Original Article

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Differences in the mode of phagocytosis of bacteria between macrophages and testicular Sertoli cells

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ABSTRACT: Sertoli cells, the sole somatic cell type in the seminiferous epithelium, play an essential role in spermatogenesis and spermiogenesis by nursing germ cells for their survival and differentiation as well as physically inhibiting the entrance of harmful substances into the seminiferous tubules. Sertoli cells possess the characteristics of immune cells; they express pattern recognition receptors, secrete antimicrobial proteins, and engulf dead or dying cells. In this study, we determined the mechanism by which Sertoli cells engulf and kill bacteria compared to that of macrophages. When the primary cultured Sertoli cells of rats were incubated with *Staphylococcus aureus*, they produced the mRNA of neutrophil protein 3, an antimicrobial peptide of the α-defensin family, but not superoxide or nitric oxide, in contrast to mouse peritoneal macrophages. Sertoli cells effectively phagocytosed *S. aureus* in a manner that was accompanied by cytoskeleton rearrangement and dependent on phosphatidylinositol 3-kinase. Engulfed bacteria appeared to stay alive in Sertoli cells, while they were rapidly killed in macrophages. These results collectively suggest that Sertoli cells eliminate bacteria that have invaded the seminiferous epithelium without evoking inflammation, unlike macrophages.

Keywords: Bacterial infection, innate immunity, macrophage, phagocytosis, Sertoli cell

1. Introduction

Mammalian spermatogenesis takes place in the luminal part of the seminiferous tubules. A permeability regulation physically protects the seminiferous epithelium from the invasion of substances that may affect tissue homeostasis. This mechanism, called the blood-testis barrier, is composed of inter-Sertoli tight junctions (*1*,*2*). Sertoli cells, a somatic cell type in the seminiferous epithelium, play an essential role in the production of sperm by helping spermatogenic cells to survive and differentiate (*3*-*6*). We previously showed the existence of immunity in the seminiferous epithelium against invading bacteria (*7*). Sertoli cells possess some of the characteristics of immune cells: they engulf apoptotic spermatogenic cells (*8*), produce antimicrobial proteins (*9*-*13*), and express pattern recognition receptors such as Toll-like receptors (*14*-*17*). The phagocytosis of apoptotic spermatogenic cells by Sertoli cells is achieved through the specific binding of phosphatidylserine exposed on the surface of target cells to class B scavenger receptor type I (SR-BI) of Sertoli cells (*18*-*20*). Scavenger receptors are known to serve as receptors in the phagocytosis of bacteria by mammalian (*21*-*23*) as well as insect (*24*-*26*) phagocytes. These findings allowed us to anticipate the direct involvement of Sertoli cells in immune responses against bacteria in the seminiferous epithelium. In the present study, we examined the phagocytic activity of Sertoli cells against bacteria and compared it to that of macrophages.

2. Materials and Methods

2.1. *Materials*

The monoclonal anti-rat SR-BI antibody, clone 3D12, was generated in mice as described previously (*27*). U0126, SB203580, and SP600125, inhibitors of signaling pathways leading to the activation of the mitogen-activated protein kinases (MAPK) ERKI/II, p38, and JNK, respectively, were purchased from Cell Signaling Technology (Beverly, MA, USA). Wortmannin and cytochalasin B, inhibitors of phosphatidylinositol 3-kinase and actin polymerization, respectively, were obtained from Sigma-Aldrich (St. Louis, MO, USA), F12-L15 medium and trypsin were from Invitrogen (Carlsbad, CA, USA), and norepinephrine was from Meiji (Tokyo, Japan).

