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Non-specific immune response of bullfrog *Rana catesbeiana* to intraperitoneal injection of bacterium *Aeromonas hydrophila**

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Abstract Non-specific immune response of bullfrog *Rana catesbeiana* to pathogenic *Aeromonas hydrophila* was studied to 60 individuals in two groups. Each bullfrog in bacterium-injected group was injected intraperitoneally (i.p.) with 0.2 ml bacterial suspension at a density of 5.2×10^6 CFU/ml, while each one in control group injected i.p. with 0.2 ml sterile saline solution (0.85%, w/v). Three bullfrogs in both groups were sampled at 0, 1, 3, 7, 11, 15 and 20 days post-injection (dpi) for the evaluation of non-specific immune parameters. It was observed that intraperitoneal injection of *A. hydrophila* significantly increased the number of leucocytes and that of NBT-positive cells in peripheral blood. Significant increases in serum bactericidal activity and serum acid phosphatase activity were also observed in the bacterium-injected frogs when compared with those in the control group. However, a significant reduction was detected *in vitro* in phagocytosis activity of peripheral blood phagocytes. No significant difference in changes in the number of peripheral erythrocytes, serum superoxide dismutase (SOD) activity, and lysozyme activity was detected between the two groups. It is suggested that bullfrogs may produce a series of non-specific immune reactions in response to the *A. hydrophila* infection.

Keyword: *Rana catesbeiana*; *Aeromonas hydrophila*; intraperitoneal injection; non-specific immune response

1 INTRODUCTION

Bullfrog (*Rana catesbeiana* Shaw), a kind of frog that extensively used as food, is remarkable for its large size, fast growth, and high economic value. Bullfrog is one of the main aquaculture animals in China (Liu et al., 1989). The crucial factor that affects bullfrog cultures is the incidence of microbial pathologies (Shu et al., 1997; Chen et al., 1999). *Aeromonas hydrophila* has been commonly associated with the epidemic, which leads to mass mortality of farmed bullfrog and causes considerable losses (Hu and Hong, 2000). *A. hydrophila*, a ubiquitous organism in freshwater and in natural flora of frogs and many other animals (Hird et al., 1983), is one of the most important opportunistic pathogens in fresh water culture (Chen and Lu, 1991; Hu and Hong, 2000). Antibiotic is the current chief means to control pathogenic *A. hydrophila*. This method has become obsolete because the extensive use of antibiotics had led to an increase in plasmid-encoded antibiotic resistance and a disorder of normal flora (Ansary et al., 1992).

Nowadays, more and more attention has been paid to the safety of food. How to control the epidemic of aquatic animals by nuisance-free method has caught public attention. An alternative is the development and use of vaccine or other immune enhancing agents. Some investigations in the prevention and cure of bullfrog disease caused by *A. hydrophila* have conducted according to the immunological theory, and some valuable results have been obtained (Crumlish and Inglis, 1999; Hu

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and Hong, 2001). However, the foundational research on bullfrog immune system is still rather unsubstantial, only a few reports about antibody and cutaneous antimicrobial peptides are available (Marchalonis and Edelman, 1966; Du Pasquier, 1982; Simmaco et al., 1998). Little is known about the non-specific immune response of bullfrog to pathogenic bacteria infection. Meanwhile, several immunity parameters such as haematocytes number, phagocytosis activity, NBT-positive cells number, bactericidal activity, acid phosphatase (ACP) activity, lysozyme activity, and superoxide dismutase (SOD) activity have been introduced into the evaluation on the non-specific immune response by some aquatic animals to bacterial infection (Secombes, 1986; Park and Wakabayashi, 1992; Moyner et al., 1993; Anderson et al., 1992; Yildiz, 1998; Benli and Yavuzcan, 2004).

It is the aim of this study to understand the innate immunity of bullfrog against the infection caused by injecting *A. hydrophila* and sterile saline and measuring several non-specific immune parameters after injection.

2 MATERIALS AND METHODS

2.1 Experimental bullfrog

Sixty healthy bullfrogs of 250–350 g were obtained from a local commercial bullfrog farm. They were divided equally into six 45 cm×50 cm×75 cm aquariums supplied with well-aerated, dechlorinated water at 24–26°C, and fed daily with commercial food pellets, which were kindly donated by Xiamen Fuxing Feed Company, throughout the experimental period. Before trial, the bullfrogs were acclimatized to laboratory conditions for a week.

