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Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*

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ABSTRACT

Eimeria bovis infections are under immunological control and recent studies have emphasized the role of early PMN-mediated innate immune responses in infected calves. Neutrophil extracellular traps (NETs) have recently been demonstrated to act as a killing mechanism of PMN against several pathogens. In the present study, the interactions of bovine PMN with sporozoites of *E. bovis* were investigated in this respect *in vitro*. For demonstration and quantification of NET formation, extracellular DNA was stained by Sytox Orange. Fluorescence images after Sytox Orange staining as well as scanning electron microscopy (SEM) showed NET formation to occur upon contact with *E. bovis* sporozoites. Exposure of PMN to viable sporozoites induced stronger NET formation than to dead or homogenized parasites. NET formation was abolished by treatment with DNase and could be reduced by diphenylene iodonium, which is described as a potent inhibitor of NADPH oxidase. After sporozoite and PMN co-culture, extracellular fibres were found attached to sporozoites and seemed to trap them, strongly suggesting that NETs immobilize *E. bovis* sporozoites and thereby prevent them from infecting host cells. Thus, transfer of sporozoites, previously being confronted with PMN, to adequate host cells resulted in clearly reduced infection rates when compared to PMN-free controls. NET formation by PMN may therefore represent an effector mechanism in early innate immune reactions against *E. bovis*. This is the first report indicating *Eimeria*-induced NET formation.

1. Introduction

Eimeria bovis infection in cattle is an important protozoan parasitosis, causing economic losses and severe clinical typhlocolitis in calves (Fitzgerald, 1980; Daugschies et al., 1998).

In general, *Eimeria* infections are under immunological control and a variety of studies performed on cell-mediated, adaptive immune responses emphasized the crucial role of lymphocytes in rodents (Rose et al., 1990, 1992; Shi et al., 2001) and calves (Speer et al., 1985; Hughes et al., 1988, 1989; Fiege et al., 1992; Hermosilla et al., 1999; Taubert et al., 2008). So far, however, relatively little is known on innate immune responses to *Eimeria* infections. The first-line defence against invading pathogens, in general, is represented by professional phagocytes, such as macrophages, dendritic cells and polymorphonuclear neutrophils (PMN). Main effector mechanisms of PMN are the killing of pathogens and the production of immunomodulatory molecules, such as cytokines or chemokines, to attract immunopotent cells to the site of infection, thereby initiating acquired immune responses. Classical PMN-conducted killing involves phagocytosis, the production of reactive oxygen species, and antimicrobial peptides/proteins. In addition, the formation of

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neutrophil extracellular traps (NETs) has been recently identified as a further effector mechanism of PMN-mediated pathogen killing. NETs act effectively against bacteria and fungi (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007) and may represent a common mechanism to eliminate pathogens.

In the case of *Eimeria* infections, PMN show distinct infiltration of parasitized tissue and accumulate very early at the site of meront formation in infected rodents (Mesfin et al., 1978; Blagburn and Todd, 1984) and in *E. bovis*-infected calves (Friend and Stockdale, 1980). The importance of PMN in *Eimeria* infections is further underlined by the observation, that PMN-depleted SCID mice significantly produce more *E. papillata* oocysts after primary infection than control mice (Schito and Barta, 1997).

For successful infection, *E. bovis* sporozoites have to traverse the mucosal layer of the ileum to reach the lymphatic capillaries for infection of the adequate host cells, lymphatic endothelial cells. In consequence, sporozoites of *E. bovis* should be exposed to the interstitial fluid and to the lymph and should be recognized as potential targets by PMN.

There is some evidence that PMN can directly interact with apicomplexan parasites in general. PMN have been shown to actively lyse *E. falciformis* sporozoites in the presence of antibodies and complement (Bekhti et al., 1992). In the case of *Toxoplasma gondii*, PMN have been shown to kill tachyzoites *in vitro* (Wilson and Remington, 1979; MacLaren and de Souza, 2002; MacLaren et al., 2004). We have recently shown that bovine PMN interact directly with *E. bovis* sporozoites (Behrendt et al., 2008) and are able to eliminate sporozoites *in vitro*. Furthermore, bovine PMN were identified as an *in vitro*-source of several pro-inflammatory cytokines (IL-6, IL-12, TNF-α), chemokines (MCP-1, GRO-α, IL-8, IP-10) and iNOS when exposed to *E. bovis* sporozoites or merozoite I antigens (Behrendt et al., 2008). The role of PMN in *E. bovis* control was also underlined by *in vitro* and *ex vivo* data showing enhanced phagocytic and oxidative burst activities of PMN either exposed to sporozoites *in vitro* or derived from *E. bovis*-infected calves (Behrendt et al., 2008).

