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A comparison of secretory response of neutrophils isolated from heifers in the course of Bovine Respiratory Disease and severity of clinical signs

Porównanie odpowiedzi sekrecyjnej neutrofilii izolowanych od jałówek w przebiegu zespołu oddechowego i stopnia nasilenia objawów klinicznych choroby

Summary. Some neutrophil products such as elastase, myeloperoxidase (MPO), alkaline phosphatase (ALKP), superoxide (O_2^-), and 5-oxo-eicosatetraenoic acid (5-oxo-EETE) may play an essential role in neutrophil mediated lung injury in the course of Bovine Respiratory Disease (BRD). The aim of this study was to evaluate how this neutrophil secretory action correlates with the severity of clinical signs on the basis of clinical score. Neutrophils isolated from 60 BRD heifers and from 30 healthy heifers were cultured, and then elastase, MPO, ALKP superoxide, and 5-oxo-EETE production were assessed. These studies revealed that the correlation between neutrophil secretory action and clinical signs exist. The clinical score correlated highly ($r = 0.65$) with elastase release, it correlated moderately with ALKP ($r = 0.44$), and correlated weakly with MPO ($r = 0.28$), 5-oxo-EETE ($r = 0.29$), and superoxide anion ($r = 0.3$). The strongest relation was observed between elastase release and severity of clinical signs.

Key words: neutrophil, elastase, lung injury, heifer

INTRODUCTION

Neutrophils (PMN) play a crucial role in resistance to infection, but under certain conditions they may exhibit prolonged or enhanced activation and then their degranulation contributes to tissue damage, and worsening the course of the disease [Whiteley *et al.* 1992, Ruchaud-Sparagano *et al.* 1998, Coomber *et al.* 2001, Wessely-Szponder *et al.* 2004, Wessely-Szponder and Bobowiec 2005, Wessely-Szponder 2006]. Elastase from neutrophil secondary granules can mediate tissue destruction by degrading elastin, collagen, and proteoglycan. This digestion finally leads to vascular and trans-alveolar protein leakage and dysfunction of lungs [Stockley 1995]. Other enzymes are also involved in the tissue injury

during inflammatory process. According to some authors, MPO and ALKP function as markers of lung injury in some species [Eppinger *et al.* 1995, McCabe 2001, Sunters *et al.* 2002]. MPO, in conjunction with HO^\cdot and Cl^- , generated hypochlorous acid (HOCl), regarded as a strong bactericidal agent, which also leads to tissue damage [Sahoo *et al.* 1998]. On the other hand, the activity of ALKP plays a role in the alveolar type II epithelial cell injuries [Sunters *et al.* 2002]. The production of oxygen-derived free radicals, such as superoxide (O_2^\cdot), by neutrophils may lead to the initiation of membrane lipid peroxidation and damage to DNA, proteins, nucleic acids and other macromolecules [Sunters *et al.* 2002]. Another neutrophil product 5-oxo-eicosatetraenoic acid (5-oxo-ETE) exacerbates neutrophil functions as it is a potent stimulant of chemotaxis and degranulation [Stamatiou 1998, Zimpfer *et al.* 2000]. Therefore, it could also play an essential role in lung injury.

The objective of this study was to investigate the relation between the secretory action of neutrophils on the basis of release of elastase, MPO, ALKP, O_2^\cdot , and 5-oxo-ETE, and the severity of the clinical signs in BRD heifers.

MATERIALS AND METHODS

Peripheral blood was collected from 60 BRD heifers and 30 healthy heifers. Physical examination of each heifer was performed before the collection of blood. Signs of clinical disease were allocated points, according to the following scoring system: body temperature greater than 39.7°C (2 points), inappetance (1 point), lethargy or depression point), marked weakness or recumbency (2 points), moribund state (3 points), cough (1–2 points), nasal discharge (1–2 points), respiratory rate greater than 60 breaths/min (1–2 points), dyspnea (2 points), and abnormal breath sounds on thoracic and tracheal auscultation (1 point) [Malazdrewich *et al.* 2004].