2.2 Bacteria and growth condition

A. hydrophila (1.927), *Micrococcus lysodeikticus* (1.634) and *Staphylococcus aureus* (1.879) were obtained from the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China) and stored at –80°C.

A. hydrophila was inoculated on nutrient agar. After overnight growth at 37°C, bacterial cells were harvested and re-suspended in sterile saline solution. The final density of bacterial suspension for challenge was adjusted to 5.2×10^6 CFU/ml, which was determined by colony counting.

2.3 Injection and sampling

Bullfrogs were categorized into bacterium-injected and control groups, each group containing 30 frogs equally in three aquariums. Each frog in bacterium-injected group was injected intraperitoneal (i.p.) with 0.2 ml bacterial suspension, while each frog in control group injected i.p. with 0.2 ml sterile saline solution.

Heparin solution (500 u/ml) was prepared by dissolving 0.1 g heparin into 28 ml sterile saline solution. 0.1 ml heparin solution was sucked into a new syringe (1 ml) to prepare heparinized syringe. Three bullfrogs, randomly took from each group, were sacrificed at 0, 1, 3, 7, 11, 15 and 20 dpi (days post injection). Blood samples were drawn twice from the heart of each frog with a heparinized syringe for 1 ml and an unheparinized syringe（5 ml）for 4 ml, respectively. Heparinized blood was used for haematocytes counting and phagocytosis activity evaluation and the unheparinized blood was allowed to clot for 1 h at room temperature and then placed at 4°C overnight to prepare serum for lysozyme, SOD, ACP, and antibacterial activities assaying.

2.4 Haematocytes counting

Leukocytes and erythrocytes were counted according to the method described by Zhao (1992) with a modification. Briefly, 20 μl heparinized blood was diluted and stained with 280 μl blood diluent (0.88 g sodium chloride, 0.3 mg neutral red, 1.5 mg crystal violet, 0.4 ml formalin in 100 ml distilled water) in a sterile Eppendorf tube. Leukocytes and erythrocytes were counted with a haemocytometer under light microscope (10×40 magnification). Each sample was examined in triplicate.

2.5 Assay of phagocytosis activity

Phagocytosis activity was evaluated by AO staining method according to Pan (2000) with a slight modification. Briefly, *S. aureus* suspension (6.5×10^7 cells/ml) was inactivated in water bathing at 100°C for 10 min. 100 μl suspension was homogenized with 200 μl heparinized blood in a sterile Eppendorf tube and incubated with a gentle shaking every 10 min in water bathing for 30 min at 30°C. 1 ml saline solution was added to the tube and the mixture was centrifuged at 1 500 r/min for 5 min, then 1 ml supernatant was discarded and the rest was homogenized. A drop of mixture was transferred onto a microscope slide and flattened. The cells were fixed with methanol for 2–3 min after drying, washed with distilled water, and dried.

Then the cells were stained with 0.28% Acridine Orange (AO, Amresco) in saline solution for 2 min, washed with distilled water, and observed under an oil immersion fluorescence microscope (*Leica*). Total phagocytes, phagocytes engulfing bacteria, and the bacteria being engulfed were counted separately. The percentage of phagocytosis (PP) and phagocytic index (PI) were calculated according to the following formulas.

$$PP=N_1/100\times100;$$

$$PI=N_2/100.$$

N_1: the total number of phagocytes engulfing bacteria among random 100 phagocytes;

N_2: the total number of bacteria being engulfed in random 100 phagocytes.

2.6 Count of NBT-positive cells

NBT-positive cells were counted according to the method described by Anderson (1992) with a slight modification. Briefly, a store solution (0.2 %, w/v) of NBT (BBI) was freshly prepared by dissolving 0.2 g NBT into 100 ml sterile saline solution and then mixed with equal volume of glucose solution (1 mg/ml) in sterile phosphate buffered saline (PBS, 0.15 mol/L, pH 7.2) to prepare an application solution. 200 μl heparinized blood was homogenized with 200 μl NBT application solution in a glass tube and incubated with a gentle shaking every 10 min in water bathing for 30 min at 30°C. A drop of the mixture was transferred into a haemocytometer and the number of NBT-positive, dark blue staining cells in the large square grid were counted under a light microscope. Each blood sample was examined in triplicate.

