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Cell lines, Md108 and Md66, from the hemocytes of *Malacosoma disstria* (Lepidoptera) display aspects of plasma-free innate non-self activities

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ABSTRACT

The innate non-self response systems of the deciduous tree pest, the forest tent caterpillar, *Malacosoma disstria* has been documented by us in terms of *in vitro* and *in vivo* reactions towards the Gram-positive nonpathogenic bacterium, *Bacillus subtilis* and Gram-negative pathogenic microbe, *Xenorhabdus nematophila* and their respective surface antigens, lipopoteichoic acids (LTA) and lipopolysaccharides (LPS). These studies, often conducted in whole and diluted hemolymph, preclude examination of plasma-free cellular (hemocyte) responses. Plasma-free hemocytes as primary cultures are difficult to obtain. The floating cell line Md66 and attached cell line Md108 from *M. disstria* hemocytes were examined as a model for plasma-free *M. disstria* hemocyte non-self responses. Herein, it was established that although both lines differed from each other and from the primary hemocyte cultures of *M. disstria* in growth parameters, cell composition and sizes both cell lines displayed granular cell-like (GL) cells and plasmatocyte-like (PL) cells according to morphological criteria and to some extent antigenic similarities based on labeling with anti-*Chrysodeixis includens* hemocyte monoclonal antibodies. Hemocyte-specific neuroglian-like protein was detected on cells of both cell lines and in the primary hemocyte cultures albeit with staining patterns differing according to culture and cell types, confluency levels and cell–cell adhesion. Both cell lines bound *B. subtilis* and *X. nematophila*, the reaction extent varying with the cell line and its cell types. LPS damaged both cell types in the two cell lines whereas LTA enhanced the adhesion of Md66 GL cells to flask surfaces followed by PL cell adhesion. PL cells of both lines, like the primary cultures, phagocytosed FITC-labeled *B. subtilis*; only Md108 GL cells phagocytosed *B. subtilis*. In either case phagocytosis was always less in frequency and intensity than the primary cultures. Proteins released from the cell lines differed in pattern and magnitude but contained bacterial binding proteins that enhanced differential bacterial adhesion to both cell types in both cell lines: the GL cells both cultures, and those of granular cells in primary cultures, were more involved than the primary plasmatocytes and PL cells. Only Md66 cells possessed lysozyme and both cell types of both lines contained phenoloxidase. Neither enzyme type was released during early phase reaction with the bacteria. LPS inhibited phenoloxidase activity. The similarities and differences between the lines and primary cultures make Md66 and Md108 useful for the systematic examination of plasma-free cellular non-self reactions.

1. Introduction

Studies of the immune system of the forest tent caterpillar, *Malacosoma disstria*, a Northern American deciduous tree pest (Furniss and Carolin, 1977), are limited to *in vitro* and *in vivo* responses of hemocytic granular cells and ameboid and stellate plasmatocytes to glass and micro-organisms and their surface molecules predominantly in the presence of insect plasma (Dunphy et al., 2007; Giannoulis et al., 2005, 2007, 2008; Gulii et al., 2009; Stoltz and Guzo, 1986). Plasma factors participate in lepidopteran cell-free immunity (Rahmanet et al., 2006) altering *M. disstria* larval adhesive cellular hemocyte responses (Giannoulis et al., 2005, 2008; Gulii et al., 2009). Although lepidopteran hemocytes have plasma-independent immunogen recognition receptors including peptidoglycan recognition protein LC (Kurata, 2004), scavenger-like receptors (Costa et al., 2005) and lipoteichoic acid–lipopolysaccharide receptors (Ohta et al., 2006), plasma-free cellular immunity is rarely

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considered including for *M. disstria* hemocytes. Hemocyte cell lines devoid of insect plasma could address this issue.

Cell lines derived from larval *M. disstria* hemocytes are used as hosts for microsporidia (Wilson and Sohi, 1977), viruses (Keddie et al., 1995), karyological analysis (Ennis and Sohi, 1976) and hormone receptor physiology (Arnold and Sohi, 1974). Ecdysteroid hormones binding to the floating cell line, Md66, initiates cell–cell and cell–substratum adhesion. Md66 and Md108 although antigenically similar to whole larvae hemolymph (Krywienczyk and Sohi, 1973), exhibit differences in morphology and cell type composition compared with primary larval hemocytes (Arnold and Sohi, 1974). However, in view of the ecdysteroid study and the importance of non-self activities to insect survival, it is possible these cell lines retain aspects of immunocyte attributes including hemocyte–microbe adhesion and responses to bacterial surface antigens as we reported previously for primary hemocyte cultures.

Herein, cell lines Md66 and Md108 are characterized in terms of morphology and antigenicity, the latter using *Chrysodeixis* (*Pseudoplusia*) *includens* monoclonal larval hemocyte antibodies (Gardiner and Strand, 1999) and antibodies to the *Manduca sexta* hemocyte proteins lacunin (Nardi et al., 2001) and neuroglian (Nardi et al., 2006). Since the independently produced Md66 and Md108 cell lines retained aspects of primary hemocyte antigenicity their physical and biochemical interactions with bacteria are defined. Despite differences between the cell lines, many of these interactions compare favorably in terms of patterns, but not always in magnitude, of previously published results for primary monolayers of *M. disstria* hemocytes. Phagocytosis of bacteria by primary hemocytes and Md66 and Md108 cell types is documented for the first time. Differences in hemocytic attributes between Md66 and Md108 will provide useful tools for exploring self–non-self responses.