Blood neutrophils were isolated according to the method of Mottola [Hoebden *et al.* 1997]. The remaining pellet was washed with phosphate-buffered saline (PBS) and the final cell pellet was resuspended in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM-Sigma). After isolation, the viability of PMNs cells was determined by trypan blue exclusion. After cell counting and differentiation, cell suspensions were adjusted to a final concentration of $2 \cdot 10^6$ cells/ml. Neutrophil degranulation was assessed by elastase, MPO, and ALKP release. Controls were performed by incubating cells in the absence of stimuli or in the presence of 0.5% CTAB to measure the background release and 100% enzyme content, respectively. Elastase activity was measured with azocasein as a substrate after 10 min. incubation at room temperature. MPO and ALKP release was measured by spectroscopy after 10 min incubation at room temperature with equal volume of o-phenylendiamine (OPD) or 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP), respectively. The elastase, MPO, and ALKP reactions were stopped by the addition of TCA, H_2SO_4 , and NaOH, respectively. Absorbance was measured at 492 nm for elastase and MPO, and at 405 nm for ALKP. All samples were assayed in duplicate [Coomber *et al.* 1997, Sahoo *et al.* 1998]. Superoxide anion production was measured by the method described by Confer [Confer and Simons 1986]. Neutrophils were incubated with 0.1% nitroblue tetrazolium (NBT) solution. Nanomoles of superoxide produced over the incubation period were calculated using the extinction coefficient 21.1 nmol [Galligan and Coomber 2000]. The generation of 5-oxo-ETE was assessed by the HPLC method. The mobile phase consisted of a

linear gradient between solvent A [water/methanol (70/30)] and solvent B [water/methanol/acetonitril/trifluoroacetic acid (20/25/55/0.0090)] as follows: 0 min 50% B; 40 min 100 B. The flow rate was 1.5 ml/min. The rate of 5-oxo-ETE production was assessed by comparison of the peak areas of UV absorbance at 280 nm with defined concentration of 5-oxo-ETE at 280 nm and the peak area of the internal standard 13-hydroxyoctadecadienoic acid (13-HODE) at 237 nm [Zimpfer *et al.* 2000].

The examined values were compared using analysis of variance and Student's t-test and differences were considered as significant at $p < 0.05$.

RESULTS

Neutrophils isolated from heifers with BRD showed a significantly increased release of their constituents as compared with values from neutrophils of healthy heifers. Especially, it was seen in case of enzyme release. Release of elastase, MPO, and ALKP by neutrophils isolated from BRD heifers was significantly greater than from neutrophils obtained from healthy heifers ($p < 0.05$). Elastase release was at the highest level in both examined groups, but differences between neutrophils from BRD and healthy heifers were the most pronounced in MPO release (Fig 1). Also, values of O_2^- (1.35 ± 0.11 nM in BRD heifers vs 1.21 ± 0.11 nM in healthy heifers) and 5-oxo-ETE (2.52 ± 1.2 nM in BRD heifers vs 2.36 ± 1.3 nM in healthy heifers) were augmented in BRD heifers. The clinical score for healthy heifers was 0, the disease score for BRD was in the range of 3 to 15 (a mean of 9.2 ± 2.95). We observed that the secretory action of neutrophils was related to the severity of disease. The clinical score correlated highly ($r = 0.65$) with elastase release, it correlated moderately with ALKP ($r = 0.44$), and weakly with MPO ($r = 0.28$), 5-oxo-ETE ($r = 0.29$), and superoxide anion ($r = 0.3$), (Fig. 2, 3).

