Developmental and Comparative Immunology 79 (2018) 137–141

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Short communication

Extracellular trap-like fiber release may not be a prominent defence response in snails: evidence from three species of freshwater gastropod molluscs

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ARTICLE INFO

Article history:
Received 14 July 2017
Received in revised form
18 October 2017
Accepted 19 October 2017
Available online 22 October 2017

Keywords:
Extracellular trap-like fiber (ET-like fiber)
Lymnaea stagnalis
Radix lagotis
Planorbarius corneus
Snail immunity
Haemocytes

ABSTRACT

The discovery that mammalian neutrophils generate extracellular chromatin fibers that entrap/kill bacteria supported a new paradigm for innate immunity in animals. Similar findings in other models across diverse taxa have led to the hypothesis that the phenomenon is ancient and evolutionary conserved. Here, using a variety of synthetic (e.g. peptidoglycan) and biological (e.g. trematode larvae) components to investigate extracellular trap-like (ET-like) fiber production *in vitro* by haemocytes of *Lymnaea stagnalis*, *Radix lagotis* and *Planorbarius corneus* snails, ET-like fibers were rarely observed. We suggest, therefore, that ET-like fibers play a marginal role in defence of these snail species and thus the fiber production may not be a critical process underpinning immunity in all invertebrate species.

1. Introduction

Reticulated DNA fibers produced by neutrophils (neutrophil extracellular traps; NETs), eosinophils (extracellular traps; ETs) and other cells of the vertebrate innate immune system are considered important structures that facilitate the elimination of bacteria and eukaryotic unicellular/multicellular parasites extracellularly (von Köckritz-Blickwede and Nizet, 2009; Zawrotniak and Rapala-Kozik, 2013; Hermosilla et al., 2014). In invertebrates, immunity typically relies on haemocytes that cooperate with humoral recognition factors such as lectins and fibrinogen-related proteins to deliver the defence response. While extracellular nucleic acids can bolster immunity as shown in the greater wax moth *Galleria mellonella* (Altincicek et al., 2008), ET-like fibers resembling NETs of vertebrates have recently also been found to mediate defence of *Litopenaeus vannamei* (Ng et al., 2013) and *Carcinus maenas* (Robb et al., 2014) haemocytes. Interestingly, mesogleal cells of the sea anemone *Actinia equina* (Robb et al., 2014), and sentinel cells of the social amoeba *Dictyostelium discoideum* (Zhang et al., 2016) have also been shown to release DNA fibers extracellularly. In molluscs, ET-like fibers have been reported in bivalves (*Mytilus edulis*, *Crassostrea gigas*) (Robb et al., 2014; Poirier et al., 2014), and gastropods (*Arion lusitanicus*, *Limax maximus* and *Achatina fulica*) in which the fibers entrapped metastrongyloid larvae (Lange et al., 2017). In the latter case, different types of ET-like fibers (i.e. aggregated, spread and diffuse) were observed, with histones and myeloperoxidase as fiber constituents (Lange et al., 2017).

In the current study, we employed haemocytes of *Lymnaea stagnalis* and two other species of freshwater gastropod snails, *Radix lagotis* and *Planorbarius corneus* to elucidate ET-like fiber production in snails that serve as intermediate hosts of trematode larvae. For comparative purposes, we used *Mytilus edulis* haemocytes that are known to release ET-like fibers.

2. Materials and methods

2.1. ET-like fiber release by Mytilus edulis haemocytes

Haemocytes of *M. edulis* were utilized for initial experiments.

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https://doi.org/10.1016/j.dci.2017.10.011
0145-305X/

Haemolymph was extracted and haemocyte monolayers were prepared as previously described (Robb et al., 2014) in 96-well tissue culture plates (Nunc) employing 250 μl haemolymph/well diluted (1:1) with 0.05 M Tris-HCl buffer, pH 7.6, supplemented with 2% glucose, 2% NaCl, 0.5% EDTA. Haemocytes were incubated with 20 μM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at 10 °C for 48 h, stained with 1 μM Sytox green (Thermo Fisher Scientific) that effectively binds DNA of dead cells (Thakur et al., 2015) and examined for ET-like fiber release under a fluorescence microscope (Olympus IX71).

