

## ULTRASTRUCTURAL STUDIES OF BOVINE PARATUBERCULOSIS (JOHNE'S DISEASE)

**T. Batbayar\*, S. Andrei, Ch. Tungalag**

The school of Veterinary medicine, MULS

\*-Corresponding author, E\_mail: bayar\_vet@yahoo.com

### ABSTRACT

*In the present study, we report the ultrastructural morphologic features of infected macrophages, epithelioid cells, Langhans' giant cells and a more detailed impression of the interaction between macrophages and engulfed bacteria in ileum and mesenteric lymph nodes of cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP).*

**y words:** *Paratuberculosis or Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), electron-transparent zone, transmission electron microscopy, scanning electron microscopy, host-pathogen interaction, cattle,*

### INTRODUCTION

Paratuberculosis (Johne's disease), a specific, chronic granulomatous enteritis due to acid fast, Gram-positive, mycobactin-dependent organism *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which is known to affect domesticated and wild ruminants. Bovine paratuberculosis has emerged as one of the most prevalent and costly disease is a major economic impact to the dairy and beef cattle industries today. In addition to the significant economic loss, MAP has also been suspected as an etiological agent of Crohn's disease in humans. The heavy economic burden on agricultural industry and potential public health concern urged scientific community to develop effective control measures for Johne's disease [10]. *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease, able to dampen or distort immune responses at the mucosal sites and co-exist with a massive infiltration of immune cells in the gastro-intestinal tract [3]. The

current view of MAP pathogenesis includes fecal-oral transmission in calves and young cattle with MAP invasion occurring in the terminal part of small intestine or ileum (portal of entry). Numerous questions remain regarding host-pathogen interactions that occur at the initial site of infection and how these interactions determine whether an animal controls a persistent MAP infection or develops Johne's disease [6]. Little is known about the host-pathogen interactions that regulate the pathogenesis of paratuberculosis (Johne's disease), particularly host cell death or survival of bacteria within infected cells [8]. *Mycobacterium avium* subsp. *paratuberculosis* is a facultative intracellular pathogen that resides within host macrophages during infection of ruminant animals. Our study was conducted to elucidate ultrastructural pathologic changes and recognize viability and damages of intracellular bacteria in macrophages, epithelioid cells, Langhans' giant cells.

## MATERIALS AND METHODS

This study was established in cattle breeding farm of Kitasato University, Japan. Five crossbred adult cows (Angus x Japanese black) with clinical signs of bovine paratuberculosis and known to be infected with MAP by the regular recovery of the bacterium from their feces were slaughtered and a full necropsy was performed. After gross observation, the tissue samples were taken from the ileum, ileocaecal valve, mesenteric lymph nodes for electron microscopic examination. 1 mm<sup>3</sup> trimming samples from tissues for transmission electron microscopy /TEM/ were prefixed in 1-2 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 and postfixed in 0.1 M phosphate buffered 1 % osmium tetroxide solution (OsO<sub>4</sub>) at pH 7.4. After fixation materials were dehydrated in alcohols, cleared in QY-1 compound and embedded in epoxy resin. 1-2 µm semithin sections were cut with an

ultramicrotome MT-1, stained with toluidine blue and examined with a light microscope. Selected ultrathin sections was cut from epon blocks with an ultratome /Reichert Nissei/, and were placed on copper grids and double stained with Sato's basic lead citrate and then examined in a Hitachi H-7000 transmission electron microscope at an accelerating voltage 80 kV. For scanning electron microscopy /SEM/ 1 mm<sup>3</sup> trimmed tissue samples using method same as for TEM procedure. Tissue was dehydrated in alcohols, critical point dried in butyl alcohol, and coated with a thin layer of aluminum and observed under a Hitachi S-450 scanning electron microscope at an accelerating voltage 15 kV. The viability of intracellular bacteria was assessed on the intactness of their electron-transparent zone, using the criteria of Armstrong J.A and Hart P.D [2].

## RESULTS

Scanning electron microscopy (SEM): SEM of the samples from clinically affected cows showed that absorptive villi over the non-Peyer's patch of terminal part of ileum was shortened and distorted.

These are irregularly ruffled and heavily depressed beneath the general contours of the villous surface and when viewed by SEM (Fig.1).

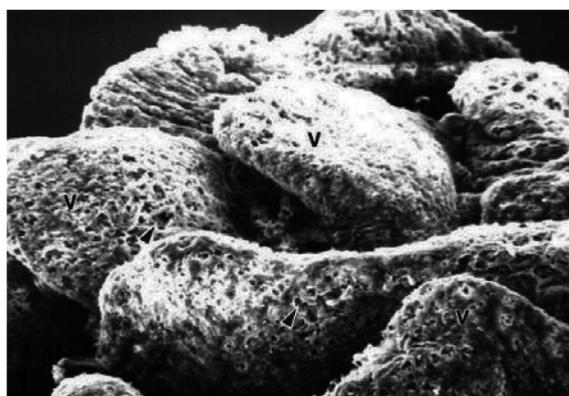


Fig. 1. Scanning electron micrograph. Affected ileum of cow infected with MAP. Absorptive villi are shortened and distorted (arrowhead). V- villus. Bar = 50 µm

Transmission electron microscopy: Transmission electron microscopic examination of ileum and mesenteric lymph nodes demonstrated intact and degraded or damaged MAP inside macrophages, epithelioid cells, Langhans' giant cells naturally

infected with *Mycobacterium avium* subsp. *papatuberculosis*. Intact-appearing MAP with a characteristic electron-transparent zone (ETZ) were seen within phagosomes or were apparently free in the cytoplasm of macrophages (Fig. 2).