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2.2. *Culturing cells and bacteria*

Rat Sertoli cells were primary cultured as described previously (*18*,*19*). Briefly, the testes of 20-day-old male Donryu rats were processed to obtain cells contained in the seminiferous tubules, and the resulting dispersed cells, which mostly consisted of spermatogenic cells and Sertoli cells, were maintained with F12-L15 medium supplemented with 10% (v/v) heat-treated fetal bovine serum and norepinephrine for 3 days at 32.5˚C. Spermatogenic cells lightly attached onto the monolayer of Sertoli cells were removed by pipetting, and the remaining Sertoli cells were used in the experiments. Peritoneal macrophages of thioglycollate-treated C57BL/6 female mice were prepared and maintained in cultures as described previously (*28*). The *Staphylococcus atureus* strains, NCTC8325 and Smith, and *Escherichia coli* strain, WN5-2, were grown in Luria-Bertani medium at 37˚C, harvested at full growth, washed with phosphate-buffered saline (PBS), and used in the experiments as described previously (*29*).

2.3. *Reverse transcription-mediated polymerase chain reaction*

Total RNA was extracted from Sertoli cells by a conventional acid-phenol method and subjected to reverse transcription (RT) with oligo d(T) as a primer. The resulting cDNA was used as a template in semi-quantitative polymerase chain reaction (PCR). The oligonucleotides used as primers in PCR were: 5'-AAGAGCGCTGTGTCTCTTGC (forward) and 5'-CAACAGAGTCGGTAGATGCG (reverse) for the mRNA of rat neutrophil peptide (RNP-3), and 5'-TGAAGGTCGGTGTCAACGGTAAAGGC (forward) and 5'-CATGTAGGCCATGAGGTCCACCAC (reverse) for the mRNA of glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

2.4. *Determination of nitric oxide and superoxide, level of phagocytosis of bacteria, and colony-formable bacteria*

S. aureus was added to cultures of Sertoli cells or macrophages (phagocytes:bacteria = 1:1000) for the assay for nitric oxide and superoxide, and the mixtures were incubated at 37˚C for 30 min. Culture supernatants were then collected by centrifugation and subjected to assays for superoxide using Diogenes (National Diagnostics Inc, Atlanta, GA, USA) and nitric acid by the Griess method, as described previously (*29*). In an assay for phagocytosis, Sertoli cells or peritoneal macrophages were incubated with *S. aureus*, which had been surface-labeled with fluorescein isothiocyanate (FITC), at a ratio of phagocytes:bacteria of 1:100 at 32.5˚C (with Sertoli cells) or 37˚C (with macrophages), and were washed with PBS to remove unengulfed bacteria. The remaining cells were treated with 3% (w/v) paraformaldehyde and ice-cold methanol followed by examination under a fluorescence phase-contrast microscope, as described previously (*18*). To determine colony-formable bacteria in phagocytes, Sertoli cells or peritoneal macrophages, which had been incubated with *S. aureus* (phagocytes : bacteria = 1:100) at 37˚C for 30 min and washed, were further maintained and lyzed by incubating with water for 10 min. The lysates were plated on agar-solidified Luria-Bertani medium, incubated at 37˚C overnight, and the number of colonies was determined.

2.5. *Data processing and statistical analysis*

Results from quantitative analyses were expressed as the mean ± SD of the data from at least three independent experiments. Other data were representative of at least three independent experiments that yielded similar results. Statistical analyses were performed using the two-tailed Student's *t*-test, and *p* values of less than 0.05 were considered significant and indicated in the figures.

3. Results

3.1. *Phagocytosis of S. aureus by primary cultured Sertoli cells*

We first examined the phagocytosis of *S. aureus* by primary cultured Sertoli cells. The *S. aureus* strain NCTC8325 surface-labeled with FITC was added to cultures of Sertoli cells, and the mixture was incubated, washed, and cytochemically examined for the occurrence of phagocytosis. An image of fluorescence microscopy showed that Sertoli cells engulfed many bacteria (Figure 1A, left panels). A numerical analysis of the data indicated that Sertoli cell phagocytosis of *S. aureus* took a relatively longer period to occur (Figure 1A, right panel). We obtained similar results with another *S. aureus* strain, Smith (data not shown). Phagocytosis of the strain NCTC8325 was not influenced by an antibody that neutralized SR-BI or the inhibitors of MAPKs, but was reduced in the presence of cytochalasin B and wortmannin, inhibitors of actin polymerization and phosphatidylinositol 3-kinase, respectively (Figure 1B). This suggested the involvement of cytoskeleton rearrangement in the phagocytosis of *S. aureus* by Sertoli cells.

