Intracellular Localization of *Staphylococcus aureus* within Primary Cultured Mouse Kidney Cells

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**Abstract** *Staphylococcus aureus* Cowan I was incubated with monolayers of cells derived from several portions of mouse kidney, and found to be ingested by all types of the renal cells. Intracellular localization of *S. aureus* was determined by resistance of intracellular cocci against lysostaphin digestion and confirmed by electron microscopy. From renal medulla, three morphological variants of the hyperosmolarity-tolerant (HOT) cells were obtained. The rate of cocci-ingesting cells varied from 16.9% to 93.4% among those of the HOT cells at the end of 3-hr incubation. From renal cortex, three morphological variants of epithelial cells grew in medium RK-1. Among them, only the cells on the edge of colony ingested Cowan I, while the epithelial cells on the center of colony ingested few cocci. Transferred from medium RK-1 to MEM supplemented with 10% FBS, part of the cortical cells changed into fibroblast-like appearance and obtained the capacity to ingest Cowan I. This result may indicate the correlation between ingesting capacity and cellular morphology. From a glomerulus, epithelial (GE) cells and fibroblast-like (GF) cells were obtained. The GE cells ingested not only *S. aureus* Cowan I but *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* after 30-min incubation, while the GF cells, like both of the HOT cells and the cortical cells, ingested only *S. aureus*. These results suggest a possibility that *S. aureus* is located within nonprofessional phagocytes during its infection and intracellular coccus plays an important role in its pathogenicity.

*Staphylococcus aureus* is an important pathogen which is responsible for significant morbidity and mortality, causing infections of soft tissue, joints, bones, and the cardiovascular system. Although *S. aureus* is regarded as one of the most representative extracellularly-growing pathogens, we observed previously that *S. aureus* Cowan I organisms not only adhere to antibody-sensitized culture cells mediated by the affinity between Fc portion of antibody and protein A on the staphylococcal cell wall, but are ingested by those cells (7, 11, Murai, unpublished result). These results suggest that *S. aureus* can be ingested by nonprofessional phagocytes in some natural conditions if it gains access to proper cells.

Since Gorrell reported that *S. aureus* frequently lodges and grows in kidney after the intravenous inoculation in an experimental animal (4), the renal lodgment
and proliferation property in mice is conveniently used as an indicator of the pathogenicity of \textit{S. aureus} (12). Although the precise mechanism of the renal lodgment property has remained to be solved, there must be a specific affinity between \textit{S. aureus} and mouse renal cells. Then, monolayers of mouse renal cells in primary cultures were subjected to a survey of the adherence of \textit{S. aureus}. During this survey, \textit{S. aureus} was found to be vigorously ingested by those renal cells. In the present communication we precisely describe the evidence for intracellular localization of \textit{S. aureus} within monolayers of mouse renal cells and discuss a possible role of staphylococcal ingestion by nonprofessional phagocytes in the pathogenesis of \textit{S. aureus}.

**MATERIALS AND METHODS**

\textit{Culture of the renal cells.} Three methods were employed to obtain various types of the renal cells. The renal hyperosmolarity-tolerant (HOT) cells were obtained according to the protocol of Sato and Ozawa (9); kidneys from a 3-week-old BALB/c female mouse were washed several times with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), and minced into pieces (diameter, 1 mm). The mince was treated in 5 ml of dispersion solution containing 0.02\% type II collagenase (Sigma, St. Louis, Mo., U.S.A.) in CMF-PBS for two successive periods of 30 min each at room temperature with constant stirring. Single cells were obtained by passing the last suspension through a mesh. Washed once with fresh medium, $5 \times 10^4$ cells in 1 ml of Eagle minimum essential medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10\% fetal bovine serum (FBS) (Biocell Lab., Carson, Calif., U.S.A.) were inoculated into a 35 mm tissue culture dish (Nunc, Roskilde, Denmark) and incubated at 37°C in 5\% CO$_2$. After one-week incubation, medium was replaced for hyperosmolar medium consisting of MEM supplemented with 1.3\% sodium chloride and 30\% FBS (556 mOsmol/liter). In this hyperosmolar medium, HOT cells including fibroblast are selectively killed (9). After 2-day incubation, hyperosmolar medium was replaced with ordinary medium, and the renal HOT cells were incubated for another 2 days, then served for ingestion assays. The HOT cells are considered to derive from renal medullary region, in particular from the collecting ducts, distal tubules and Henle's loop (9).