2.7 Assay of antibacterial activity

Antibacterial activity to *A. hydrophila* was determined according to Yan et al. (2001) with a slight modification. Briefly, *A. hydrophila* was harvested in sterile phosphate buffered saline (PBS, 0.1 mol/L, pH 6.4) after overnight growing. 3 ml bacterial suspension ($OD_{570}\approx0.3$) was homogenized with 50 μl fresh serum in tube. The primary photodensity (A_0) of the mixture was measured at 570 nm immediately. After incubating in water bathing at 37°C for 30 min, the secondary photodensity (A) was measured. Serum antibacterial activity (U_a) was calculated according to the following formula.

$$U_a= [(A_0-A)/A_0]^{1/2}$$

2.8 Assay of lysozyme activity

Lysozyme activity was determined following the method described by Yan et al. (2001) with a slight modification. Briefly, overnight grown *M. lysodeikticus* was harvested and suspended in sterile phosphate buffered saline (PBS, 1/15 mol/L, pH 6.4). 3 ml bacterial suspension ($OD_{570}\approx0.3$) was homogenized with 50 μl fresh serum in tube. The primary photodensities (A_0 for the mixture of bacterial suspension and serum, A_1 for the mixture of sterile PBS and serum, which was used as blank control) were measured at 570 nm immediately. After incubation in water bathing at 37°C for 30 min, and the secondary photodensities (A for the mixture of bacteria and serum, A_2 for the mixture of sterile PBS and serum) were measured. Serum lysozyme activity (U_L) was calculated according to the following formula:

$$U_L= [(A_0-A) + (A_2-A_1)]/A_0.$$

2.9 Assay of acid phosphatase (ACP) activity

ACP activity was determined with an ACP reagent kit (Nanjing Jiancheng Bioengineering Institute) according to instructions of the manufacturer. The activity unit of ACP was defined as 1 mg hydroxybenzene produced by 100 ml serum acting on substrate at 37°C for 30 min.

2.10 Assay of superoxide dismutase (SOD) activity

SOD activity was determined with a SOD reagent kit (Nanjing Jiancheng Bioengineering Institute) according to instructions of the manufacturer with some modification to fit the 96-wells plate. The photodensity of each well was read at 550 nm by using a microplate reader. The activity unit of SOD was defined as the quantity of SOD when the depression rate of SOD was up to 50 % in 1 ml reactant.

2.11 Statistical analysis

All values are expressed as means ± SD. Student's *T*-test was used to determine the difference at each sampling time. One-way ANOVA was introduced to analyze the trend in the changes of the immune parameters. Differences were considered significant when $p<0.05$.

3 RESULTS

The effects of *A. hydrophila* injection on several non-specific cellular immune parameters are shown in Fig.1. Bullfrogs in two groups exhibited different trends in the change of leukocytes. The numbers of leucocytes in peripheral blood of bacterium-injected bullfrogs at 1, 7 dpi were observed to be significantly higher than those in the control group (P<0.05), and then leveled off at 11, 15, 20 dpi. At 3 dpi, a sharp decrease in the number of leucocytes in bacteria-injected bullfrogs was observed compared with those at 1, 7 dpi (P<0.05) (Fig.1a). The numbers of erythrocyte did not change greatly after injection. No significant difference in the number of erythrocyte in peripheral blood was detected between the bullfrogs from bacterium-injected group and control group at each sampling time (Fig.1b). After injection, bullfrogs from both groups showed a trend of gradual reduction in the percentages of phagocytosis of peripheral blood. A significant reduction (P<0.05) in the bacterium-injected bullfrogs was observed at 1, 3, 7 dpi compared with those in the control group. The percentages of phagocytosis in the two groups leveled off at 12, 15, 20 dpi (Fig.1c). In both groups, the values of phagocytic index at the initial stage (0, 1, 3 dpi) were higher than those at the anaphase. Though no significant difference was detected between the two groups, the values of bacterium-injected bullfrogs were found lower than those of control group (Fig.1d). The numbers of NBT-positive cells of bacterium-injected bullfrogs increased at the initial stage, and then fell at the anaphase. Significantly higher values were observed at 1, 3, 7 dpi in bacteria-injected bullfrogs than those of control (P<0.05).

