem for humans was reported by a surgeon at the Western Infirmary in Glasgow Scotlandin 1913, T. K. Dalziel, who called attention to the emergence of cases of a new form of enteritis with the clinical features of tubercular

*Correspondence to: Kun Taek Park, Department of Biotechnology, Inje University, Injero 197, Kimhae-si, Gyeongsangnam-do, Republic of Korea.
E-mail: ktpark@inje.ac.kr

enteritis caused by *Mycobacterium tuberculosis* (*Mtb*). Although the pathology looked identical to tubercular enteritis, no bacteria could be identified by microscopy. Also, the diagnostic use of guinea pigs to determine whether the causative agent was *Mtb* yielded negative results. This led to the consideration that another pathogen might be the causative agent. Based on studies reported by a veterinarian, McFadyan, in the Journal of Comparative Pathology and Therapeutics, Dalziel proposed the enteritismight be attributable to a mycobacterium, referred to as pseudotuberculosis, the causative agent of enteritis in cattle. By visual inspection, the gross appearance of affected tissues from cattle and humans appeared indistinguishable, strongly supporting the proposition that the same mycobacterium might be the causative agent in cattle and humans. The inability to detect *Map* in feces or tissue, however, raised issues as to whether *Map* was the causative agent of this new unique enteritis beginning to appear in humans. Further characterization of this form of enteritis occurred during the ensuing years led by B. B. Crohn (a leading gastroenterologist at the time) and associates. They described the clinical features of the emerging new form of enteritis that set it apart from other disorders involving the gastrointestinal track (reprinted article first published in 1932[3]). He and his colleagues were aware of the suggestion made by Dalziel that a mycobacterium might be the causative agent, but they, like other contemporary investigators, were unable to detect a mycobacterium in feces or tissue with available technologies. This gave rise to the supposition that this new form of enteritis, which became known as Crohn's disease (CD), was attributable to other causative factors. This thought became entrenched in the minds of the medical community and slowed progress in determining the etiological basis for the emerging new form of enteritis that paralleled the spread JDin the US and other countries. It was not until 1984, that Chiodini, a graduate student at the time, succeeded in isolating a mycobacterium from patients with the clinical features of CD[4]. Unlike *Map* isolated from cattle, the initial isolates obtained from humans were a spheroplastic form of a mycobacterium without a cell wall, not detectable by acid fast staining[5]. On culture, however, the isolates regained the capacity to form a cell wall (a feature of mycobacteria reviewed in[6]). A preliminary report by Van Kruinigan et al. demonstrated passage of one of the isolates, Linda, into goats led to development of a clinical enteritis identical to JD[7]. Concurrent studies by McFadden et al. revealed the unclassified mycobacterium isolated from humans was *Map*[8,9].

Although the data were convincing, technical difficulties in duplicating the initial results reported by Chiodini, caused the medical community to challenge the findings and postulate CD was attributable to other environmental or immunologically based factors. The medical and research communities split into two factions, those that believed *Map* was the etiologic agent of CD and those that believed CD was caused by other factors. This division biased the studies on the infectious and immunological basis of CD, resulting in few investigators looking at the immune response to *Map* in patients with CD[10]. The majority of studies conducted by investigators that believed *Map* is the etiologic agent of CD focused on improving methods for detection of *Map* in patients diagnosed with CD (reviewed in[11]). Studies by investigators that believed *Map* was not the etiological agent of CD focused on investigating factors that could explain the basis for the chronic inflammation of the intestine associated with the immunopathological characteristics of CD (reviewed in[12]).

The recognition that JD had become a major disease problem and a potential zoonotic pathogen, led to the development of the International Association for Paratuberculosis, Inc. in 1989 http://www.paratuberculosis.net/index.php. Shortly thereafter, the National Academy of Sciences, Engineering and Medicine was commissioned to review the current status of information on the diagnosis and control of paratuberculosis and make recommendations. The commission included a request to review available evidence that *Map* might be the etiologic agent of CD[1]. Based on the recommendations resulting from the review, the USDA sponsored a multi-institutional research program on *Map* to advance knowledge on methods to diagnose and control of JD in 2003. Both events facilitated development of collaborative efforts at the national and international level to study *Map*. As reviewed by K. Stevenson, collaborative studies led to the sequencing of available isolates of *Map* obtained from cattle, sheep, and humans[13]. The studies included use of multi-locus sequence polymorphisms to distinguish and compare isolates obtained from domestic species, undomesticated wild species, and humans. Of interest, results from cumulative comparative studies of isolates from different species revealed the presence of isolates of the same phenotype in humans and animals, providing evidence of interspecies transmission as well as susceptibility of humans to infection with *Map*[13].