The renal cortical tubular cells were obtained by a modification of the method of Taub and Sato (13); mouse renal cortex was separated from medulla through the corticomedullary junction under a dissecting microscope. The cortical tissues were digested using the same method as described above and free nephron fragments were incubated for 11 days in a serum-free medium, RK-1, consisting of a 50: 50 mixture of Dulbecco's modified Eagle MEM (Nissui Pharmaceutical Co., Ltd.) and Ham's F12 (Biocell Lab.) supplemented with 5 $\mu$g/ml of insulin (Sigma), $5 \mu$g/ml of transferrin (Sigma) and $5 \times 10^{-8}$ M of hydrocortisone (Sigma). In medium RK-1, tubular epithelial cells grow without fibroblast overgrowth (2).

The glomerular cells were obtained by a commonly used method (5) as follows: the mince of renal cortex was buttered through a 94 $\mu$m nylon membrane, and rinsed
with CMF-PBS through successive 86 μm and 40 μm nylon membrane. Tissues on the lowest fine screen were transferred into a centrifuge tube, centrifuged at 800 rpm for 5 min, and resuspended in MEM supplemented with 10% FBS. The last suspension was inoculated into 35 mm dishes and incubated for 3 weeks. Since glomeruli obtained from 3-week-old mouse kidney were too small to be completely isolated by this protocol, some tubular fragments contaminated the last suspension. However, a glomerulus is easily distinguished from a tubular fragment under light microscope. Therefore, only the cells outgrowing from glomeruli were regarded as the glomerular cells.

**Bacteria.** *Staphylococcus aureus* Cowan I and three clinical isolates of other two staphylococcal species, *S. epidermidis* H155 and *S. saprophyticus* 10308 and 10312, kindly provided by Prof. Kosei Kurosaka of Aoto Hospital, the Jikei University School of Medicine, were used in this study. Bacteria were grown on heart infusion agar (Difco Lab., Detroit, Mich., U.S.A.) at 37°C overnight, harvested, washed several times with saline, suspended in MEM supplemented with 1% FBS at the concentration of 1 × 10⁸ CFU/ml, and served for ingestion assays.

**Staphylococcal ingestion assay.** The monolayer of the renal cells prepared in a 35 mm tissue culture dish was washed twice with serum-free MEM and incubated with 1 ml of bacterial suspension for 1 hr at 37 C in 5% CO₂. Bacterial suspension was replaced with fresh medium, and the renal cells were incubated for another 2 hr. Then floating bacteria were washed out with serum-free MEM and the renal cells were fixed with methanol, stained with Giemsa’s solution and served for light microscopy. Extracellular *S. aureus* and *S. saprophyticus* within some of those cultures were digested with 10 μg/ml and 50 μg/ml of lysostaphin (Sigma) respectively at 37 C for 30 min before the fixation. Lysostaphin lyses extracellular staphylococci but not intracellular ones (15). Each experiment was performed three times.

**Electron microscopy.** The renal cells were incubated with cocci in the same manner as described above, then fixed with 2% glutaraldehyde-0.1 M phosphate buffer at 4 C overnight. Specimens were rinsed with phosphate buffer, and post-fixed with 1% osmium tetroxide, dehydrated with a series of ethanol and embedded in Epon. Ultra-thin sections were stained with lead nitrate and uranyl acetate, and examined with a Hitachi H-500 electron microscope.

**RESULTS**

**Ingestion of *S. aureus* by the Renal Hyperosmolarity-Tolerant Cells**

Three morphological variants of cells were recognized in primary culture after selection in the hyperosmolar medium, and classified as follows: Type I—middle fibroblast-like cells; Type II—small epithelial cells with dome formation in culture, indicative of the retention of differentiated ion-transport properties; Type III—large epithelial cells with a paving-stone appearance. These cells were incubated with 1 × 10⁸ CFU of *S. aureus* Cowan I for 3 hr, then ingestion of Cowan I by all types of the HOT cells was determined by digestion of extracellular staphylococci with 10 μg/ml of lysostaphin at 37 C for 30 min (Fig. 1). Furthermore, intracellular
localization of Cowan I was confirmed by transmission electron microscopy (Fig. 2).

All types of the HOT cells ingested Cowan I in a time-dependent manner, although the percent of cells ingesting cocci varied among the above-mentioned three types of HOT cells. At the end of 3-hr incubation, 93.4±3.9% (mean±S.D.) of type I cells and 68.8±8.9% of type II cells and 16.9±3.4% of type III cells contained at least one or more cocci (Fig. 3). The mean number of intracellular cocci per cell for each cell type also increased in a time-dependent manner (Fig. 4). However, the number of intracellular cocci per cell at 3 hr varied from 0 to over 100 within the same type of the cells, and no difference was observed in the style of distribution of number of intracellular cocci among those of the HOT cells except for frequencies of noningesting cells (Fig. 5).
Fig. 2. Transmission electron micrograph of the HOT cell after 3-hr incubation with *S. aureus* Cowan I. Apparently intracellular organisms enclosed within tight or loose vesicle membrane are observed. Bar indicates 2 µm.