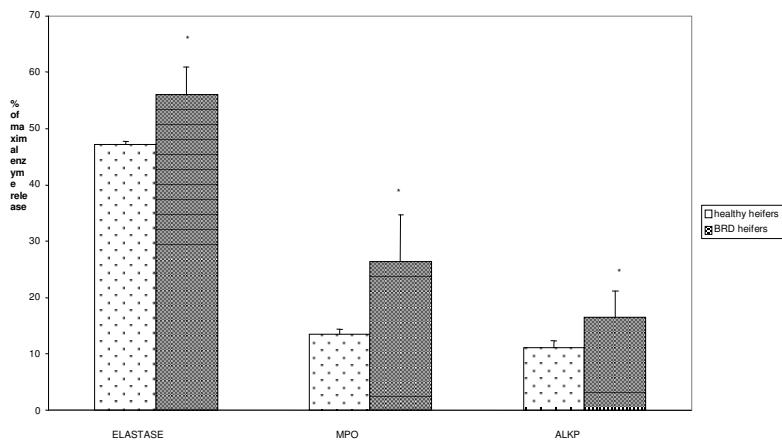


Fig. 1. Release of enzymes by neutrophils from healthy and BRD heifers. ** $P < 0.01$ versus neutrophils from healthy heifers (mean \pm SD)

Rys. 1. Uwalnianie enzymów przez neutrofile uzyskane od zdrowych jałówek i w przebiegu BRD

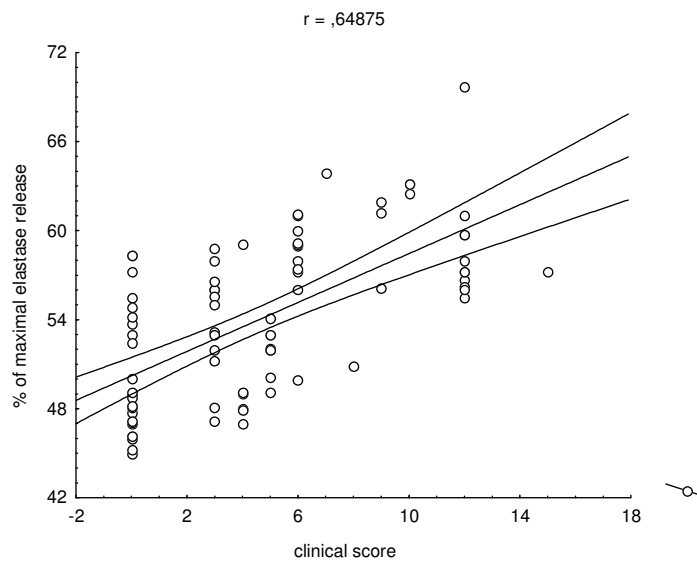


Fig. 2. Correlation between elastase release (% of maximal elastase release) by neutrophils and clinical score of heifers

Rys. 2. Zależność pomiędzy uwalnianiem elastazy (% maksymalnego uwalniania) przez neutrofile i stopniem nasilenia objawów klinicznych

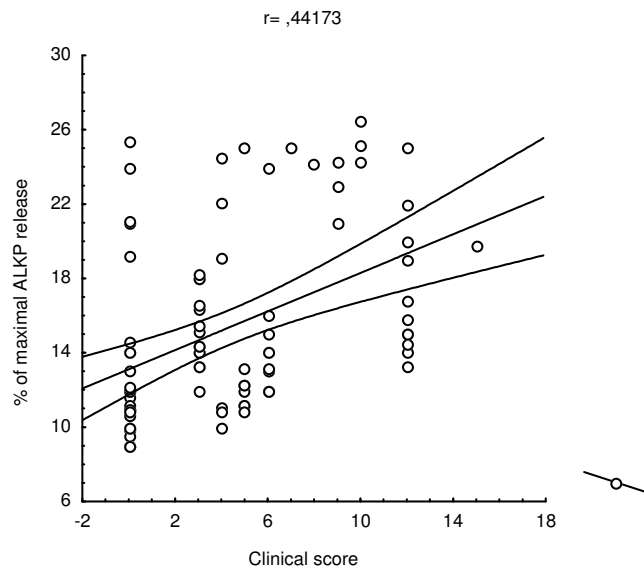


Fig. 3. Correlation between ALKP release (% of maximal ALKP release) by neutrophils and clinical score of heifers