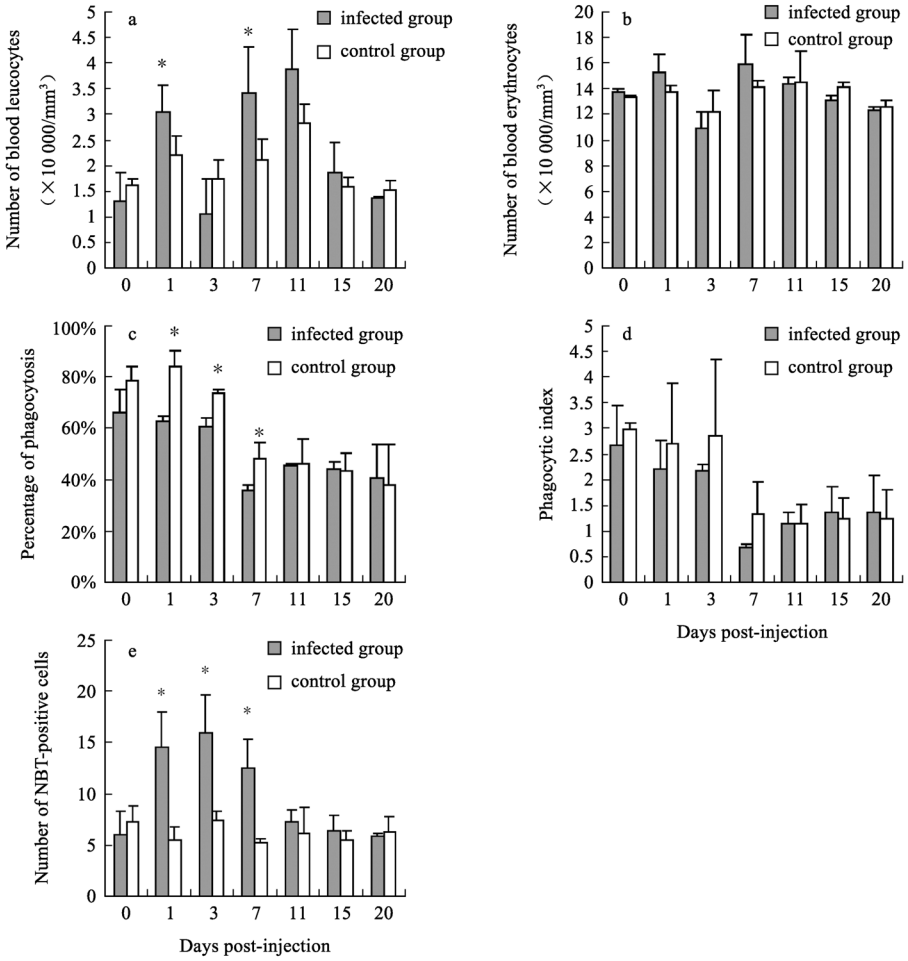

Fig.1 The effect of *A. hydrophila* injection on the cellular parameters in peripheral blood of bullfrogs

a. Leukocytes number; b. erythrocytes number; c. percentage of phagocytosis; d. phagocytic index; e. number of NBT-positive cells. *: Significant difference (P < 0.05)