Fig. 3. Kinetics of the HOT cells ingesting *S. aureus* Cowan I. Original inoculum was $1 \times 10^8$ CFU per 35 mm tissue culture dish. The cells were incubated at 37 C in 5% CO$_2$ for the indicated time, and subjected to digestion of extracellular staphylococci by lysostaphin, then fixed, stained and counted intracellular cocci for at least 100 cells per dish under light microscope. Values are based on three independent experiments per point, and bar indicates standard deviation.
Ingestion of S. aureus by the Renal Cortical Cells Cultured in Medium RK-1

Three morphological variants of cells outgrew from renal cortical tubular fragments in medium RK-1, and were classified as follows: Type IV—small epithelial cells with many cytoplasmic granules, and some of the cells in a high concentrated region were round in shape; Type V—middle epithelial cells with dome-forming capacity; Type VI—large epithelial cells.

These cells were incubated with Cowan I suspension in the same manner as described above. Photomicrographs of the cells after 3-hr incubation and following lysostaphin treatment are shown in Fig. 6, in which only the cells on the edge of type IV and type V colonies contain many cocci. In particular, all of the cells and approximately half of the cells on the edge of type IV and type V colony, respectively, contain cocci. On the other hand, the epithelial cells on the center of both colonies contain few cocci. In addition, some of the round cells in a high concentrated region type IV colony also contain many cocci. The type IV cells contain fewer Cowan I organisms than did other type of the cells.

Effects of Medium Shift on Cellular Morphology and Staphylococcal Ingestion by the Renal Cortical Cells

The renal cortical cells grown in medium RK-1 for 9 days were transferred into MEM supplemented with 10% FBS. After 2-day incubation, the cells on the edge of type IV colony and the whole cells of type V colony changed into fibroblast-like appearance and got a capacity to ingest Cowan I, while the cells on the center
of type IV colony retained an epithelial appearance, few of which ingested Cowan I (Fig. 7). The cells of type VI also retained an epithelial appearance and did not enhance ingestion of Cowan I.

Ingestion Assay of Other Staphylococci

Both of the HOT cells and the cortical cells were incubated with S. epidermidis H155, S. saprophyticus 10308 and 10312 for 3 hr. No adherence of S. epidermidis H155 to any type of the cells was observed. On the other hand, adherence of S. saprophyticus 10312 to type IV of the cortical cells grown in medium RK-1 and a little adherence of both strains of S. saprophyticus to the HOT cells were observed. Then, 50 µg/ml of lysostaphin was applied to lyse extracellular S. saprophyticus. In a culture dish within the cortical cells incubated with medium RK-1, although many of S. saprophyticus organisms adhering to the surface of culture dish were incompletely lysed by lysostaphin, cell-associating cocci disappeared completely after lysostaphin
Fig. 6. Photomicrographs of the renal cortical cells grown in medium RK-1 after 3-hr incubation with *S. aureus* Cowan I and subsequent lysostaphin treatment. Three morphological variants of cells were classified as follows: Type IV—small epithelial cells (A); Type V—middle epithelial cells (B); Type VI—large epithelial cells (C). Photographs of higher magnification demonstrate that Cowan I ingestion was restricted to the cells on the edge of colony (D), and to the cells on the high concentrated region (E).
Fig. 7. Photomicrograph of medium-transferred cortical type IV cells after 3-hr incubation with *S. aureus* Cowan I and subsequent lysostaphin treatment. The cortical cells grown in medium RK-1 were transferred into 10% FBS-MEM and incubated for 2 days, then subjected to ingestion assay. Almost all of the cells taking a fibroblast-like morphology contain Cowan I organisms.

Fig. 8. Photomicrograph of the glomerular cells after 30-min incubation with *S. aureus* Cowan I. The epithelial cells (GE) contain many Cowan I organisms while the fibroblast-like cells (GF) have contained few coccci yet.
digestion. In a culture dish within the HOT cells, all *S. saprophyticus* organisms adhering to the surface of culture dish were lysed by lysostaphin and a few of cell-associating cocci escaped from lysostaphin digestion, though the number of those *S. saprophyticus* organisms were much fewer than that of those *S. aureus* Cowan I organisms.

