LEUKOTOXIN AS A VIRULENT FACTOR OF *PASTEURELLA HAEMOLYTICA*

S.K. Maheswaran, L.O. Whiteley, E.L. Townsend, T.R. Ames, D.J. Weiss, H.S. Yoo, C. Gonzalez, and M.S. Kannan.

Departments of Veterinary PathoBiology, Clinical and Population Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108, U.S.A.

INTRODUCTION

Pasteurella haemolytica biotype A, serotype 1 (A1) has been established as the primary agent responsible for the clinical disease and pathophysiologic events leading to acute lobar fibrinonecrotizing pneumonia associated with bovine pneumonic pasteurellosis(1,2,3).

The bacteria produces several potential virulent factors, of which the leukotoxin (LKT) has received the most attention(4). The LKT is a heat-labile proteinaceous exotoxin, that is oxygen-stable, non-dialyzable, water soluble, and is produced in high concentrations by *P.haemolytica* during the logarithmic phase of growth(5). The genes that code for the synthesis and secretion of this LKT have been cloned(6). All 15 serotypes of *P. haemolytica* produce LKT(7). It has a molecular weight of 101-105kDa when determined by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis(8). Attempts to purify the LKT has been futile because it is tightly bound to the LPS and there is no efficient way to remove the LPS without inactivating the LKT. It is one of a family of RTX(repeat toxins)-pore forming cytolysin which has a unique specificity, in that it is only cytocidal to ruminant leukocytes(9). This cytotoxicity is caused by the formation of pores in the cell membrane which allows the influx of calcium and results in a sequence of cell-damaging events(10). Since this organism produces disease only in ruminants, this would support the role of LKT as a virulence factor. Indeed, several observations point to the central role that LKT has in the pathogenesis of pneumonic pasteurellosis. For example, in experimental pasteurellosis, the clinical and pathophysiologic events are dose-dependently reproduced by the intatracheal administration of live logarithmic phase *P. haemolytica* and not by stationary phase organisms(11). This enhanced pathogenicity may be related to the amount of LKT produced by these different populations. Indeed, we (5) and others(12) have shown that the logarithmic phase cells produce far greater amounts of this LKT than stationary cells. In other studies, cattle with high LKT neutralizing antibody titers have higher survival rates in the natural disease and experimental pasteurellosis than animals with low antibody titers(13).

Although advances have been made in describing the cytolytic properties of the *P.haemolytica* LKT, the mechanisms by which it brings about lung injury in cattle are poorly understood. Our laboratory has long been interested in elucidating the contributions and mechanisms by which the LKT induce this acute lung injury in pneumonic pasteurellosis. Three experiments were designed to study if the LKT contributed in the genesis of lung injury in pneumonic pasteurellosis.

Experiment 1: Determination of the cellular and subcellular sites of interaction of LKT in bovine pneumonic lungs.

This study was undertaken to demonstrate the presence of LKT in situ in acute lung lesions from experimental and natural disease of pneumonic pasteurellosis. Calves suffering from experimental or the natural disease were euthanized with a

barbiturate overdose. Representative samples of lung lesions were taken for light and electron microscopic immunohistochemistry. Monospecific antiserum directed against the LKT was produced by immunizing pasteurella-free rabbits with purified LKT(14). The antiserum containing anti-LKT antibodies was then used to localize the LKT in these lung lesions. For light microscopy the avidin-biotin peroxidase and immunogold silver enhancement techniques were used. The periodate-lysine-paraformaldehyde (PLP) and methacarn fixatives gave the most intense reaction. At the ultrastructural level, Lowicryl embedded, PLP-fixed tissues preserved both tissue morphology and immunoreactivity. Our study demonstrated that the LKT was present in inflammatory exudate and bound to the membranes of degenerating neutrophils and macrophages located in the alveolus. This study also indicated that the LKT was not found within the alveolar endothelial cells or epithelial cells. This was the first study that demonstrated the presence of LKT in the lung lesions from cattle affected with natural or experimental pneumonic pasteurellosis.

Experiment 2: Determination of the in vitro effects of LKT on bovine polymorphonuclear neutrophils(PMNs).

We and others have characterized the progression of the microscopic and ultrastructural pneumonic lesions that occur in natural and experimentally induced pneumonic pasteurellosis(15,16,17). These studies suggest that early in the infection, an influx of PMNs occurs, followed by accumulation of extensive edema fluid containing fibrin in the alveoli, pleural surface, and interlobular septa. Other experimental data also suggest that PMNs play a key role(18). If PMNs play a critical role in mediating the acute lung injury, then what factors released from the PMNs are responsible for this injury? This study was undertaken to determine the effects of LKT on bovine PMNs.

Results from this study showed that the LKT from *P.haemolytica* in addition to its cytocidal activity on bovine PMNs, also stimulated a respiratory burst and released various proteolytic enzymes. Generation of oxygen-derived free radicals, such as superoxide anion(O_2^-) and hydrogen peroxide (H_2O_2) was used as a measure of stimulation of respiratory burst. Release of myeloperoxidase and arylsulfatase B was used as a measure of lysosomal degranulation. Our results showed that stimulation of the respiratory burst was an immediate event, while cytolysis and release of proteolytic enzymes by degranulation occurred over a period of time.

We believe that the toxic oxygen radicals and the proteases released by the PMNs, can initiate harmful inflammatory reactions and lung damage which may be relevant in the pathogenesis of the disease.

Experiment 3: Determination if the leukotoxin augmented the neutrophil-mediated injury of the pulmonary vascular endothelium.

Morphological and immunohistochemical studies from our laboratory, using bovine pneumonic lung sections, indicated that damage to pulmonary endothelium was observed beneath the sites of neutrophil(PMN) attachment suggesting that PMNs play a key role in the vascular leakage and the ensuing alveolar edema(16). It is unclear which virulence factor from *P.haemolytica* contribute to this PMN-mediated endothelial cell injury. We used an in vitro PMN-endothelial cell coculture system to address this question. Our results demonstrated that the LKT was indeed responsible for augmenting the PMN-mediated killing of endothelial cells. This augmented killing was related to the stimulation of PMNs by the LKT. Furthermore, the hydroxyl radical(HO·) and proteases released by LKT-exposed PMNs damaged the vascular endothelial cells.

We conclude that the acute lung injury seen in the disease is caused by interactions between various cellular constituents in the lung and inflammatory mediators inititated by the *P.haemolytica* leukotoxin and endotoxin. Elucidation of the

mechanisms underlying these interactions is crucial to control the disease by various therpeutic measures.

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