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Effects of infection of EGFP-expressing *Escherichia coli* on haemocytes in *Ciona intestinalis*

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Abstract

The effects of infection of EGFP-expressing *Escherichia coli* on the haemocytes of the ascidian *Ciona intestinalis* were investigated. The results showed that THC of the infected individuals changed significantly. Hyaline amoebocytes phagocytosed *E. coli* in 5 min and excreted lysosome particles that attached to the surface of the bacteria. Granular amoebocytes released lots of particles for humoral immunity while stem-cell-like haemocytes remained intact. With the increase in THC, the stem-cell-like haemocytes showed division and proliferation. A small portion of hyaline amoebocytes was at early apoptosis stage 1 h after infection and typical apoptosis bodies emerged in granular amoebocytes. A few of the infected haemocytes showed DNA damage using SCGE assay. Flow cytometry analysis revealed an obvious apoptosis peak in infected haemocytes. In conclusion, apoptosis was found to be an important immune response of ascidian haemocytes response to bacterial infection. To our best knowledge, this is the first report of the occurrence of apoptosis of haemocytes in ascidians.

Keywords: Apoptosis; Cellular response; *Ciona intestinalis*; EGFP-expressing *E. coli*; Haemocytes; Infection

1. Introduction

Ciona intestinalis, which is the representative species of the subphylum Urochordata, belongs to a critical taxonomic position between invertebrates and vertebrates and is considered to be the most primitive members of the phylum Chordata (Dehal et al., 2002; Satoh, 2003). In the last decades, researchers showed a surge of interest in ascidians because of their critical evolutionary position and representative early developmental mode (Satoh and Jeffery, 1995; Simmen et al., 1995; Di Gregorio and Levine, 1998; Corbo et al., 2001; Dehal et al., 2002; Nishida, 2002). At present, the whole genome of *C. intestinalis* has been sequenced, analyzed and annotated (Dehal et al., 2002; Satoh, 2003); hundreds of thousands of ESTs were disclosed; a great number of development involved genes were cloned and

Abbreviations: EGFP, enhanced green fluorescence protein; TUNEL, terminal-deoxynucleotidyl transferase mediated nick end labeling; THC, total haemocytes count; SCGE, single cell gel electrophoresis.

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hundreds of gene expression patterns were published (Nishikata et al., 2001; Satou, 2001; Inaba et al., 2002; Kusakabe et al., 2002; Satou et al., 2002; Fujiwara et al., 2002; Shida et al., 2003; Satoh, 2003). The ascidians were regarded as a "biology's rising star" (Pennisi, 2002), and was also considered as one of the best experimental system for the study of evolutionary and comparative immunology in invertebrates (Nomaguchi et al., 1996; Du Pasquier, 2004; Kasahara et al., 2004) because the animals are semi-transparent, embryogenesis is rapid, so that most of the inner organs can be easily distinguished and thus facilitating mutagenesis and genetic screens (Sordino et al., 2001); they have relatively small genome size and compact gene distribution (Dehal et al., 2002), have rapid growth and short lifespan (Nomaguchi et al., 1996) and the draft genome and ESTs are now free for scientific use (Satoh, 2003).

In the last few decades, interest in invertebrate immune responses has led to a great increase in the scientific production dealing with mutual relationships between haemocytes and non-self materials (Simth and Söderhäll, 1983; Smith and Söderhäll, 1983; Söderhäll et al., 1986; Person et al., 1987; Lee and Söderhäll, 2002; Arala-Chaves and Sequeira, 2000). In the ascidians *C. intestinalis*, relatively few experimental results are available on the haemocytes immune responses (Shida et al., 2003; Di Bella and De Leo, 2000); nevertheless there are bioinformatic results that reveal some specific immune characteristics (Dehal et al., 2002; Azumi et al., 2003; Fujita et al., 2004; Du Pasquier, 2004; Kasahara et al., 2004). In this study, EGFP-expressing *Escherichia coli* was used as infectious material to study the cellular responses of the haemocytes of *C. intestinalis*.

2. Materials and methods

2.1. Animals

Adult ascidians were collected from the Jiao Zhou Bay, the Chinese Yellow Sea. They were maintained in filtered and aerated seawater at 18 °C in the laboratory and were fed with the diet combination of dry *spirulina*, egg yolk, *Dicrateria* sp. (Liu et al., unpublished data). They were acclimated at least 1 week prior to use. Only apparently healthy adult animals were used.

2.2. Transformation of the plasmid pEGFP into E. coli

Transformation of pEGFP into *E. coli* was performed using standard techniques (Sambrook and Russell, 2001). In brief, plasmid DNA of pEGFP was transformed into the competent TOP10, dam-host strain of *E. coli,* and then selected a positive clone by PCR, using the EGFP primers GP1: 5′-ATGGTGAGCAAGGGCGAGGAG-3′; GP2: 5′-TTACTTGTACAGCTCGTCCATG-3′. The reaction was carried out at 94 °C for 40 s, 57 °C for 45 s, 72 °C for 1 min, and 35 cycles with a 3-min initial 94 °C denaturation step, and a 10-min 72 °C final elongation step. The positive clone was cultured in LB broth with 100 mg/l ampicillin for 6 h and the cells were collected. By these means, the bacteria could expression EGFP, which was confirmed by simultaneously observation under fluorescence and phase contrast field microscope (Zeiss, Germany). Finally EGFP expressing *E. coli* was cured from the plasmid by repeated subculture to antibiotic-free media until disappearance of fluorescence and loss of resistance to ampicillin.

2.3. Injection of bacterial suspension into ascidians

The bacteria *E. coli* were washed twice using sterile artificial seawater (ASW, 400 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 10 mM Hepes, pH 8.0) and adjusted to a final density of 1–3×10^7 ml^{-1}. To make sure that the bacterial suspension was successfully injected into the body of the ascidians, 0.1% phenol red in Tris–HCl buffer was mixed into the bacterial suspension as indicator at a ratio of 1:3. Totally 50 μl of mixture, namely about 1×10^6 bacteria, was injected into every animal. The controls were injected the same mixture without bacteria. After injection, the ascidians were maintained singly in 200 ml of filtered and sterile seawater. The successful challenge was made if the red bacterial fluid could be seen to spread quickly and stay in the body for not less than 30 min instead of being discharged from the out-current and in-current siphons of the injected ascidians.

2.4. Haemocytes sampling

When sampling, the tunica of ascidian was discarded and the heart was exposed and punctured. Haemolymph were collected with a 6-ml sterile syringe containing sterile anticoagulant (sodium citrate 27 mM, NaCl 336 mM, glucose 115 mM, $EDTA\cdot Na_2$ 9 mM; Rodriguez et al., 1995) and filtered through polyamide gauze with a mesh width of 40 μm. After centrifugation at 500×*g* for 5 min at 4 °C, the supernatant was removed and the haemocytes were washed three times in sterile anticoagulant.