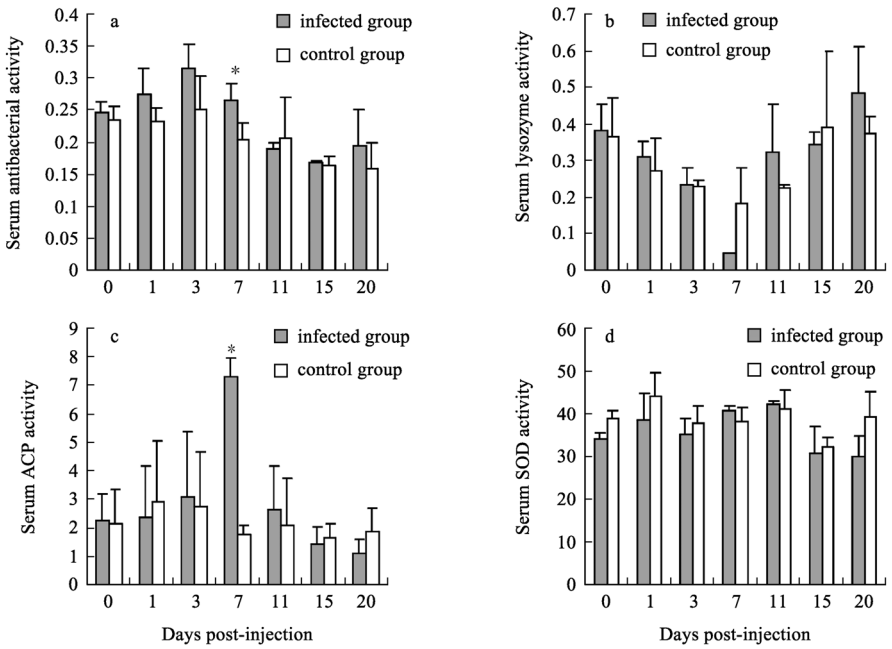

Fig.2 The effect of *A. hydrophila* injection on humoral non-specific immune parameters of bullfrogs

a. Bactericidal activity; b. lysozyme activity; c. ACP activity; d. SOD activity. ***:** Significant differences ($P < 0.05$).

The effects of *A. hydrophila* injection on several non-specific humoral immune parameters are shown in Fig.2. No obviously trend in the changes of serum antibacterial activities was found in the bullfrog from the two groups. Serum antibacterial activities of bacterium-injected bullfrogs were found higher than those in the control group at 1, 3, 7 dpi, and significant difference was detected at 7 dpi ($P<0.05$), then the value of antibacterial activity in bacterium-injected bullfrogs was found dropped to the level of control at the latter stage (Fig.2a). After injection, the serum ACP activities of bacterium-injected bullfrogs exhibited an increase at the initial stage and reached its peak at 7 dpi, and then fell at the anaphase. No obvious trend was found in control bullfrogs. Serum ACP activities of bacterium-injected bullfrogs were also found significantly higher than those in the control at 7 dpi ($P<0.05$), and no significant difference was detected between two groups at all other sampling times (Fig.2b). After injection, bullfrogs from both groups exhibited the same trend in the changes of lysozyme activity. The serum lysozyme activity gradually reduced at the initial stage, and then gradually increased at the anaphase. No significant difference was detected between the two groups at all sampling times (Fig. 2c). For serum SOD activity, no obvious trend was found after injection. No significant difference was detected between two groups at all sampling times (Fig.2d).

4 DISCUSSIONS

Animal has evolved a series of defense mechanisms to protect itself from bacterial infection. As an amphibian species, bullfrog has developed a specific immunity (Marchalonis and Edelman, 1966), but the specific immune response functions several days or weeks after bacteria invasion, while non-specific immunity plays a major role in the anti-infection immune response at the initial stage. The non-specific immunity includes mechanical defense, complement fixation, phagocytosis, lysozyme, and local inflammation mediated by cytokine (Nahm et al., 1999).

Macrophage, or mast cells activated by invasive bacteria produce inflammatory mediators, and promote the transfer and accumulation of leukocytes to the infection site (Lydyard et al., 2000). Increases in the numbers of leukocytes during fish defense against the invasive pathogenic bacteria were well known (Yildiz, 1998; Caruso et al., 2002). The number of leukocytes in bacterium-injected bullfrogs was observed greater than those in control at 1, 7 dpi. After maturation, most of these leucocytes transferred to the infection site and resulted in a sharp decrease in the number of peripherally leukocyte in bacterium-injected

bullfrogs at 3 dpi. The remarkable increase in the number of leucocytes in bacterium-injected bullfrogs at 7 dpi was perhaps due to the continual proliferation of leucocytes and the elimination of invasive bacteria.

As a type of immunocyte, erythrocyte has a great number of receptors of complement fragment (C3b), which can mediate the bonding of erythrocyte with pathogenic granule, and facilitate the bacteria clearing by phagocyte (Passantino et al., 2002). For tilapia infected spontaneously with *Edwardsiella tarda* or experimentally infected with *Mycobacterium marinum*, the declines in the number of erythrocyte were detected (Benli and Yavuzcan, 2004; Ranzani-Paiva et al., 2004), while in the present study, no significant difference in the number of erythrocytes between two groups was detected, which perhaps was due to the small dosage of bacteria used for injection or the lower hemolysis activity of *A. hydrophila* to the erythrocytes of bullfrog. However, it is worthy of being studied further.