The introduction of *Map* into different countries and the appearance of the new form of enteritis is not well documented for most countries, as well as evidence that *Map* is the etiologic agent of CD in humans (more appropriately referred to as JD in humans). As reviewed by Hruska and Pavlik, however, the emergence of *Map* in livestock in the Czech Republic is well documented[14]. Until the fall of Soviet Union in 1989, the Czech Republic was isolated from other European countries where *Map* was becoming

endemic in livestock. Hruska and Pavlik document the appearance of JD as a consequence of movement of latently infected animals into the Czech Republic. As reported, there was a concurrent appearance of the new form of enteritis associated with the spread of *Map* in livestock, supporting the premise that *Map* was the causative agent. More definitive data have been obtained from India where *Map* is endemic in livestock and the environment[15]. A survey of samples of blood, serum, and stool from subjects in apparently good health and subjects visiting clinics for various illnesses including gastrointestinal disorders (N = 42,200) revealed the presence of *Map* in a large number of the subjects, regardless of health status. Some *Map* positive subjects had clinical signs of inflammatory bowel disease (IBD). Later follow up studies reported in a case report of a subject presenting with advanced stages of an inflammatory enteritis including emaciation and continuous bouts of diarrhea, revealed the presence of mycobacteria in his feces detectable by microscopy and acid fast staining. The mycobacterium obtained following culture proved to be the bison strain of *Map*, the primary strain of *Map* detected in humans, domestic and wild animals, and the environment in India[16]. Treatment of the subject with antibiotics cleared the infection and clinical enteritis, providing the strongest evidence that *Map* is zoonotic and the causative agent of the enteritis first described by Dalziel in 1913[16].

Progress in control of Johne's disease in humans and livestock

Control of JD in humans.

As a result of the cumulative findings obtained in recent years, the medical community hasbecome more fully aware of *Map* being a zoonotic pathogen, causing major problems in the livestock industries and becoming a public health risk factor. The incidence of inflammatory bowel disease has increased in countries where *Map* has become endemic in livestock and the environment, as clearly revealed in a study by Singh et al.[15]. As a consequence of this awareness, a more focused effort is underway to improve methods of diagnosis and treatment of patients infected with *Map* presenting with the clinical signs of paratuberculosis[11,17-18]. A human paratuberculosis foundation has been established to draw together clinicians and investigators dedicated to controlling disease caused by *Map* in humans and animals. https://humanpara.org/. Programs are being expanded to develop improved methods of detection of *Map* in patients with enteritis and other diseases where *Map* may be involved in pathogenesis[19]. Further efforts arealso being made to optimize the use of antibiotics to clear infections with *Map*[18]. Considerably more research is needed to understand the mechanisms of immunopathogenesis of paratuberculosis in humans, in patients with the classic clinical features of inflammatory enteritis and patients with other immune related disorders where *Map* may be playing an indirect role, through antigenic mimicry, in exacerbating diseases caused by other factors e,g. type 1 diabetes, ulcerative colitis, multiple sclerosis, sarcoidosisand rheumatoid arthritis[17].

Control of Johne's disease in livestock

Control of *Map* in livestock, the main source of exposure of humans to *Map* has, until now, proven difficult because of the lack of understanding of the immune response to *Map* and the lack of a vaccine that elicits a protective immune response. When studies of the immune response to *Map* were initiated, it was not clear whether there was an age related susceptibility to *Map* and whether animals could be exposed to *Map* without becoming infected (reviewed in[1]). This is attributed to a lack of understanding of the similarity of the immune response to tuberculosis where infection initially leads to development of an immune response that controls but does not clear the infection. The sensitivity of available diagnostic assays was low, obscuring the actual prevalence of infected animals in dairy herds (reviewed in[1]). The variation in the duration of the latency period made it appear exposure didnot lead to infection. This still remains a problem with interpretation of susceptibility to infection with *Map* (reviewed in[20-21]). Also, limited information was available on the immune system in cattle. Monoclonal antibodies (mAbs) specific for leukocyte differentiation molecules were just becoming available to analyze the immune response in cattle and to *Map*[22]. When direct studies were conducted by exposure of neonatal calves under experimental conditions and followed to determine when humoral and cellular responses were first detectable, the results showed that all exposed calves became infected as detected by appearance of an antibody response at ~5 months. A proliferative response could be detected by flow cytometry dominated by CD4 T cells in the same time frame[23,24]. Although it was not possible to conduct studies with older animals, the data indicated that the difficulty with determining if exposure always leads to infection, is associated with the lack of sensitivity of assays used to assess whether exposure always leads to infection. It is also associated with a lack of recognition that exposure leads to development of a latent infection under immune control. The duration of the latency period in older animals exposed to *Map,* in addition to the low sensitivity of the assays, accounts for not identifying all exposed animals infected with *Map.* The early studies did not include assessment of development of an immune response following exposure.