2.2. Snails and haemocytes

Laboratory-reared *L. stagnalis* and *R. lagotis* were maintained at 19–22 °C in aerated aquaria, and fed fresh lettuce *ad libitum*. *Planorbarius corneus* snails were obtained from a local pond (Prague) and examined for cercarial shedding; infected snails were excluded from experiments. Haemolymph from snails was extracted according to Sminia (1972). Samples from L. *stagnalis* and *P. corneus* were pooled on ice, diluted 2:1 with sterile snail saline (SSS; Adema et al., 1991) and 250 μl transferred into individual wells of a 96-well plate. Experiments with *P. corneus* were also conducted in Chernin's balanced salt solution (CBSS; Chernin, 1963). Haemolymph from *R. lagotis* was handled as described previously (Skála et al., 2014). The haemocyte number per well was approx. 2.8×10^5 for *L. stagnalis*, 6×10^4 for *R. lagotis* and 1.2×10^5 for *P. corneus*, enumerated using a Bürker haemocytometer.

2.3. Preparation of parasite material

Miracidia of *Trichobilharzia regenti* were obtained *via* the laboratory life cycle according to Horák et al. (1998), fixed in 2% (v/v) paraformaldehyde for 30 min and free aldehyde groups blocked in 1% glycine at 4 °C overnight (Zahoor et al., 2008). The larvae were then washed twice with SSS and stored at −20 °C. Homogenised miracidia were prepared by sonicating miracidia for three cycles (7W, 20 s each, Vibracell-72405 100-W ultrasonicator, Bioblock Scientific, France) in SSS followed by determination of protein concentration using Quant-iT Protein Assay Kit (Invitrogen).

2.4. Haemocyte exposure

Lymnaea stagnalis haemocyte monolayers were treated with SSS containing peptidoglycan (PGN; 0.1, 1.0 and 10.0 μg/ml), *E. coli* lipopolysaccharide serotype 0111:B4 (LPS; 0.1, 1.0 and 10.0 μg/ml), PMA (0.1, 1.0, 10.0 μM), D-galactose or L-fucose (200, 400, 800 nM, 1 and 10 μM), D-galactose-L-fucose in combination (800 nM of each in SSS) (all purchased from Sigma-Aldrich), live/heat-killed *Staphylococcus saprophyticus* at ~10, ~100 and ~1000 bacteria/haemocyte, or miracidial homogenate (1 or 10 μg/ml). All incubations were performed at room temperature (e.g. Plows et al., 2005) for 3 h and 24 h. Three independent experiments were performed with one replicate for each condition/duration. The haemocytes were then stained with 1 μM Sytox Green in SSS for 20 min and the entire cell populations examined visually under the fluorescence microscope; haemocytes producing ET-like fibers were enumerated.

For intact parasite exposure, 200 miracidia in 100 μl SSS were transferred to individual wells of a chamber slide (Lab-Tek); 200 μl complete *L. stagnalis* haemolymph were added and after 1 h incubation, 100 μl supernatant were replaced by 100 μl fresh haemolymph. This step was done to enhance the continuous migration of haemocytes towards the parasite. Incubation times/Sytox green staining were as above; the experiments were performed twice independently. Finally, specimens were embedded in Vectashield (Vector Laboratories), examined using a Zeiss LSM880 laser scanning confocal microscope, and images analysed using FIJI Image J (Schindelin et al., 2012).

Haemocyte monolayers obtained from *R. lagotis* and *P. corneus* were incubated in SSS containing PMA (0.1, 1, 5, 10 μM), LPS (0.1, 1.0, 10.0 μg/ml), or heat-killed *S. saprophyticus* at ~100 bacteria/haemocyte.