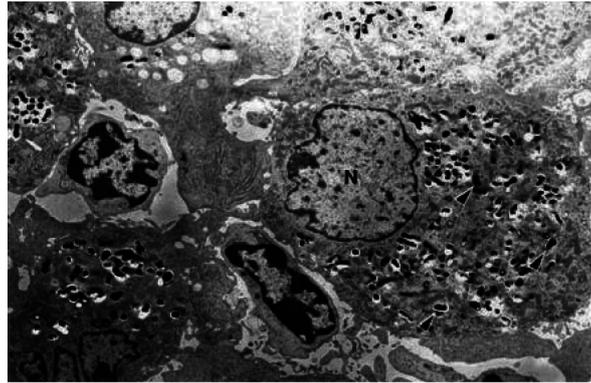


Fig. 2. Transmission electron micrograph. Ileum. Macrophages in the lamina propria of ileum contains large numbers of bacteria (arrowhead). N- nucleus. Bar = 3  $\mu$ m

In the lamina propria, macrophages containing bacteria in phagosomes and phagolysosomes were common. MAP was most often an intact, small clusters of bacteria could be seen. Fusion of phagosomes with lysosomes (dense granules) to

form phagolysosomes was apparently seen. Intact and partially degraded bacteria were observed in large phagosomes and phagolysosomes of the macrophages (Fig 3).

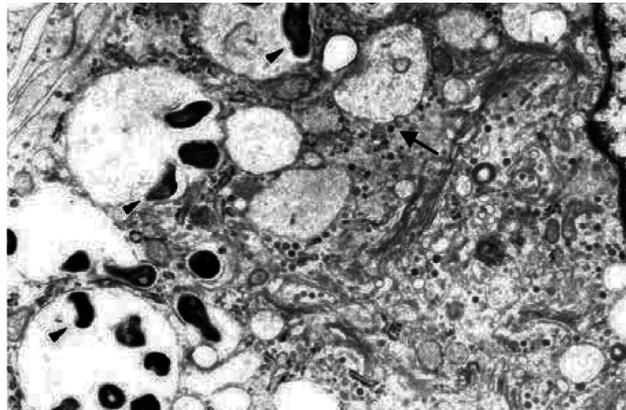


Fig. 3. Transmission electron micrograph. Ileum. Phagosomes contains many intact or degraded in varying manner bacteria (arrowhead) and visibly damaged MAP in phagolysosomes (arrow). N-nucleus. Bar = 1  $\mu$ m

*Mycobacterium avium* subsp. *papatuberculosis* were seen intact with ETZ in epithelioid cell arised from macrophages and inflammatory Langhans' giant cell derived from macrophages in granulomatous

inflammation. The typical appearance of granulomatous inflammatory epithelioid cell and multinucleate Langhans' giant cell are shown in Fig. 4.

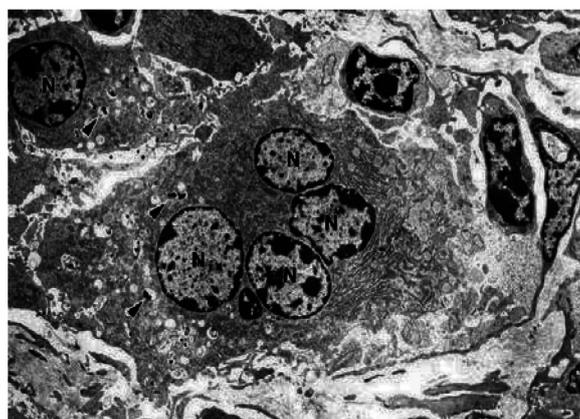


Fig. 4. Transmission electron micrograph. Ileum. Engulfed bacteria in epithelioid cell (Left up) and Langhans' giant cell (center). N-nucleus. Bar = 5  $\mu$ m

In electron micrograph of mesenteric lymph nodes most bacteria inside the macrophages had the same intact appearance. However, the phagolysosomes, varying in frequency from cell to cell, contained

intact or damaged MAP. Degraded (damaged) and intact bacteria permits the pattern of fusion of lysosomes (dense granules) with phagosomes (Fig. 5).