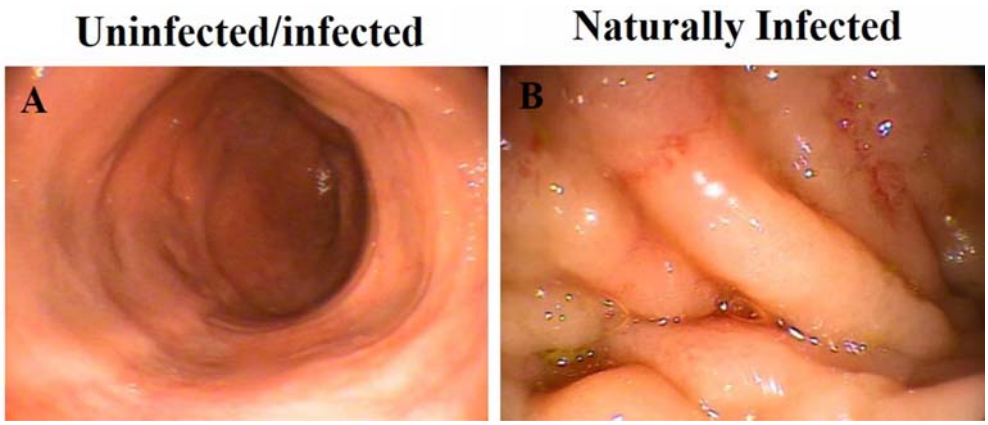

Fig. 1. Illustration of the cannulated ileum model used to evaluate the early stages of infection in calves experimentally infected with *Map* (A) and the lateclinical stages of infection cowsnaturally infected with *Map* (B). A) No lesions were evident during the early stages of infection with *Map*. B) Alterations were evident during late stages of infection with *Map* characterized by thickened mucosa[27].

Further advances in understanding the early events of infection were made with the development of a cannulated ileum model (Fig. 1)[25]. *Map* directly introduced into the ileum were rapidly taken up by enterocytes and then dispersed to other sites. Direct visualization of the ileum revealed uptake of bacteria did not lead to persistent or transient colonization of the ileum. Gross lesions in the ileum were not evident up to 18 months following direct inoculation. Protective immunity transitioned from innate to adaptive immunity during the early stages of infection controlling but not eliminating the infection with *Map*. The cannulated ileum model also proved useful in demonstrating the immune response to *Map* is resilient even though not able to eliminate the pathogen. Direct inoculation of high concentrations of *Map* directly into the ileum did not break down the immune response and accelerate progression to the clinical stage of disease. Likewise, transient depletion of CD4 T cells did not disrupt the immune response to *Map*[26]. Of interest, use of the model with the original isolates of *Map* taken from patients with CD demonstrated that passage in humans did not alter their capacity to infect cattle or establish a persistent infection[27].

The paradox of an immune response with the ability to control but not eliminate the pathogen has not been elucidated. This has slowed progress in development of a vaccine. Early studies with killed vaccines demonstrated some efficacy in reducing the incidence of infected animals becoming clinical during their productive life span[28] and (reviewed in[1]). More recent studies with killed vaccines have shown such vaccines can be useful as a first step in reducing the incidence of animals progressing to clinical disease where *Map* is endemic in animals and the environment[29-30]. The sequencing of the genome has provided an opportunity to explore the potential of developing a live attenuated vaccine. Transposon mutagenesis has been used to develop deletion mutants for testing as attenuated vaccines[31-32]. A summary of the recent studies with mutants submitted to a multi-institutional study (Johne's disease integrated program, JDIP) for evaluation has concluded that indirect measurements of potential of a vaccine are not sufficient for evaluating the potential efficacy of a candidate mutant as a vaccine[33]. The conclusion is based on evaluation of results with use ofa three part protocol to obtain predictive information on the potential efficacy of a deletion mutant as a vaccine for JD. The protocol involved using: 1) reduction in the capacity to survive in macrophages as the first criterion for selecting a mutant for further evaluation, 2) reduction in the capacity to survive in a mouse model as the second criterion, and 3) loss of ability to establish a persistent infection in goats, one of the natural hosts of *Map*. The criteria used in the first 2 steps of the protocol were not predictive of efficacy in the natural host. The final mutants selected for evaluation were able to establish persistent infections in goats used as the third part of the protocol. Many of the mutants submitted for evaluation were excluded based on results with macrophages and results in the mouse model. The recommendation was to focus on the use of the natural host to fully evaluate the predictive efficacy of the next generation of candidate vaccines[33].