**Ingestion Capacity of the Glomerular Cells**

Two types of cells outgrew from a glomerulus: contact-inhibited polygonal epithelial cells with many cytoplasmic granules (GE cells), and multi-layered fibroblast-like cells (GF cells) (Fig. 8). Both types of the cells were incubated with Cowan I suspension in the same manner as described above. Then, all of the GE cells were found to efficiently ingest Cowan I after only 30-min incubation (Fig. 8). The style of distribution in number of intracellular cocci per GE cell was apparently different from that of the HOT cells (Fig. 9).

![Fig. 9. Distribution in number of intracellular cocci per GE cell at the end of 30-min incubation with three species of staphylococci. Procedures are the same as described in legend of Fig. 3, except that fifty cells per dish were counted. Top: *S. aureus* Cowan I. Middle and bottom: *S. saprophyticus* 10308 and 10312, respectively. The data presented is representative one of three independent experiments.](image-url)
The GE cells also ingested both S. saprophyticus and S. epidermidis after 30-min incubation. Ingestion of S. saprophyticus was determined by digestion of extracellular cocci with 50 µg/ml of lysostaphin. In this case, S. saprophyticus organisms adhering to the surface of dish were completely lysed by lysostaphin. The mean number of intracellular cocci per cell and the style of distribution in number of intracellular cocci per cell of 10312 were similar to those of Cowan I and the mean number of intracellular cocci per cell of 10308 was less than that of Cowan I (Table 1 and Fig. 9). Ingestion of S. epidermidis was determined by transmission electron microscopy (data not shown).

On the other hand, the GF cells ingested only S. aureus Cowan I among three staphylococcal species examined. It took 3 hr for approximately 80% of the GF cells to ingest Cowan I (Table 1), and the style of distribution of number of intracellular cocci per GF cell resembles that of the HOT cells (data not shown).

**DISCUSSION**

*S. aureus* is usually considered to express its pathogenicity largely in the form of extracellular proliferation. Therefore, intracellular localization of *S. aureus* within cells other than phagocytic leukocytes has been negligible in staphylococcal infections. However, several studies recently reported the damage to endothelial monolayer by intracellular *S. aureus* and discussed that it plays a role in the initiation of infectious endocarditis by *S. aureus* (3, 6, 15, 16). In addition, Schmidt et al described that *S. aureus* has a potential to invade into HEp-2 cells, although the potential of *S. aureus* is lower than that of *S. saprophyticus* (10).

*S. aureus* Cowan I was efficiently ingested by all types of the renal cells cultured in the present investigation. Ingestion by the tubular cells and the GF cells appears to be accomplished by parasite-directed endocytosis, of which concept is proposed by McGee et al (8) to be similar to the term parasite-specified phagocytosis used by Byrne and Moulder (1), because of the following two reasons: the efficient phagocytosis of *S. aureus* was achieved by the renal cells which are nonprofessional phagocytes, and the efficient phagocytosis by the renal cells, except for glomerular GE cells, was directed to only *S. aureus*.

The GE cells ingested staphylococci more actively than the other renal cells in
terms of the time required for efficient ingestion and staphylococcal species ingested. In addition to staphylococci, preliminary experiments revealed that the GE cells can also ingest Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli (unpublished results). Moreover, there is apparent difference in the style of S. aureus ingestion between the GE cells and the other renal cells (Figs. 5 and 9). Thus the mechanism of bacterial ingestion by the GE cells may differ from that by the other renal cells.

Among the other renal cells, there were several morphological variants which varied in a capacity to ingest S. aureus; this may be reflected by cellular origin. Alternatively, an ingesting capacity may depend on morphological variation of the cells in vitro. The latter possibility is suggested by the result of medium shift experiment in which morphological change from epithelial to fibroblast-like appearance seems to be required for the enhanced ingestion of S. aureus (Fig. 7). However, both cellular origin and morphological variation cannot explain why only the cells on the edge of epithelial colony ingested a large number of cocci while the cells on the center of the same colony ingested few cocci (Fig. 6). Further studies on the cellular condition responsible for the efficient ingestion of S. aureus are now in progress.

It has not been well-documented yet whether S. aureus is located within host cells in vivo at infected kidney and other tissues. We believe, however, that intracellular localization of S. aureus in nonprofessional phagocytes reflects certain important pathogenic properties, because intracellular S. aureus organisms appear to be still alive and part of them seem to proliferate intracellularly. Hence, not only the ability for S. aureus to be located within cells but intracellular events should be investigated to reveal the pathogenicity of S. aureus. Furthermore, since kidney is not a primary site for infection by S. aureus, a survey of cells (other than the renal cells) that are able to ingest S. aureus is now in progress. So far, primary-cultured mouse skin fibroblast cells are found to ingest S. aureus specifically (14).

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REFERENCES


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