Developmental and Comparative Immunology 45 (2014) 10–20

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

# A continuous cell line, SYSU-OfHe-C, from hemocytes of *Ostrinia furnacalis* possesses immune ability depending on the presence of larval plasma

Jian Hu *, Xiangping Feng, Zhongguo Yang, Zhuoxin Chen, Wenqing Zhang

*State Key Laboratory of Biocontrol, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China*

ARTICLE INFO

*Article history:*
Received 10 November 2013
Revised 29 January 2014
Accepted 31 January 2014
Available online 7 February 2014

*Keywords:*
Cell line
Hemocyte
Immune
Encapsulation
Spreading
Lepidoptera

ABSTRACT

A continuous cell line, SYSU-OfHe-C, from larval hemocytes of corn borer, *Ostrinia furnacalis* was established. With increasing passages, the cells grew increasingly faster, and approximately 45% of the cells were in division at passage 55. The culture was mainly composed of two types of cells, granulocytes and plasmatocytes, which showed different division and proliferation behaviors, but possessed similar phagocytic ability. Its spreading ability was significantly weaker than that of hemocytes from naïve larva; however, it could be promoted by larval plasma. Furthermore, its encapsulation ability was also promoted by larval plasma to form multilayer capsules on Sephadex A-25 beads. Finally, the expression of several immune-related genes was verified after provocation by microbes or Sephadex beads. These results indicated that the cell line possessed immune ability depending on the presence of plasma of naïve larvae and are beneficial to studies of insect cellular systems.



## 1. Introduction

Insect resist attack of foreign objects such as microbes by humoral and cellular immune reactions. Cellular immune reactions are hemocytic behaviors that clear foreign objects such as phagocytosis, nodulation and encapsulation; however, how they are regulated is less understood compared to the knowledge about humoral immune reactions. Generally, there are five types of hemocytes, prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids in Lepidoptera (Hu et al., 2003; Lavine and Strand, 2002; Ribeiro and Brehelin, 2006), but only granulocytes and plasmatocytes have been shown to participate in immune reactions, and they play different roles. For example, granulocytes form the first layer of capsules on beads and closure encapsulation, while plasmatocytes constitute the main body of capsules in the encapsulation of *Pseudoplusia includens* hemocytes (Pech and Strand, 1996; Schmit and Ratcliffe, 1977). Until now, the separation of immune hemocytes has been difficult in most Lepidoptera, although it was successful in a few species such as *P. includens*, in which granulocytes and plasmatocytes were successfully separated by a Percoll gradient (Pech et al., 1994). First, the small size of insect larvae restricts the quantity of hemocytes that can be collected from them. Secondly, hemocytes coagulate when they are collected in vitro under the effect of plasma factors. An anticoagulant buffer may inhibit the coagulation of cells to some extent, but it affects the immune behavior of hemocytes in further analysis, which deeply limits the study of the molecular mechanisms of cellular immune reactions. Furthermore, some plasma factors participate in cellular immune reactions and play very important roles in modulating the behavior of hemocytes. Their identification and separation are also not easy, because hemocyte behavior is affected by residual traces of plasma, so there is no effective method that can be used to assess which part of plasma affects hemocytes. However, some insect cell lines from non-hemocytic tissues were used to study the molecular mechanisms of insect immunity with good results. For example, an embryonic cell line, NISESBoMo-Cam1, was used as an in vitro model of immune system in the silkworm (Taniai et al., 2006). In addition, a cell line with granulocytic characteristics were established with embryos of *Chrysodeixis includens* and was thought to has several valuable attributes for studying immune interactions with polydnavirus and potentially other pathogens (Johnson et al., 2010). A sustained and stable cell line from insect hemocytes would be suitable for further study of immune mechanisms of hemocytes. Compared to lepidopteran larvae, cell lines can provide a large quantity of uniform cells in a short time and are not easily affected by plasma factors such as those promoting adhesion or phenoloxidase. At the same time, a cell line is suitable for identifying and separating

* Corresponding author. Tel.: +86 20 39332962; fax: +86 20 39943515.
*E-mail address:* lsshj@mail.sysu.edu.cn (J. Hu).

http://dx.doi.org/10.1016/j.dci.2014.01.025
0145-305X/

factors that modulate hemocytic immune behavior in the absence of larvae plasma in the culture medium.

