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Sub-clinical necrotic enteritis in broiler chickens: Novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue

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Abstract

The present study revealed several previously not recognized etiological details in the development of necrotic enteritis (NE) in broilers. We provide evidence that the pathological process leading to mucosal epithelium necrosis follows morphologically distinct phases commencing at the basal domain of the mucosal epithelium and then progressively invading the entire lamina propria. Initially mucosal epithelium appears normal, but as the pathological changes progress throughout the lamina propria, the adjacent enterocytes begin to show features of necrotic cell death and the necrotic process of the epithelium progresses from being focal to locally extensive.

Ultra-structural examination showed that primary changes occur at the level of basal and lateral domains of the enterocytes, whereas the apical domain of enterocytes remains intact even in the face of advanced necrotic changes. This indicates that the mucosal necrosis does not result from direct damage to the mucosal epithelium. Rather, the necrotic death of enterocytes is a consequential effect of the destruction of lamina propria, the extra-cellular matrix, and intercellular junctions.

The nature of these morphological changes indicates that initiation of the pathological process leading to NE involves proteolytic factors affecting the extra-cellular matrix and cellular junctions. Further studies revealed that, indeed, the elevated activity of collagenolytic enzymes in the mucosal milieu and in intestinal tissue represents an integral component of the pathological process leading to NE. In the first instance we discovered that *Clostridium perfringens* strains isolated from field cases of NE secrete several potent collagenolytic enzymes. In the second instance we observed that, in comparison to controls, broilers challenged with *C. perfringens* isolated from field cases of NE show high levels of several collagenolytic enzymes in the intestinal tissue. A major component of the overall collagenolytic activity detected in the intestinal tissue was identified by zymography as matrix metalloproteinases (MMPs). Dominant activity was associated with MMP-2. We confirmed using immuno-histochemistry that this enzyme is expressed at high levels in mucosal tissue showing signs of NE.

The high levels of collagenolytic activities, in particular associated with MMP-2, demonstrated in our studies are consistent with the nature of morphological changes observed primarily in extra-cellular matrix (ECM) at the basal domain of enterocytes, as well lateral domains of enterocytes. The lack of changes at the level of apical domain of mucosal epithelium indicates that the lipolytic aspect of alpha toxin in NE is not an essential factor in primary lesions development. Taken together, our findings indicate that the early lesions leading to NE are associated with virulence factors that induce proteolytic activity, rather than lipolytic activity.

Keywords: Broiler; *Clostridium perfringens*; Necrotic enteritis; Morphological changes; Electron microscopy; Extra-cellular matrix; MMP

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1. Introduction

Necrotic enteritis (NE) remains an important disease in poultry (for recent review see Van Immerseel et al., 2004). Despite considerable research over the last 30 years, the issues regarding the underlying etiology of necrotic lesions in broiler chickens still remain unresolved. The pathogen most commonly associated with necrotic enteritis in poultry is *Clostridium perfringens* type A. These bacteria produce the alpha toxin (phospholipase C), which has been shown to hydrolyze some phospholipid substrates such as phosphatidylcholine and sphingomyelin (Krug and Kent, 1984; Titball et al., 1999). As such, alpha toxin has the potential to disrupt cellular membranes having specific phospholipid composition (Flores-Diaz et al., 2004; Nagahama et al., 1996).

Historically, the alpha toxin has been recognized as the virulence factor of central significance in the pathogenesis of NE (Al-Sheikhly and Truscott, 1977; Fukata et al., 1988). However, the validity of this tenet has been challenged by recent research. Gholamiandekhordi et al. (2006) reported that alpha toxin production by *C. perfringens* does not show a positive correlation with the clinical outcome. Furthermore, the study of Keyburn et al. (2006), using defined chromosomal mutants, provided evidence that that alpha toxin is not an essential virulence factor in NE.

Previously, we reported that early changes in the mucosal tissue associated with *C. perfringens* infection initially occur at the interface of the basal domain of enterocytes and the lamina propria (Olkowski et al., 2006). Further work in our lab confirmed that the primary morphological changes commence at the level of basement membrane of enterocytes. It was also discovered that the pathological process progresses towards the center of the villus, eventually leading to the complete obliteration of the lamina propria matrix, while the mucosal epithelium architecture remains largely unaffected.

Since this pattern of NE lesion development is not consistent with the commonly assumed effects of alpha toxin, our findings raised questions regarding the role of other putative factors in the pathogenesis of NE. Our initial observations led us to hypothesize that: (1) disruption of structural integrity of the basal domain of enterocytes may be the primary cause of mucosal epithelium deterioration and necrosis, and (2) the pathological process induced by *C. perfringens* affects the extra-cellular matrix at the interface of enterocytes with lamina propria. Further, we hypothesized that proteolytic factors may be involved in the etiology of NE. Accordingly, our goals were focused on detailed evaluation of primary changes in the basal and lateral domains of extra-cellular architecture of the enterocytes induced by *C. perfringens*, and to examine whether the development of the lesions is associated with matrix degrading enzymes.

In the present study we were primarily interested in subclinical NE. Fulminant NE is relatively well characterized both clinically and pathologically, whereas the natural history of lesion development in NE is poorly understood. There is a general consensus that the sub-clinical form of the disease is more important than the clinical form because it may persist in broiler flocks without overt clinical manifestation. Moreover, because of large hidden economic costs associated with sub-clinical NE, and also because of the high risk of pathogen transfer to the food chain, and public health concerns, this problem is perceived among the industry experts as a major issue (for review see Van Immerseel et al., 2004).

2. Materials and methods

2.1. General

The basic research material for the present study was obtained from a commercial broiler flock affected by NE, confirmed upon post mortem examination at the Prairie Diagnostic Services, University of Saskatchewan. Several live birds from this flock showing clinical signs consistent with NE including lack of appetite, depression, somnolence, ruffled feathers, diarrhea, and dehydration, were tentatively diagnosed with necrotic enteritis. The affected broilers were then euthanized, and the presence of necrotic lesions was confirmed upon post mortem examination. Specimens collected from the birds diagnosed *in vivo* were used as reference material for a comparative study of morphological changes using light and electron microscopy and for pathogen isolation. The experimental work was focused on the evaluation of morphological and biochemical changes in the intestinal tissue following oral administration of wild type cultures of *C. perfringens* isolated from field cases of necrotic enteritis. The experimental protocol was approved by the Animal Care Committee and the procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

2.2. Isolation and identification of bacteria from field cases of NE

Sections of the intestines from broilers diagnosed with NE were cultured following standard procedures using two plates of Columbia blood agar and one of MacConkey agar (BBL, Beckton Dickinson). One blood agar plate was incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h, at which time subcultures of the bacterial colonies were done for further identification and the plates were re-incubated for another 24 h to detect possible slow growers. The other blood agar plate was incubated anaerobically at 37 °C for 48 hours. The MacConkey agar was incubated for 24 hours aerobically at 37 °C. All bacteria were identified by standard methods (Carter and Cole, 1990).

The bacteria isolated from NE specimens anaerobically showing the characteristic colony types of *C. perfringens*