This study was conducted to characterise early reactions of bovine PMN against *E. bovis* with respect to NET formation. We show here that direct contact of PMN with viable sporozoites triggers the formation of NETs, which, in turn, immobilize these invasive stages of the parasite thus preventing the infection of host cells. As such, sporozoite-induced NET formation was proven as additional effector mechanism directed against *E. bovis* sporozoites *in vitro*.

2. Materials and methods

2.1. Parasite

The *E. bovis* strain H used in the present study was maintained by passages in Holstein–Friesian calves for oocyst production. Calves were infected orally with 5×10^5 sporulated *E. bovis* oocysts. Collection of oocysts, oocyst sporulation and excystation of sporozoites were performed as previously described (Hermosilla et al., 2002). Free sporozoites were collected and suspended in medium free of FCS (RPMI 1640, Gibco, 1% penicillin/streptomycin, v/v).

2.2. Isolation of bovine PMN

Calves were bled by puncture of the jugular vein and blood was collected in 50 ml plastic tubes (Nunc), containing 0.1 ml heparin (Sigma) as anticoagulant. Heparinized blood was diluted in an equal amount of PBS containing 0.02% EDTA (Sigma), layered on Biocoll Separating Solution (Biochrom AG) and centrifuged at $800 \times g$ for 45 min. After removal of plasma, lymphocytes and monocytes, the pellet was suspended in distilled water and shaken for 40 s to lyse erythrocytes. Osmolarity was immediately re-adjusted by adding the appropriate amount of Hanks Salt Solution (10×, Biochrom AG). PMN were washed twice with RPMI 1640 medium, resuspended in medium RPMI 1640 (6.6×10^6 cells/ml) and incubated at 37 °C and 5% CO_2 for at least 30 min before use.

2.3. Scanning electron microscopy

Bovine PMN were incubated with freshly isolated *E. bovis* sporozoites at a ratio of 10:1 for 2, 3 and 4 h on poly-L-lysine pre-coated coverslips. After incubation, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 15 min and afterwards washed with 0.1 M cacodylate buffer. The cells were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, washed three times in distilled water, dehydrated in ascending ethanol concentrations, critical point dried with CO_2 and sputtered with gold. Specimens were examined using a Philips XL20 scanning electron microscope.

2.4. Co-culture of bovine PMN and Eimeria bovis sporozoites

PMN were placed in flat bottom 96-well plates at a density of 5×10^5 cells/well. For different experimental conditions, the wells were filled up to a final volume of 200 μl with various incubation mixes and incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. All compounds used were diluted or suspended in medium RPMI 1640 whilst plain medium served as negative control. For positive controls, PMN were stimulated with phorbol 12-myristate 13-acetate (PMA, ORPEGEN-Pharma) at a final concentration of 10 ng/ml. To obtain maximum values for extracellular DNA, PMN were lysed with Triton X100 (0.1%), added 15 min before the end of incubation.

To test for sporozoite-induced NET-formation, 10^5 sporozoites (vital or heat inactivated at 60 °C for 30 min) were added. To test a sporozoite-homogenate for it's ability to induce NET-formation, sporozoites underwent three freeze and thaw cycles (freezing in liquid nitrogen for 1 min and complete thawing at 37 °C) and subsequent sonication (15 min, 50 kHz). The amount of homogenate per well corresponded to 10^5 sporozoites.

For DNase treatment 90 U DNase I (Roche Diagnostics) per well were added at the start of incubation. Inhibition assays were performed using 5 μM diphenylene iodonium (DPI) or 10% neonatal calf serum throughout the incubation period.