In 1962, the first sustained insect cell line was established, and there were already 260 cell lines in Lepidoptera in 2007 (Lynn, 2007). Most of these cell lines come from the embryos, reproductive organs and fat body of insects, among others. There were only four established hemocytic cell lines in Lepidoptera until now, and they originated from hemocytes of *Helicoverpa zea* (Miltenburger et al., 1984), *Mamestra brassicae* (Lehman and Weilepp, 1989), *Spodoptera exigua* (Chisa et al., 2004), and *Ostrinia nubilal* (Belloncik et al., 2007). Although the phagocytic ability of the cells was analyzed in *S. exigua*, all others were devoted to improving viral production levels and/or viral stability. Overall, no hemocytic cell line aimed to study the insect immune mechanism has been established until now.

The corn borer *O. furnacalis* is a serious agricultural pest distributed worldwide and the immune relationship between it and it's natural enemy *Macrocentru cingulum*, a polyembryonic developmental parasitoid have been studied for several years (Hu et al., 2008, 2010, 2003; Xu et al., 2012). Until now, no cell line was established in *O. furnacalis*. In its relative *O. nubilalis*, cell lines from embryos (Trisyono et al., 2000), fat bodies (Goodman et al., 2001) and hemocytes (Belloncik et al., 2007) have been established, but no immune-related reactions were analyzed in the hemocytic cell line AFKM-On-H. In this study, a continuous hemocytic cell line SYSU-OfHe-C from *O. furnacalis* was established, and it possesses immune abilities depending on the presence of plasma of larvae, so it should be a good material to study the mechanisms of the insect cellular immune system.

## 2. Materials and methods

### 2.1. Establishment and maintenance of the cell line, SYSU-OfHe-C

*Ostrinia furnacalis* were reared in lab with artificial food as previously described (Hu et al., 2003). Larvae at the late stage of the 4th instars, the first day and the second day of 5th instars were sterilized with 75% alcohol for three times before bleeding. Hemolymph from one larva was dropped directly into the center of a 35 mm culture plate (Corning, Tewksbury, MA, USA) with 2 ml of Ex-Cell 420 medium (Sigma–Aldrich, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA) by cutting off the middle legs with scissors at super clean bench. Culture plates were then sealed with Parafilm® (BEMIS, Neenah, WI, USA) and cultured in incubator (SHEL LAB, Cornelius, OR, USA) at 27 °C. Nine plates of hemocytes from each larva at each developmental stage were cultured. Hemocytes were observed under inverted microscope (Olympus X71, Olympus, Tokyo, Japan) twice a day. If melanization occurred, the plates were discarded. When obvious hemocyte proliferation was observed and the cells in the center part formed were more than one layer, the hemocytes were transferred to a 25 $cm^2$ flask (Nalge Nunc International, Penfield, NY, USA) with medium as above for further culture. Cells were classified into two groups: floating cells and strongly attached cells. After gently shaking, floating cells containing in 1.2–1.8 ml medium were transferred to a new 25 $cm^2$ flask (3.6 ml medium in all) for further culture every four to six days. Trypsin (0.4%) (Sigma–Aldrich) was used to detach attached cells, which were transferred to a new flask.

At passage 56, a single or several cell(s) with the same shape under a phase contrast microscope were picked out using a glass caterpillar and placed into a well of a 96-cell culture plate (Corning, Tewksbury, MA, USA) with Ex-Cell 420 medium with 10% FBS and 10%, 20%, 30% or 40% culture medium of cells in the logarithmic growth phase to build some cell strains.

Aliquots were stored in liquid nitrogen in FBS with 10% DMSO (Sigma–Aldrich) for further use.