3.2. *Increase in α-defensin mRNA, but not superoxide and nitric oxide in Sertoli cells upon incubation with S. aureus*

We next examined the response of Sertoli cells to bacteria in terms of the production of antimicrobial substances. RNP-3, an antimicrobial peptide of the α-defensin family, has been shown to be produced in mammalian testes (*9-13*). Therefore, we determined the level of RNP-3 mRNA in Sertoli cells before and after incubation with

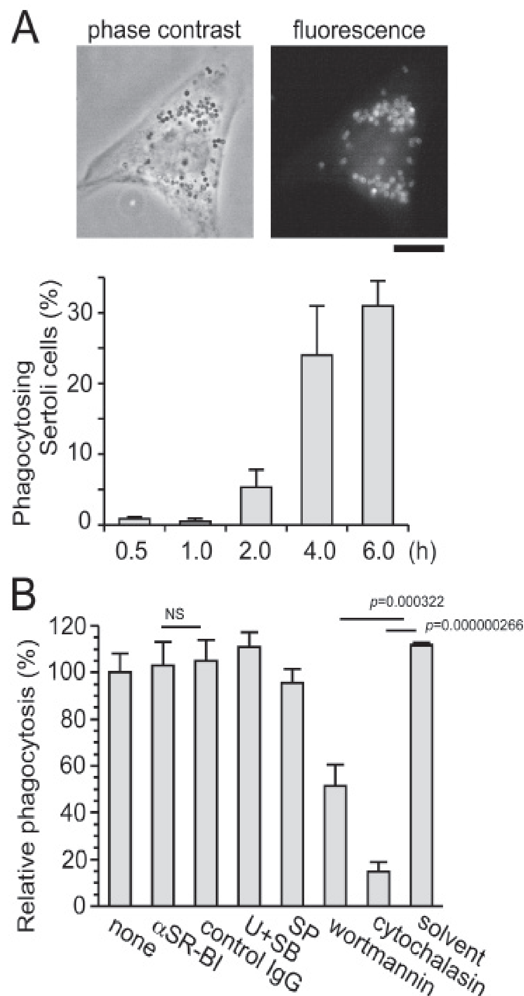

Figure 1. Phagocytosis of *S. aureus* by Sertoli cells. Primary cultured rat Sertoli cells were subjected to an assay for phagocytosis using FITC-labeled live *S. aureus* (strain NCTC8325) as targets. (**A**) Sertoli cells incubated with bacteria for the indicated periods were washed and examined under a fluorescence-phase contrast microscope for the ratio of Sertoli cells that engulfed bacteria. Shown at the top are phase-contrast and fluorescence views of the same microscopic field (scale bar, 10 μm). Results after numerical analysis of the data are shown at the bottom. Representative data from one of three independent experiments that yielded similar results are shown. (**B**) Sertoli cells were pre-incubated with the indicated proteins and chemicals for 30 min prior to being subjected to an assay for phagocytosis for 2 h. The concentrations of the supplements are: 50 μg/mL for anti-SR-BI IgG (αSR-BI) and normal mouse IgG (control); 10 μM for U0126 (U), SB203580 (SB), SP600125 (SP), and cytochalasin B; and 0.1 mM for wortmannin. The solvent for the chemicals was dimethyl sulfoxide. Representative data from one of two independent experiments with similar results are presented. NS, difference not significant.

the *S. aureus* strain NCTC8325 and the *E. coli* strain WN5-2. Total RNA extracted from Sertoli cells was analyzed by RT-mediated PCR for the level of mRNA. The intensity of a signal derived from RNP3 mRNA increased upon incubation with *S. aureus* and, to a lesser extent, with *E. coli*, while G3PDH mRNA, analyzed as an internal control, did not change after incubation with either bacterium (Figure 2A). An increase in the level of RNP-3 mRNA was not influenced when Sertoli cells were incubated with bacteria in the presence of an anti-SR-BI antibody, the inhibitors of MAPKs, or an inhibitor of phosphatidylinositol 3-kinase (Figure 2B).