In addition to recognition that further studies should focus on use of the natural host to assess the potential efficacy of candidate vaccines, was the need to improve the methods of assessing the functional activity of effector lymphocytes elicited in response to vaccination with mutants or the proteins encoded by the genes. Approaches taken thus far to address this critical need have involved assessment of methods to examine the response to a killed vaccine developed for use in sheep[34], a response to a 2526 base pair

fusion construct encoding a polypeptide (HAV) comprised of 4 *Map* genes[35], and responses to a relA deletion mutant and a membrane protein expressed by *Map*[36-37]. Comparison of the intracellular killing in the in vitro killing assay monitored with a rapid qPCR technique for enumeration of viable bacteria revealed killing activity was reduced in preparations of PBMC from vaccinated sheep compared to unvaccinated sheep. The findings indicate the assay successfully demonstrated the commercial vaccine failed to elicit a protective response and importantly that the assay may prove useful in screening candidate vaccines for efficacy[34]. Bull et al. explored the use of the immune response to a polypeptide (HAV) expressed in two engineered virus vectorscurrently in use to develop vaccines to other pathogens, the human Adenovirus 5 (Ad5) and the modified vaccinia virus Ankara (MVA)[35,38]. As mentioned, the HAV polypeptide encoded the predicted peptide sequences of 4 genes:MAP2444c (encodes a cytoplasmic protein), MAP1235 (data suggest it encodes a molecule expressed in the membrane), MAP1589c (data suggest it encodes a molecule expressed at the surface of the cell membrane), and MAP1234 (available data suggest it encodes a secreted protein). The HAV did not induce sterile immunity but did induce an immune response that reduced the bacterial load in tissues. It also reduced the frequency of live bacteria present in blood as detected with a bacterium viability assay[35,38]. The immune response to the individual gene products was not examined. As part of the multiple institutional research program, Park et al. used site-directed mutagenesis to examine the effect of gene deletion on the capacity of *Map* to establish a persistent infection and interfere with establishment of an infection with wild type *Map*. Three genes associated with virulence were selected for the initial studies:*relA* (a global regulator), *pknG* (a gene encoding a kinase that interferes with phagosome lysosome fusion), and *lsr2* (a gene regulating lipid biosynthesis and antibiotic resistance)[39]. Comparison of the response to the deletion mutants in cattle and goats revealed deletion of *relA* disrupted the capacity of the mutant to establish a persistent infection whereas deletion of the other genes did not impair their capacity to establish an infection[36,40]. Challenge exposure with wild type *Map* in cattle and goats vaccinated with the Δ*relA* mutant demonstrated the immune response to Δ*relA* impaired the capacity of *Map* to establish an infection[40]. Subsequent studies focused on analysis of the immune response to the mutant revealed the reason why the mutant couldn't establish an infection was that it elicited an immune response that cleared the infection. Two important assays were developed in the most recent follow up studies to fully characterize the immune response to the *relA* mutant: 1) a method to distinguish live from dead bacteria,

