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Research paper

Effects of dexamethasone and *Mycoplasma bovis* on bovine neutrophil function *in vitro*

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ABSTRACT

It is well established that exposure either to elevated levels of glucocorticoids, or to *Mycoplasma bovis* (*M. bovis*), has a negative effect on bovine neutrophil function. The objective of this research was to determine whether *in vitro* treatment of bovine neutrophils by *M. bovis* strains ($n = 4$) and glucocorticoids would additively impair phagocyte function. Twenty, healthy, dairy cows were enrolled. Whole blood was collected from all cows for neutrophil isolation. Phagocytosis and the generation of superoxide anion (O_2^-) were tested *in vitro* by incubation of neutrophils with FITC labeled *Escherichia coli* (*E. coli*) and cytochrome c after treatment. Treatments included: NM1-4D (neutrophils treated with dexamethasone and exposed to one of the four *M. bovis* strains); NM1-4 (neutrophils exposed to one of the four *M. bovis* strains only); ND (neutrophils treated with dexamethasone only); and N (non-treated control neutrophils). The overall percentages of neutrophils phagocytizing *E. coli* were: 32%, 51%, 37%, and 53% ± 5.25% for treatments NM1-4D, NM1-4, ND, and N, respectively. The overall statistically transformed means of phagocytized *E. coli* per neutrophil were: 1.37, 1.72, 1.33, and 1.67 ± 0.057 for treatments NM1-4D, NM1-4, ND, and N, respectively. The overall statistically transformed means of neutrophil O_2^- production were: 8.60, 11.91, 9.01, and 12.21 ± 0.21 nmol/10^6 for treatments NM1-4D, NM1-4, ND, and N, respectively. Exposure of neutrophils to *M. bovis* plus dexamethasone had an additive effect on generation of reactive oxygen species ($p = 0.0057$), but not on the percentage of neutrophils phagocytizing *E. coli* ($p = 0.0817$) or number of *E. coli* phagocytized per neutrophil ($p = 0.2946$). Only one of the four *M. bovis* strains had a negative effect on neutrophil phagocytic function. Dexamethasone treatment consistently decreased neutrophil function as indicated by decreased percentage of neutrophils phagocytizing *E. coli*, decreased number of *E. coli* phagocytized per neutrophil, and decreased neutrophil O_2^- production, compared to controls ($p < 0.0001$). Results suggested a synergistic effect of *in vitro* incubation of glucocorticoids and *M. bovis* on reduction of bovine neutrophil function as measured by generation of reactive oxygen species. These findings may explain in part the interaction between stressful events and outbreak of *Mycoplasma bovis* associated bovine disease.

Abbreviations: ACD, acid citrate dextrose; cytochrome c, cytochrome complex; *E. coli*, *Escherichia coli*; FITC, fluorescein isothiocyanate; M, molar concentration; *M. bovis*, *Mycoplasma bovis*; MbAD, *Mycoplasma bovis* associated diseases; NM1-4D, neutrophils treated with dexamethasone and exposed to one of four *M. bovis* strains; ND, neutrophils treated with dexamethasone only; NM1-4, neutrophils exposed to one of four *M. bovis* strains only; N, non-treated control neutrophils; O_2^-, superoxide anion; *p*, probability; PI, phagocytic index; % Phag. Cells, percentage of cells phagocytizing; ROS, reactive oxygen species.

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1. Introduction

Mycoplasma bovis associated diseases (MbAD) in cattle include pneumonia, otitis media, mastitis, arthritis and reproductive disorders (Pfutzner and Sachse, 1996; Maunsell and Donovan, 2009). *Mycoplasma bovis* (*M. bovis*) has the ability to induce both immune response and immunosuppressive effects (Razin and Yogev, 1998). Immune responses against MbAD include innate and adaptive humoral and cellular (Razin and Yogev, 1998). Although activation and recruitment of immunocytes are thought to contribute to the lesions during MbAD (Howard et al., 1987), control of MbAD infections is mediated through an integrative action of adaptive humoral and innate immune responses (Bennett and Jasper, 1978; Howard et al., 1979). These include the interactions of various components such as different classes of immunoglobulins, complement components, and different phagocytic cells (Howard et al., 1976; Howard, 1981, 1984). A key component of the innate immune response against MbAD involves proper function of the phagocytes including macrophages and neutrophils (Howard et al., 1976; Marshall et al., 1995). In the absence of *M. bovis* infection, alveolar macrophages are the predominant phagocytic cells to clear *M. bovis* from the lung (Allen et al., 1992). However, during *M. bovis* infection, it is thought that the neutrophils are the major phagocytic cells, resulting from the effect of TNF-α released by hyper-activated macrophages (Jungi et al., 1996). Neutrophils are the major phagocytic cell type found in tissue or secretions in systems infected with *M. bovis* (Gagea et al., 2006; Al-Abdullah and Fadl, 2006; Chima et al., 1981; Maunsell et al., 2012). There are a limited number of studies that have investigated the interaction between *M. bovis* and neutrophil function, both *in vivo* and *in vitro* (Jain et al., 1969; Howard et al., 1976). Although neutrophils had the capacity to phagocytize *M. bovis in vitro* (Howard et al., 1976), the capacity to phagocytize did not appear to be sufficient to clear the infection as tested *in vivo* (Jain et al., 1969). Neutrophils exposed to unopsonized *M. bovis* have altered *in vitro* bactericidal functions including impaired ability to phagocytize *M. bovis* and *E. coli*, decreased production of reactive oxygen species (ROS), and decreased degranulation (Howard and Taylor, 1983; Finch and Howard, 1990; Thomas et al., 1991; Wiggins et al., 2010).

