Pathogenesis of Johne’s disease; a possible role of cell-wall deficient forms of Mycobacterium avium subsp. paratuberculosis

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SUMMARY

Background: Mycobacterium avium subsp. paratuberculosis (Map) is the cause of Johne’s disease and has been implicated in Crohn’s disease. Cell wall deficient (CWD) forms have been suggested as the pathogenic form of the organism in humans. It is uncertain if CWD forms are present in tissue of animals with Johne’s disease. We used a previously developed an in situ hybridization assay for CWD Map to study animals. Purpose: To determine whether CWD Map were present in diseased animals and to validate the in situ hybridization assay in animal tissue.

Methods: Paraffin-embedded tissues (intestines, liver and lymph nodes) from 36 coded archival specimens (representing 14 cows and one sheep) were tested by in situ hybridization assay using a digoxigenin-labeled IS 900 probe. Positive and negative controls were beef tissues injected with Map CWD forms and acid fast bacilli, respectively. Results: Six of the 10 Johne’s disease infected animals were positive for Map (60 % sensitivity); 4 intestinal and 2 intestinal/lymph nodes. The single positive liver specimen originated from a cow with Johne’s disease whose intestinal specimen was also positive. None of the 5 control cows was positive (100 % specificity).

Conclusion: The presence of CWD Map in animals with Johne’s disease may explain why Map isn’t readily cultured from some animals and/or why pathology is seen in tissues like the liver when no acid fast bacilli are detected. CWD forms may represent a highly evolved survival strategy as the lack of the bacterium’s outermost structural layer could assist in evading or delaying the host’s defense system, thereby allowing it to quietly establish itself within the host.

INTRODUCTION

Mycobacterium avium subsp. paratuberculosis (Map) is the cause of a chronic granulomatous enteritis in ruminants known as Johne’s disease. The disease occurs worldwide with enormous economical losses (2,19,20). It develops in two stages: A transient subclinical (symptomatic) stage, and a severe clinical (symptomatic) stage. This process of transformation may last for several years during which the animals are highly contagious (Map-carrier). The infection is difficult to detect in the early stages of disease due to the low sensitivity of the current diagnostic tests including serology, cultures, and PCR (4,20). Map is mainly spread through the fecal-oral route; animals in both subclinical and clinical stages shed bacteria in their feces and are likely to contaminate the herd’s feed and water. The organism can also be transferred from cow to calf through colostrum or milk and intrauterine infection (2,20). Hence, it has recently been suggested that viable Map can be found in the food chain (meat, milk, dairy products) and water (5). It is therefore possible that Map can be transmitted from animals to humans and there is evidence to suggest that it may cause the Johne’s-like disease (Crohn’s disease) in genetically predisposed humans (7).

Two forms of Map have been observed in Johne’s disease tissues. The acid fast form that contains a lipid-rich cell wall and the cell wall deficient (CWD) form that contains intact cell membrane without surrounding cell wall. The lack of cell wall is difficult to detect. CWD forms have been observed by electron microscopy within macrophages and giant cells in a granuloma of tissue from a subclinical Johne’s disease case (6). CWD forms of Map have also been isolated primarily by long term cultures from Crohn’s disease tissue and milk from mothers with Crohn’s disease (3,11,14-16,18). They have also been observed by in situ hybridization in tissues from Crohn’s disease patients (8,9,17). Finding these forms inside the tissue is consistent with a potential role in pathogenesis and/or transmission of disease. The aim of this study is to utilize our in situ hybridization adapted specifically for the detection of these forms in tissue specimens to determine whether
CWD forms are present in tissue from animals with Johne’s disease.

MATERIALS AND METHODS

Animal Tissues. Archival, formalin fixed and paraffin embedded tissue samples from Holstein cows and one sheep were tested in a blinded study. Samples were derived from the gut, lymph node, and liver tissues.

In situ hybridization. Four µm sections were placed on Silane-Prep slides (Sigma-Aldrich, Milwaukee, WI), deparaffinized with xylene and rehydrated in phosphate buffered saline (PBS) before in situ hybridization. The in situ hybridization method combined with the specific probe (a digoxigenin labeled 251 bp DNA fragment derived from the 5’ region of the IS 900 fragment) was used as previously described in detail (9). The hybridized product was detected by immunological detection with alkaline phosphatase (AP)-conjugated anti-DIG antibodies and color development was performed using nitro-blue-tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim Corp., Indianapolis, IN). Slides were incubated for 4 h at room temperature, until the reaction of the positive control slide was appropriate. After washing in distilled water for 2 min, cover slips were mounted using water based mounting medium (GelMount, Biomeda, Foster City, CA). The slides were read by a pathologist (H.M.T. El-Zimaity). Negative slides were not further studied. But the positive slides were repeated with and without probe. To ascertain that false positive signal due to a non-specific binding or staining was not observed, the anti-DIG-AP-antibodies step was excluded. A sample containing non-specific staining was considered negative. The location of positive signal and the status of cells (intact or leaky due to apoptosis) were determined by counter staining with hematoxylin and eosin (H&E).