### 2.2. Cell morphology analysis of SYSU-OfHe-C

Cells were seeded in 25 $cm^2$ flask at $1 \times 10^4$ /ml and cultured overnight, and then they were washed with PBS three times, fixed in 4% paraformaldehyde in 0.1 M PBS for 40 min, treated with 0.3% Triton X-100 for 5 min, blocked with 1% BSA in PBS for 1 h, and stained with Alexa Fluor® 532 phalloidin (Invitrogen, Carlsbad, CA, USA), which was specifically conjugated with F-actin, the main structure of cytoskeleton, for 2 h in the dark. Finally, cells were washed with PBS 3 times and stained with Hoechst 33342 (10 μg/ml in PBS, Invitrogen) for 10 min at room temperature in the dark. Cells were observed and imaged under phase contrast and fluorescence inverted microscope (Olympus X71, Tokyo, Japan). To follow the cell division, cells were seeded in a 25 $cm^2$ flask at $1 \times 10^3$ /ml and cultured under an inverted microscope (Olympus X71) at 22 °C. Photos were taken every 20 min for 48 h. Finally, movies (.avi) were made from the c photos taken.

### 2.3. Ultra microstructure of SYSU-OfHe-C

Cells from SYSU-OfHe-C-G5 or SYSU-OfHe-C-D5 or Sephadex A-25 beads covered by one layer or more than one layer of hemocytes were collected separately into 1.5 ml aseptic eppendorf tubes and centrifuged at 5000 rpm/min for 10 min on a horizontal centrifuge (ROTOFIX 32 A, Hettich, Kirchlengern, Germany). The pellets were washed three times with PBS and fixed in 1 ml fixation buffer (2% glutaraldehyde (w/v)) at 4 °C for 24–48 h and then post-fixed in 5 ml washing buffer containing 1% (w/v) $OsO_4$ at 4 °C for 2 h. Samples were dehydrated in a series of acetone, immersed in a mixture of pure acetone and embedding medium (Epon 812, Sigma–Aldrich) at a ratio of 1:1 at 37 °C for 30 min and then kept in pure embedding medium for 2 h, and finally 60 °C for 48 h. Extremely thin sections (60–80 nm) were cut on a microtome (Leitz, Oberkochen, Germany). Sections were transferred onto copper grids and stained with lead-citrate, then washed once in 0.02 mol/ml sodium hydroxide solution and three times in distilled water. Finally, sections were observed under an electron microscope (EM-902, ZEISS, Oberkochen, Germany).

### 2.4. Cell growth and proliferation analysis of SYSU-OfHe-C

The growth curve of the cell line was measured at the 37th passages. Three bottles of cells (25 $cm^2$ flask) at $1 \times 10^5$ cells /ml were seeded and the cell density were measured every 48 h for 12 days with a hemocytometer and Trypan blue staining (Invitrogen, Grand island, NY, USA). For each flask, three independent samples were counted. The tests were replicated three times. Finally, the population doubling time during logarithmic growth was calculated using the formula: $DT = \Delta t \times Lg^2/(Lg^{Nt} - Lg^{N0})$ ($t$: time for culture; $N_0$: initial cell numbers; $N_t$: cell numbers after culture).

The rates of cells proliferation at passage 19 and 55 were measured with an EdU Dying Kit (RIBO BIO, Guangzhou, China) following the manufacturer's protocol. Approximately $1 \times 10^5$ cells in logarithmic growth phase at passage 19 and 55 were seeded in 6-cells plates and then dyed with 50 μm EdU for 4.5 h. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min. 1× Apollo reaction buffer was added followed by incubation in the dark for 30 min, and then cells were washed with PBS with 0.5% TritonX-100 three times and methanol for 5 min twice. Finally, cells were dyed with 1× Hoechst 33342 in the dark for 30 min, washed with PBS for three times and resuspended in 800 μl PBS. Dyed cells (100 μl) were observed under an inverted fluorescence microscope (Olympus X 71) and 700 μl of cells were