Rys. 3. Zależność pomiędzy uwalnianiem ALKP (% maksymalnego uwalniania) przez neutrofile i stopniem nasilenia objawów klinicznych

DISCUSSION

Neutrophil action in the pathogenesis of respiratory disease is a double-edged sword. Neutrophil influx into pulmonary tissues is important for the complete resolution of bacterial and viral infections of the lung. However, as a potent source of enzymes, reactive oxygen species, and eicosanoids, activated neutrophils are simultaneously cytotoxic to pathogens and to host tissues [Stockley 1995]. Some authors [Whitley *et al.* 1992, Malazdrewich *et al.* 2004] mentioned an important role of neutrophils in pathogenesis of lung injury in cattle. Whitley *et al.* [1992] discovered that neutrophils enter the lung during the first few hours after bacterial infection and are responsible for the lung tissue injury. After neutrophil depletion the pulmonary injury decreases in comparison with pathological alterations that occur in the intact animal. Therefore, neutrophil depletion protected animals from acute inflammatory injury. According to Malazdrewich *et al.* [2004] because neutrophils and the host inflammatory response are important factors in pathogenesis of bovine *mannheimiosis*, it may be possible to treat or prevent this disease through pharmacological modulation of pulmonary inflammatory responses. However, the role of particular neutrophil products in pathogenesis of lung injury in the course of BRD still remains unclear.

Our previous studies pointed out that there is a greater release of elastase, MPO and ALKP by neutrophils isolated from heifers with respiratory tract infections in comparison with neutrophils from healthy heifers [Wessely-Szponder *et al.* 2004]. These studies revealed that generation of other neutrophil products, namely superoxide and 5-oxo-ETE also significantly increased during BRD.

Some neutrophil constituents were previously estimated as lung injury markers for other species [Eppinger *et al.* 1995, Hagio *et al.* 2001, McCabe *et al.* 2001, Kawabata *et al.* 2002], thus an increased production of these compounds may also lead to worsening of the course of respiratory disease in cattle. In these studies a correlation between a release of some neutrophil constituents i.e. elastase, MPO, ALKP, superoxide, and 5-oxo-ETE; and the clinical severity of BRD was shown, which confirmed their influence on the course of disease. Because of a close relation between elastase release and the severity of clinical signs, this enzyme may be considered as crucial in the pathogenesis of BRD.

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Streszczenie. Niektóre produkty neutrofilowe, takie jak: elastaza, mieloperoksydaza (MPO), zasadowa fosfataza (ALKP), anion nadadtlenkowy (O_2^-) oraz kwas 5-okso-eikozatetraenowy (5-oxo-EET) mogą odgrywać istotną rolę w wywoływaniu przez neutrofile uszkodzeniu płuc w przebiegu zespołu oddechowego u bydła (BRD). Celem pracy było stwierdzenie, w jaki sposób aktywność wydzielnicza neutrofilii koreluje ze stopniem nasilenia objawów klinicznych określonym na podstawie zastosowanej skali. Neutrofile wyizolowane od 60 jałówek w przebiegu BRD i od 30 zdrowych jałówek poddano hodowli *in vitro*, a następnie oceniono uwalnianie elastazy, MPO, ALKP oraz wytwarzanie anionu nadadtlenkowego i 5-oxo-EET. Przeprowadzone badania wykazały, że istnieje wysoka korelacja pomiędzy uwalnianiem elastazy a stopniem nasilenia objawów klinicznych. Zależność ta była średnia dla ALKP ($r = 0.44$) i mała dla MPO ($r = 0.28$), 5-okso-EET ($r = 0.29$) oraz anionu nadadtlenkowego ($r = 0.3$).

Słowa kluczowe: neutrofil, elastaza, uszkodzenie płuc, jałówka