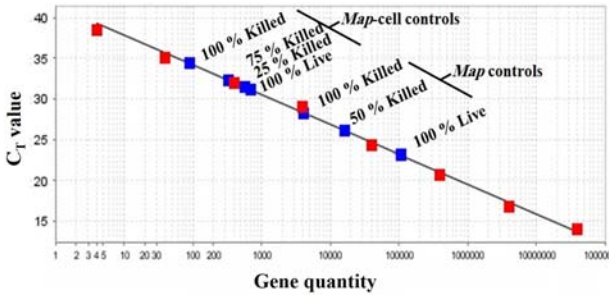

Fig. 2. Illustration of the bacterial viability assay used to distinguish live from dead bacteria isolated from infected macrophage target cells. The *Map* viability assay was adapted from a method developed by Kralik et al. to distinguish and quantify the concentration of live *Map* present in food products and samples from the environment[42]. The method involves the use of Propidium monoazide (PMA), a membrane impermeant fluorescent compound similar to Propidium iodide. It only enters dead cells and intercalates into DNA. When it is activated by light, it binds covalently to DNA and blocks binding of probes used to detect *Map* genes. The concentration of live *Map* in a mixture of live and dead bacteria is determined from a standard curve generated with known concentrations of pure DNA from live *Map* (red squares). The number of live bacteria present in a sample preparation is determined by using quantitative PCR (qPCR) with a probe specific for a single copy gene, F57, specific for *Map*[42]. Two types of controls are used to adapt the PMA method for measuring *Map* killing.The first set of controls is used to demonstrate the concentration of live *Map* in a defined mixture of live and dead bacteria.The controls are prepared from pure DNA from live *Map* for use as reference standards for extrapolating the extent of killing mediated by antigen-specific cytotoxic T cells (CTL). In the first set of controls (lower set of blue squares), tubes are prepared to contain 2×10^7*Map*/ tube, containing 100% live, 50% live/50% dead, and 100% dead *Map*. DNA is isolated for analysis. The second set of controls (upper set of blue squares) are used to quantitate the number of live *Map* present in *Map*-infected MoMΦ. Tubes are prepared to contain 2×10^7*Map*/ tube, containing 100% live, 75% live/25% dead, 50% live/50% dead, 25% live/75% dead, and 100% dead *Map*. This set of controls is essential for determining the extent of killing by CTLs since it covers the dynamic range for detection of live vs dead *Map* obtained from infected MoMΦ before and after incubation with CTL. Aliquots of *Map* mixed in the four ratios are added to the cultures of MoMΦ at a MOI of 10 respectively and incubated for 3 hours. The cultures are then washed to remove free bacteria. In this set of controls, cells are lysed following infection and incubation with *Map* for 3 hours and at 6 hours, the same period of time as used with infected macrophages incubated with lymphocytes from control uninfected steers and vaccinated steers stimulated with antigen presenting cells pulsed with the *relA* mutant or membrane protein MMP) (Manuscript under review).

in a mixed population of bacteria, was developed to replace the use of the colony forming unit (CFU) assay to study the cellular response to *Map* (Fig. 2) and 2) a method to analyze