2. Materials and methods

2.1. Bacterial cultures

Nonpathogenic *Bacillus subtilis* (Boreal Biological Company) was subcultured on tryptic soy agar. Entomopathogenic *Xenorhabdus nematophila* in the phase one form, which infects insects (Akhurst, 1980), was cultured on tryptic soy agar supplemented with triphenyl tetrazolium chloride (30 mg/l) and bromothymol blue (25 mg/l). Both species incubated at 25 °C in darkness were subcultured every two weeks.

Test bacteria, grown in tryptic soy broth (5 ml) in scintillation vials (20 ml) on a gyratory shaker (250 rpm, 25 °C) until achieving an optical density of 0.75 at 660 nm, were killed to preclude ongoing bacterial metabolism influencing primary hemocytes and various cell types from the established cultures Md66 and Md108 from influencing insect cell–bacterial interaction (Alavo and Dunphy, 2004). Killing involved UV-irradiation (203 nm, 3 h) after which bacteria were washed by centrifugation (12,000*g*, 2 min, 25 °C) and resuspension in phosphate-buffered saline (PBS, pH 6.5, 1 ml; Alavo and Dunphy, 2004). Bacterial death was based on the absence of bacterial colonies from aliquots (100 μl) of suspension plated on tryptic soy agar. Samples were incubated at 25 °C and 35 °C for 96 h to detect any UV-generated mutants capable of growth at different temperatures.

2.2. Insect cell lines and insects

Stock cultures of *M. disstria* cell lines, Md66 and Md108 (provided by Forestry Canada, Sault Ste. Marie, Ontario), were maintained at 21 °C in modified Grace's insect tissue culture medium (5 ml) in polystyrene tissue culture flasks (25 cm^2) in darkness. The lines were from the same geographical source as larvae used in previous innate cellular non-self publications (Giannoulis et al., 2005, 2007, 2008; Gulii et al., 2009). Md108 and Md66 were used because their cell composition differs and Md108, unlike Md66, grows attached to flasks (Arnold and Sohi, 1974). Culture medium consisted of filter-sterilized (0.22 μm) Grace's medium (Gibco, Ontario) supplemented with heat-inactivated (56 °C, 30 min) fetal calf serum (8% v/v, Gibco) and tryptose broth (2.5 g/l medium, Difco) at pH 6.5. Both Md66 and Md108 cells were subcultured at 80–90% confluency (Ennis and Sohi, 1976).

For experimental purposes cell lines were grown at their optimal temperature, 28 °C and characterized by exoenzyme production at 50% confluency (Giannoulis et al., 2008) ensuring culture quality. Unless stated otherwise both cell lines were used at 50% confluency in all experiments.

Laboratory-reared *M. disstria* larvae (supplied by Forestry Canada) were maintained on a casein dextrose diet at 25 °C (Addy, 1969). Fourth instar larvae, as used in our previous publications, were herein used to compare larval hemocyte and cell line antigenicity.

2.3. Morphological and antigenic characterization of the cell lines

Morphologically, cells in both cell lines were compared with primary hemocyte monolayers (Giannoulis et al., 2005) using a phase contrast tissue culture microscope. Cells in both cell lines and primary cultures were analyzed according to Price and Ratcliffe (1974).

The antigenicity of both cell lines and primary hemocyte monolayers were compared using anti-*C. includens* hemocyte monoclonal antibodies (Gardiner and Strand, 1999) and anti-neuroglian and anti-lacunin *M. sexta* monoclonal antibodies (Nardi et al., 2001, 2006). The former group of antibodies was chosen based on the similarities of the innate cellular and humoral responses between *C. includens* and *M. disstria* (Beckage, 1998; Giannoulis et al., 2005, 2007, 2008; Strand, 1994; Stoltz and Guzo, 1986). The latter antibody group was selected because these hemocyte-specific epitopes occur on hemocytes from Diptera and Lepidoptera (Nardi, 2004; Zhuang et al., 2007).

Briefly, Md66 cells (500 μl culture) were gravity-sedimented in microcentrifuge tubes (40 min, 4 °C) and resuspended in *Galleria mellonella* anticoagulant buffer II (200 μl, Mead et al., 1986; henceforth referred to as anticoagulant buffer). After sedimentation (30 min, 4 °C) the cells were resuspended and fixed in anticoagulant containing formaldehyde (8% v/v anticoagulant buffer, 200 μl, 20 min, 4 °C). Between subsequent steps, the cells were centrifuge-washed (750 g, 4 min, 20 °C) in PBS (200 μl) three times. These cells, blocked with bovine serum albumin [BSA (3% w/v PBS), or where appropriate, permeabilized with 0.2% Triton-X-100 while blocked in BSA–PBS buffer)] for 1 h, were labeled with 200 μl of primary antibody (the anti-neuroglian and anti-lacunin antibodies being diluted 1:1000 in PBS; all other antibodies were diluted 1:2 in PBS) for 2 h (150 rpm on a horizontal gyratory shaker, 20 °C) or overnight (4 °C). Cells were then probed with secondary goat anti-mouse IgG conjugated to Alexafluor 488 (1:100 in PBS, 200 μl, Molecular Probes) for 1 h with shaking (150 rpm, 20 °C). After final washing the labeled cells were suspended in PBS (20 μl) and Immumount (5 μl), placed on slides and allowed to settle prior to microscopy.

Md108 (200 μl), detached from flasks by trypsinization (Ennis and Sohi, 1976), were added to polystyrene cover slips (previously disinfected in 70% ethanol, 1 h) in 24-well tissue culture plates. Cells were incubated overnight (28 °C) to allow cell–substratum adhesion after which culture medium (500 μl) was added and the cells incubated for 72 h at 28 °C to allow recovery. Cells were chilled (30 min, 4 °C) prior to medium removal, and subsequently washed (500 μl anticoagulant buffer, 4 min on a horizontal gyra-