Controls were sections of previously prepared formalin fixed and paraffin embedded beef tissue injected with Map acid-fast (negative control) and CWD forms (positive control) (9). The presence of acid-fast organisms were confirmed by Kinyoun’s staining (1).

RESULTS

Thirty-six archival, formalin fixed and paraffin embedded tissue samples from 9 cows and one sheep (at least 2 years old) with clinical Johne’s disease and 5 calves (5 to 6 days old) with no signs of Johne’s disease were used. The availability of paraffin blocks varied between animals and consisted of two blocks from 9 animals including the sheep and 3 blocks from 6 animals.

The results of in situ hybridization are summarized in Table 1. CWD forms were detected in 6 out of ten animals with Johne’s disease by in situ hybridization. Positive signals were observed in intestinal tissues from 3 cows, in 2 intestinal and 2 lymph node tissues of the same two cows, and in one intestinal tissue and the only one liver tissue from the same cow. No signal was observed from the intestinal or liver tissues from the sheep. None of the five control samples from the calves was positive. The positive and negative beef controls gave the expected results, respectively.

Sections that hybridized with the IS900 probe showed dark purple to black granules corresponding to DNA from the presumed CWD Map (Figure 1A). The histologic examination of granulomatous lesions revealed positive signals in both macrophages and giant cells indicating the presence of Map DNA or their CWD cells in these intact chronic inflammatory cells (Figure 1B). In Kinyoun’s stain sections, the acid-fast bacilli were observed in aggregates and inside intact chronic inflammatory cells (not shown). Thus, we found both of the acid fast and spheroplasts forms in animals with clinical Johne’s disease.

Table 1. Results from in situ hybridization in 14 Holstein cows and one sheep.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of animals</th>
<th>Number of positive animals</th>
<th>Total positive/total tested (%)</th>
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<tr>
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<td>Controls e</td>
<td>5 Holstein cows</td>
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a Paraffin blocks contained tissue specimens from specified organs.
b Animals were at least 2 years old with clinical Johne’s disease.
c Positive signals were observed in the intestinal and lymph nodes of the same two cows.
d Positive signals were observed in the intestinal and liver of the same cow.
e Animals (5 to 6 days of age) with no signs of Johne’s disease.
DISCUSSION

CWD-forms, L-forms, L-variants, and mycoplasma-like organisms (MLO’s), are few of the many different names describing a bacterial stage where more or less of the cell-wall has degenerated (12,13). These aberrant forms may be a result of imbalance in the continuous degradation and regeneration of the cell wall. Organisms that lack their cell wall also lack many immunogenic antigens and are likely to trigger a reduced immune response. This trait could be a successful strategy for an organism to establish itself within a host. It has been stated that CWD forms are the predominant forms of mycobacteria in vivo, possibly because of the lower energy demands (10,13). Little is known about the role that CWD forms of mycobacteria play in various diseases.

Our goals in this investigation was to determine whether the CWD forms of Map are present in cattle with Johne’s disease. We applied an in situ hybridization method on blinded specimens from animals with Johne’s disease and controls. In order to reduce the risk of false positive results, the assay was repeated on the positive tissue samples in parallel with controls by excluding either the probe or the anti-digoxigenin-AP antibodies. This precaution was taken since the specimen size and formalin fixation time prior to embedding can affect the target accessibility and cause background variations. Positive signals were detected in intact-live chronic inflammatory cells such as giant cells (observed after H&E counter stain) in tissue specimens obtained only from the majority of animals with Johne’s disease. The hybridization signals were also found in the same cellular locations as previously found with some cases of Crohn’s disease (i.e. in intact-live chronic inflammatory cells such as macrophages, polymorphonuclear (PMN) cells and fibroblast-cells in the lamina propria). As with all methods targeting the DNA, it is difficult to prove that the signal indeed came from intact bacteria and not from free DNA or dying bacteria. Based on the fact that the signal was found within healthy, intact inflammatory cells known for their non-professional capacity of engulfing bacteria but lacking the cell-deestroying activities (such as lysosomes), we postulate that the signal was indeed derived from CWD forms.

The presence of CWD forms in animals with Johne’s disease may explain why Map isn’t readily cultured from some animals and why pathology is seen in tissues like the liver when no acid-fast bacilli are detected. Map CWD forms may also represent a highly evolved survival strategy immunologically. It is possible that the lack of cell wall layer could assist in evading or, at least delaying the host’s defense system, thereby allowing the bacteria to quietly establish itself within the host. It is also possible that these forms are metabolically active with stable entities that could themselves lead to the production of a significant pathology. Therefore, these Map CWD-forms may play an important role in transmission and/or pathogenesis of these diseases.

In summary, our study shows the presence of Map CWD forms in cattle with Johne’s disease as we previously shown in humans with Crohn’s disease. Further studies addressing the importance of CWD forms in the development of disease could be of great importance not only for understanding and controlling Johne’s disease but may also help in preventing its transmission to other animals and perhaps to humans.

ACKNOWLEDGMENT

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REFERENCES