Similar to the effect of *M. bovis* on neutrophil function, exposure to glucocorticoids during stress or following exogenous glucocorticoids administration results in alteration of phagocyte function *in vitro*. *In vitro* exposure of isolated bovine neutrophils to elevated levels of glucocorticoids reduced phagocytic ability to engulf *E. coli* and reduced production of ROS compared to controls (Moiola et al., 1994; Hoeben et al., 1998). Similarly, increased peripheral blood glucocorticoid levels during physiologic stress or *via* exogenous glucocorticoid administration negatively impairs *in vitro* phagocyte function, oxidative burst activity and neutrophil degranulation (Mateus et al., 2002; Roth and Kaeberle, 1981; Weber et al., 2006).

Stressful events including overcrowding, mixing calves, parturition, transportation, and inclement weather have all been associated with outbreaks of MbAD (Boothby et al., 1983; Bayoumi et al., 1988; Allen et al., 1992; Woldehiwet et al., 1990; Reinhold and Elmer, 2002; Wilson et al., 2007). Exogenous glucocorticoid administration has been used in livestock disease models to mimic stress (Roth and Kaeberle, 1981). Recently, we demonstrated that calves fed milk containing *M. bovis* had increased nasal pharyngeal colonization after intramuscular dexamethasone administration (Alabdullah et al., 2011). Both the duration and number of sites colonized by *M. bovis* are increased in treated calves *versus* controls (Alabdullah et al., 2011). Our overarching hypothesis is that susceptibility to MbAD disease results from a combination of glucocorticoid-induced immunosuppression in conjunction with the immunomodulation effects of *M. bovis*. The purpose of this study was to determine if the combination of glucocorticoids and *M. bovis* exposure had an additive effect on neutrophil function *in vitro*. Additionally, we examined whether different strains of *M. bovis* incubated *in vitro* with neutrophils had different immunomodulation effects.

2. Materials and methods

2.1. Animals

Twenty healthy Holstein dairy cows past peak milk production, within 100–350 days in milk, the Washington State University Dairy Center were used to obtain neutrophils for the study. Two weeks prior to blood collection, milk samples and moistened swabs from accessible body sites (nose, ear, and eye) were collected and cultured to determine that the subject animals were free of *M. bovis* infection as described by Punyapornwithaya et al. (2010). Forty ml of venous blood was collected in sodium/lithium heparin and acid citrate dextrose (ACD) containing vessels for phagocytic and superoxide anion (O_2^-) analysis, respectively. Blood was collected from only a single cow per day.

2.2. Neutrophil isolation

Neutrophils were isolated *via* density gradient centrifugation, as described by Weber et al. (2001) with some modifications. Briefly, 40 ml of anti-coagulated whole blood was centrifuged at 4 °C for 20 min at 1000 × *g*. Plasma, buffy coat and half of the red layer were aspirated and discarded. The cells were re-suspended in 4 ml of an isotonic, ice-cold phosphate buffer saline solution (PBS) (pH 7.2) and transferred into 50 ml centrifuge tubes. Remaining erythrocytes were lysed by the addition of 20 ml, sterile deionized water. Isotonicity was restored within 30 s by addition of 10 ml of a 3-fold concentration of PBS. Cells were pelleted by centrifugation at 200 × *g* for 10 min, the supernatant were discarded, and the remaining cells of the pellet were reconstituted with 30 ml PBS with 20% sterile, heat-treated, fetal calf serum. Granulocytes were separated by density gradient centrifugation after addition of 10 ml of 1.084 g/ml saline-Percoll solution (Sigma Chemical Company, St. Louis, Mo) and centrifuged at 22 °C for 40 min at 400 × *g*. After aspiration, the remaining granulocyte pellet was washed, pelleted and then reconstituted with 4 ml of PBS with 20% sterile, heat-treated fetal calf