3. Results and discussion

Initial experiments were performed with haemocytes of *M. edulis*, previously shown to produce ET-like fibers (Robb et al., 2014), to demonstrate fiber release in our laboratory. Similar to Robb et al. (2014), PMA clearly induced ET-like fiber release (Fig. 1A and B in Supplementary Materials) that was ETotic.

Next, snail haemocytes were exposed to PMA or LPS, compounds that were shown previously to stimulate effective NETs/ET-like fiber formation (von Köckritz-Blickwede and Nizet, 2009; Robb et al., 2014; Ng et al., 2013). Other components (e.g. L-fucose/D-galactose) were employed because they are linked to snail-trematode interactions (Plows et al., 2005).

The screening assays revealed that *L. stagnalis*, *R. lagotis* and *P. corneus* haemocytes produced only low numbers of extracellular DNA fibers (Table 1) and, therefore, other components associated with ET-like fibers such as histones (Ng et al., 2013; Robb et al., 2014) were impossible to investigate. However, given that occasional DNA fibers were observed in all species studied (Fig. 1) we define the fibers as 'ET-like' as in other invertebrates (Ng et al., 2013; Robb et al., 2014; Poirier et al., 2014; Lange et al., 2017).

That compounds such as PGN or PMA failed to elicit robust ET-like fiber production in *L. stagnalis* was surprising (Table 1). Similarly, PMA did not stimulate ET-like fiber formation by *C. gigas* haemocytes (Poirier et al., 2014). Exposure of haemocytes to 20 μM PMA in SSS or in modified SSS (SSS supplemented with D-trehalose (1 g/L), D-glucose (1 g/L) (Sigma-Aldrich) and antibiotics (penicillin/streptomycin; Lonza)), enabling longer-term *L. stagnalis* haemocyte survival for 48 h also did not evoke haemocyte ETotic responses (data not shown). On the other hand, *M. edulis* haemocytes produced fibers when exposed to 50 μM PMA for 48 h (Robb et al., 2014). In *R. lagotis*, haemocytes exposed to PMA produced only few ET-like fibers (Table 1, Fig. 1H). This finding was unexpected because PMA induces the respiratory burst in *R. lagotis* haemocytes (Skála et al., 2014), a reaction considered essential for ET-like fiber formation (Robb et al., 2014; Poirier et al., 2014).

Although LPS significantly induced NETs/ET-like fiber formation in mammalian neutrophils or shrimp haemocytes (von Köckritz-Blickwede and Nizet, 2009; Ng et al., 2013), only two ET-like fibers were produced by *L. stagnalis* haemocytes (Table 1, Fig. 1A). Additionally, no ET-like fibers were observed when these haemocytes were treated with 25 μg/ml LPS in modified SSS for 24 h, and the protocol of Brinkmann et al. (2010) was used to visualise the fibers (data not shown). With *P. corneus*, one ET-like fiber was observed when haemocytes were exposed to 10 μg/ml LPS in CBSS for 24 h (Fig. 1F) whereas nine fibers were observed in SSS (Table 1). Thus, these different culture media did not seem to largely influence the outcome with respect to ET-like fiber formation.

PMA and LPS activate protein kinase C (PKC) in *L. stagnalis* haemocytes (Walker and Plows, 2003; Wright et al., 2006), which stimulates NO production (Wright et al., 2006). Such responses might, at least in part, explain the inability of PMA and LPS to effectively promote ET-like fiber production. However, D-galactose and L-fucose attenuate PKC and extracellular-signal regulated kinase (ERK) activation in *L. stagnalis* haemocytes, with subsequent suppression of phagocytosis (Plows et al., 2005). These sugars are present on the surface of the helminth *T. regenti* (Blažová and Horák, 2005; Chanová et al., 2009), an incompatible parasite that