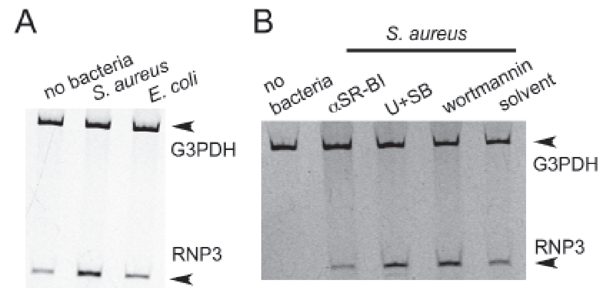

Figure 2. Levels of RNP-3 mRNA in Seroli cells incubated with *S. aureus* and *E. coli*. Primary cultured Sertoli cells were incubated in the presence and absence of *S. aureus* (strain NCTC8325) and *E. coli* (strain WN5-2) at ratios of phagocytes: targets of 1:1000 and 1:3000, respectively, at 32.5°C for 30 min. Total RNA extracted from Sertoli cells was subjected to RT-mediated PCR, and the final products were separated on a polyacrylamide gel followed by staining with ethidium bromide. (**A**) PCR products derived from the mRNA of RNP-3 and G3PDH, analyzed as an internal control, were shown. Data from one of five independent experiments with similar results are presented. (**B**) Incubation of Sertoli cells with *S. aureus* was performed in the presence of the indicated protein and chemicals. The symbols and concentrations of the supplements are the same as those shown in Figure 1B. Representative data from one of two independent experiments that yielded similar results are shown.

We next examined the production of radicals in Sertoli cells after incubation with bacteria. Cultures of Sertoli cells and mouse peritoneal macrophages were supplemented with NCTC8325, and culture supernatants were then examined for the production of nitric oxide and superoxide. We found that the production of either substance could not be detected in Sertoli cell cultures, while both nitric oxide and superoxide were detectable in the culture supernatants of peritoneal macrophages (Figure 3), as we reported previously (*29*). All these phenomena observed with Sertoli cells after the phagocytosis of the *S. aureus* strain NCTC8325 were reproduced when Smith, another *S. aureus* strain, was used as target bacteria (data not shown).

3.3. *Impaired killing of S. aureus in Sertoli cells*

We then examined how soon engulfed bacteria were killed in Sertoli cells. Sertoli cells or peritoneal macrophages were incubated with NCTC8325, washed to remove unengulfed bacteria, and further maintained. The cells were then lyzed and analyzed for the presence of colony-formable bacteria (Figure 4). We found that the number of colonies obtained from the lysates of Sertoli cells was almost constant in the 2-h incubation after washing. Similar results were obtained in the reaction using Smith, another *S. aureus* strain, as target bacteria (data not shown). In contrast, *S. aureus* engulfed by macrophages appeared to be killed in 1 h. These results indicated that *S. aureus* engulfed by Sertoli cells is resistant to killing.

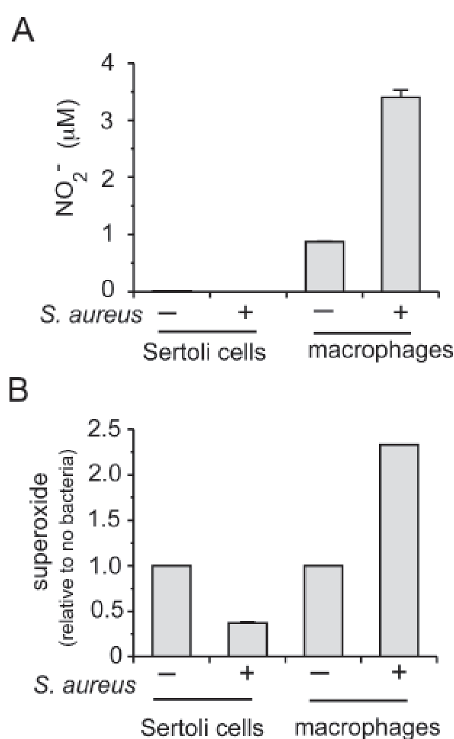

Figure 3. Production of nitric oxide and superoxide by Sertoli cells and macrophages incubated with *S. aureus*. Sertoli cells and peritoneal macrophages were incubated in the presence and absence of *S. aureus* (strain NCTC8325) (phagocytes:targets = 1:1,000) for 30 min, and culture supernatants were analyzed for the levels of nitric oxide and superoxide. Representative data from one of three independent experiments that yielded similar results are shown.