Many types of frog leucocytes, especially monocyte and neutrophil have pseudopodia to ingest particles (phagocytosis) (Pan, 1999). Though the enhancement in the phagocytosis activity of fish peripheral blood neutrophil during bacteria infection has been reported (Park and Wakabayashi, 1992), a significant decrease in the phagocytosis after infection was observed in this study. The results suggest that *A. hydrophila* infection increased the number of active phagocytes in bullfrog but did not increase the ability to phagocytose *in vitro*, which was perhaps due to the lower phagocytosis activity of neonatal phagocytes or vast consumption of phagocyte *in vivo*.

During phagocytosis, the membrane-bound enzyme NAD(P)H-oxidase transforms the molecular oxygen into the superoxide anion (O_2^-) radicals (Babior et al., 1973), which is termed respiratory burst. This reactive oxygen is a potent killer for the foreign bacteria, independently or associated with other lysosomal enzymes (Grant and Loake, 2000). The NBT reduction product obtained after reaction with superoxides was a good indicator of the health status or the immunization effectiveness in fish (Anderson et al., 1992). *A. hydrophila* infection had been reported enhanced NBT reaction activity of Javanese carp (Shariff et al., 2001). Similar result in the present study suggested that the portion of phagocytes in state of killing bacteria increased after injection.

Part of the O_2^- generated by the phagocytes is dismutated to hydrogen peroxide (H_2O_2), either spontaneously or catalysed by superoxide dismutase (SOD) (Fridovich, 1978), which protects the organism from protein denaturation or DNA fragmentation (Halliwell and Gutteridge, 1989). In the present study, the activity of serum superoxide dismutase did not change significantly after *A. hydrophila* injection. This result shows that *A. hydrophila* infection did not influence the level of bullfrog serum superoxide dismutase perhaps due to the balance between the consumption and production of SOD.

Phagocytes excrete many types of molecules into blood during phagocytosis (Lydyard et al., 2000). These molecules kill bacteria directly or indirectly. Our results indicate that serum antibacterial activity of bacterium-injected bullfrogs was higher than those of control bullfrogs, suggesting that the intraperitoneal injection with *A. hydrophila* could induce phagocytes of bullfrog to excrete bactericidal substance into blood in order to eliminate the bacteria. Serum acid phosphatase (ACP), a marker enzyme of lysosome, is able to catalyze the hydrolysis of phosphormonoester under acid condition. ACP had been reported existed in the leukocyte of grass carp (Guo and Lu, 1994). In some cases, ACP was secreted out from phagocytes (Davies et al., 1974). ACP activity is widely believed to be a good indicator to macrophage activation in fish (Secombes, 1986). The increase in ACP activity of bullfrogs after *A. hydrophila* injection suggested the importance of ACP in non-specific humoral immunity.

Lysozyme is a cationic enzyme defined as muramidase that catalysed the hydrolysis of β-1, 4-glycosidic bond, and might cause the lysis of bacteria (Blake et al., 1965). Leucocytes, especially monocytes, neutrophils, and macrophages, can produce lysozyme (Yildirim et al., 2003). *A. salmonicida* infection increased serum lysozyme activities of Atlantic salmon in different degrees (Moyner et al., 1993). However, in the present study, there was no significant difference in serum lysozyme activity between the two groups at all sampling times. This perhaps was due to the various levels of serum lysozyme among different animals and different pathogenesis of infected bacteria. The enhancement of bactericidal activity might be due to other agents such as ACP or some other complements. The variation of several parameters such as the percentage of phagocytosis, phagocytic

indexes, and lysozyme activity at different sampling times may be due to the stress in bullfrogs to the injection and frequent disturbance.

5 CONCLUSION

The results obtained in this study suggest that: after intraperitoneal injection of *A. hydrophila*, bullfrog would trigger a series of non-specific immune responses including the proliferation of leukocytes, phagocytosis of phagocytes, and humoral bactericidal agents such as ACP, to eliminate the invasive pathogenic bacteria.

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