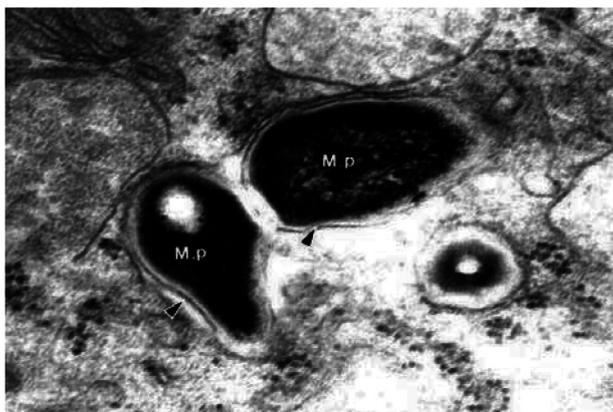


Fig. 5. Transmission electron micrograph. Mesenteric lymph node. Intact bacteria with electron-transparent zone (arrowhead) and damaged or degraded bacteria contained within a phagolysosome in an macrophage (arrow). Bar = 1  $\mu$ m

These results suggest that the cell wall with electron-transparent zone (ETZ) is one of the characteristics of the acid-fast bacteria. In macrophages, epithelioid cells and Langhans' giant cells, most phagosomes and phagolysosomes containing apparently intact bacteria and others containing visibly damaged or degraded bacteria. The results of assessment of

MAP electron-transparent zone intactness, using the criteria of Armstrong J.A and Hart P.D [2] indicated that the most often engulfed bacteria was intact and others were degraded by lamination and herniation manners. MAP that were not considered to be damaged were classified as intact.

## DISCUSSION

After ingestion, MAP organisms are taken up by M cells (and by enterocyte to a lesser extent), pass through the cells by transcytosis, and are then engulfed by submucosal macrophages residing at the basolateral side of the intestinal epithelial cells. The influence of the granulomatous inflammatory response on the development of the lesions in intestinal lumen could not be assessed from previous studies for MAP examined by SEM. In normal ileum of calf most absorptive villi were long and fingerlike, but some were flattened and leaf shaped [5]. Our results that the absorptive villi over the non-Peyer's patch of terminal part of ileum was shortened and distorted, and luminal surface of ileum is ruffled. We suggest that results of lesions in ileal lumen and ileal absorptive villi are commonly influenced from granulomatous inflammation in the lamina propria of small intestine. Results of transmission electron microscopic observation (TEM) indicate that phagosomes and phagolysosomes of the macrophages, epithelioid cells and Langhans' giant cells most commonly contains apparently intact bacteria. A small number

of specialized microorganisms can survive inside macrophages designed specifically to kill bacteria. However, a hallmark of mycobacterial pathogenesis is their ability to survive, and even replicate, within macrophages [1]. Intracellular mycobacteria were surrounded by a electron-transparent zone (ETZ) of 70-100 nm, which protected the mycobacteria from host-mediated killing mechanisms. The ETZ prevents direct contact between the bacilli and the lysosomes [9]. The killing is not always successful and MAP can persist in macrophages by inhibiting phagosome maturation. A recent study indicated that MAP infection suppresses apoptosis of primary bovine macrophages, which may further allow the intracellular MAP to live and replicate in the host cells [10]. The crucial role of macrophages in the development of resistance to infection with this bacterium might be induction of cell-mediated immunity. A failure in this afferent function could result in a blockade of the subsequent functions of macrophages, killing and lysis of bacteria [2]. These ultrastructural studies indicated that some of engulfed MAP are degraded or damaged. Their are

was degraded by lamination and herniation manners. The interaction *Mycobacterium avium* subsp. *paratuberculosis* (Map) with bovine macrophages was found to be a complex processes involving strategies for survival of bacteria or host cell death depending upon the number of bacteria infected per macrophage (multiplicity of infection, MOI). MAP at equal bacterial burden per cell (MOI=1) was not harmful for macrophages, but at MOI of 10 induced apoptosis. Interestingly, MAP at higher bacterial burden (MOI=50 or greater) induced both apoptosis

and necrosis in macrophages. It is concluded that under higher bacterial burden and spatial stress, MAP induced apoptosis and necrosis of macrophages by complex mechanisms as to find a new niche for survival and replication [8]. Eventually, the heavily infected macrophage membranes rupture and the organisms are released into to the surrounding tissue where the phagocytosis cycle will be repeated [4].

## SUMMARY

1. The absorptive villi over the non-Peyer's patch of terminal part of ileum was shortened and distorted, and luminal surface of ileum is ruffled. These lesions may be influenced from granulomatous inflammation in the lamina propria of ileum of cattles infected with MAP.
2. Transmission electron microscopic observation (TEM) indicate that phagosomes and phagolysosomes of the macrophages, epithelioid cells and Langhans' giant cells in ileum and mesenteric lymph nodes most commonly contains apparently intact bacteria. The killing or degradation of MAP is not successful and MAP can persist in macrophages, epithelioid cells and Langhans' giant cells.
3. Some of engulfed MAP are degraded or damaged. Their are was degraded by lamination and herniation manners.
4. In conclusion, once remained intactly MAP can replicate in macrophages, epithelioid cells and Langhans' giant cells, wich is lead to clinical signs of the Johne's disease in cattle naturally infected with MAP include diarrhea, emaciation, lethargy, eventually death

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