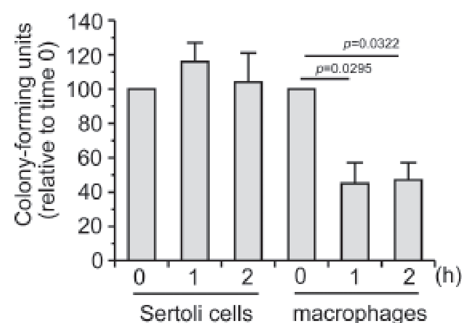

Figure 4. Survival of bacteria after engulfment by Sertoli cells. Sertoli cells and peritoneal macrophages were incubated with *S. aureus* (strain NCTC8325), washed, and further incubated for the indicated periods. The cells were lyzed and examined for the level of colony-formable bacteria. Representative data from one of five (with Sertoli cells) and three (with macrophages) independent experiments that yielded similar results are presented.

4. Discussion

We previously reported the existence of immune responses against invading bacteria in the seminiferous epithelium (*7*). Sertoli cells, the sole somatic cell type in the seminiferous epithelium, possess the characteristics of immune cells. In the present study, we examined the mode of action of Sertoli cells in response to bacteria and compared it to the action of peritoneal macrophages. We found that Sertoli cells isolated from rat testes and maintained in primary cultures effectively engulfed *S. aureus*, as did macrophages, in a manner accompanied by cytoskeleton rearrangement. However, engulfed bacteria were not efficiently killed in Sertoli cells, unlike those engulfed by macrophages. Sertoli cells produced an antimicrobial peptide, and not nitric oxide or superoxide upon incubation with *S. aureus*, while macrophages secreted both radical species. Such actions of Sertoli cells against *S. aureus* should account, at least partly, for immunity in the seminiferous epithelium. Regarding a comparison between the two types of phagocytes, the responses of Sertoli cells to bacteria were less pro-inflammatory than those of macrophages.

Authentic immune cells with phagocytic activity such as macrophages and neutrophils produce radicals when they encounter and phagocytose invading bacteria. Our findings indicate that Sertoli cells, tissue-restricted phagocytes, engulf bacteria, but do not actively kill them. Sertoli cell phagocytosis of bacteria may be interpreted as a defense mechanism by which pathogens are cleared without disturbing homeostasis in the seminiferous epithelium, in which spermatogenesis and spermiogenesis take place. A certain type of defensin plays a role in sperm maturation besides antimicrobial activity (*30*). Therefore, it is possible that RNP-3 is involved in spermatogenesis and/or spermiogenesis rather than killing *S. aureus*.

Sertoli cells retain the apical-basolateral polarity in primary cultures with the apical side toward the medium (*27*). It is thus most likely that Sertoli cells engulfed bacteria at their apical side in our assay for phagocytosis. Sertoli cells are responsible for the phagocytic elimination of spermatogenic cells undergoing apoptosis (*18*), and this action of Sertoli cells is necessary for the effective progress of spermatogenesis (*31*,*32*). Sertoli cell phagocytosis of apoptotic spermatogenic cells takes place in the seminiferous epithelium, and SR-BI serves as a receptor that recognizes phosphatidylserine exposed on the surface of target cells (*18-20*,*32*). However, SR-BI may not be involved in the phagocytosis of *S. aureus* by Sertoli cells because the antibody that neutralized the activity of SR-BI had no effect on phagocytosis. Whatever the receptor for phagocytosis is, it should recognize bacteria with no aid from immunoglobulin as an opsonin in the seminiferous epithelium, a site of immune privilege (*33*).

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