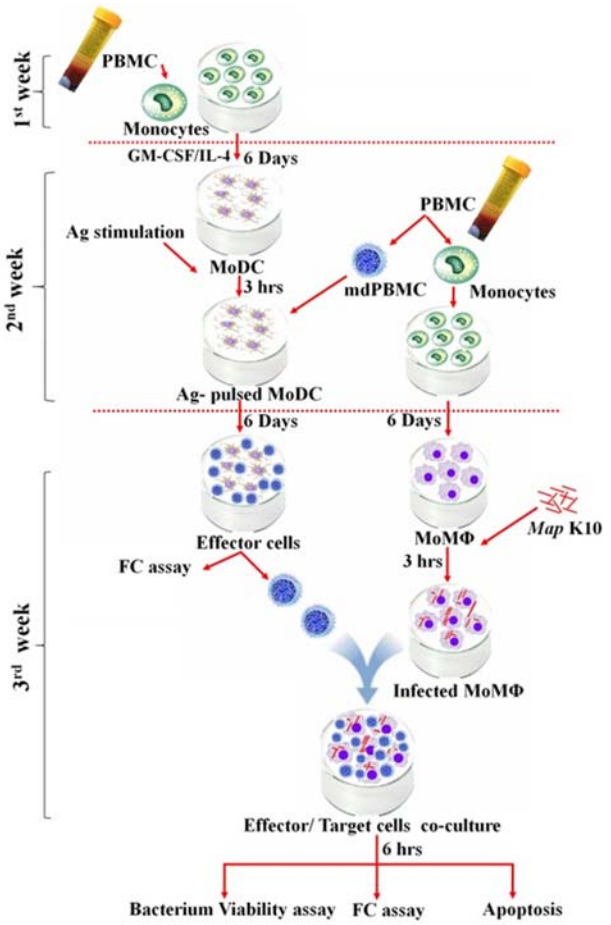

Fig. 3. Flow diagram illustrating the protocol for isolation and culture of peripheral blood mononuclear cells (PBMC) to study the memory T cell recall response to *Map* mutants and peptide antigens *ex vivo*. In the first step of the assay, magnetic beads coated with a mAb to CD14 are used to obtain monocytes from a preparation of PBMC to generatemonocyte derived dendritic cells (MoDC). Following 6 days of culture, the MoDC are pulsed with a mutant (*relA* in this example) or antigen for 3 hours. Concurrently, a second preparation of fresh PBMC is separated into monocyte depleted PBMC (mdPBMC) and monocytes. The mdPBMC are added to the antigen pulsed MoDC. The monocytes are cultured to generate macrophagesfor use as infected target cells. Following an additional 6 days of culture, the MoMΦ are infected with a target bacterium (*Map* in this illustration). The antigen primed mdPBMC are placed in culture with the infected target cells and incubated for 6 or 24 hours to determine their ability to kill intracellular bacteria. The bacterium viability assay is performed to determine the extent of killing by effector T cells. Flow cytometry is performed to determine the phenotype and activation status of effector T cells proliferating in response to antigenic stimulation. An apoptosis assay is performed to determine the mechanisms used be the effector cells to mediate intracellular killing of bacteria (Manuscript under review).

the functional activity of memory T cell subsets generated by vaccination with Δ*relA* in vivo and ex vivo (Fig. 3). Analysis of the recall response of memory T cells proliferating in response to antigen presenting cells (APC), pulsed with Δ*relA*, demonstrated the response included both CD4 and CD8 T cells. Little or no proliferative activity was evident in natural killer cells (NK) or γδ T cells following ex vivo stimulation with Δ*relA*-pulsed APC. Further analysis revealed the CD8 memory T cells, elicited in response to antigenic stimulation, developed ability to kill intracellular bacteria. The response of the CD8 cytotoxic T cell (CTL) was directed against a 35 kDa major membrane protein (MMP) encoded by MAP1272c[41] (Fig. 4). Results from the most recent studies, where the response to APC pulsed with the Δ*relA* mutant and MMP were compared ex vivo, revealed a comparable CD8 CTL response was elicited by Δ*relA* and MMP (in press).

Although a vaccine that induces sterile immunity to *Map* has not been achieved, methods have been developed that should facilitate further studies in the natural host. Killed vaccines elicit an immune response that reduces the frequency of infected animals progressing to clinical disease. Recent data suggest the immune response can be improved with the use of attenuated mutants of *Map* that can't establish an infection and peptide-based vaccines that elicit development of CTL that kill intracellular bacteria. The development of a method to distinguish live from dead bacteria present in a mixed culture of live and dead bacteria now provides a way to examine the activity of CTL against intracellular bacteria present in target cells in real time, replacing the CFU assay. Development of an ex vivo platform to study the immune response to *Map* now provides a way to examine antigen processing by APC in detail and importantly, the signaling mediated by APC driving differentiation of naïve T cells into effector cells with the ability to kill intracellular bacteria. The ex vivo platform also provides a way to examine the phenotype and functional activity of CD4 and CD8 T cells responding to antigenic peptides presented by APC. The ex vivo assays overcome some of the major hurdles that have been impeding progress in developing a vaccine against *Map*.

Acknowledgment

The study was partly supported by the project titled "Evaluation of food safety and development of novel processed meat products for application of deep sea water", funded by the Ministry of Oceans and Fisheries, Republic of Korea. Additional support was provided by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the High Value-addedFood Technology Development Program, funded by Ministryof Agriculture, Food and Rural Affairs (No.

1150043).

국문요약

19세기말 *Mycobacterium avium* subsp. *paratuberculosis* (*Map*)이 요네병(Johne's disease)의 원인균임이 밝혀진 이후, 불현성 감염된 동물들의 국제적 이동은 요네병을 전 세계로 퍼뜨리기 시작하였다. 이러한 요네병이 축산분야에 나타남과 동시에 새로운 형태의 대장염으로서 요네병과 같은 증상을 나타내는 질병(크론병)이 사람에게서도 나타나기 시작하였다. 그러나 *Map*이 이러한 새로운 대장염의 원인균이며 인수공통전염병의 원인체라는 인식은 20세기 후반 이러한 질병을 앓고 있는 사람의 조직으로부터 *Map*을 검출할 수 있게 되고서야 나타나기 시작했다. 본 총설은 어떻게 *Map*이 축산분야와 사람의 공중보건 측면에서 심각한 문제를 야기시키게 되었고, *Map* 감염에 의한 대장염 환자의 치료가 어떻게 발전되어 왔는지 간단히 요약하고, 축산에서 *Map*의 통제를 위한 새로운 백신개발 전략에 대하여 소개한다.

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