Jikeikai Med J 2005 ; 52 : 21-9

Effect of Lysostaphin on Establishment of Staphylococcal Infectious Foci in Mouse Kidney

Keiko SEKI[1], Hitomi SHINJI[1], Miyo MURAI[2], Akiko TAJIMA[1], and Shogo MASUDA[1]

[1]*Department of Microbiology (II), The Jikei University School of Medicine*
[2]*Department of Medical Technology, Saitama Prefectural University Junior College*

ABSTRACT

To clarify the mechanisms how *Staphylococcus aureus* organisms proliferate and how the infectious foci are established in mouse kidney, we carried out the *in vivo* experiments using mice and the *in vitro* experiments using kidney homogenate of mice and lysostaphin.

We demonstrated that the number of *S. aureus* organisms in the kidneys of mice treated with lysostaphin 1 hr after injection of the bacteria was one-third of that without lysostaphin treatment. The major reason of the diminution seemed that the bacteria had been directly killed by lysostaphin in the bloodstream before they came to the kidney. The bacteria that could escape from lysostaphin-attack were thought to exist inside leukocytes or some renal cells, or the very narrow intracellular space of the kidney. We also demonstrated that the leukocytes got a higher phagocytic activity according to the injection of heat-killed bacteria. In the formation of infectious foci, leukocytes were suspected to play two conflicting roles, killing and protecting bacteria, and renal cells might be a hiding place for *S. aureus* organisms. (Jikeikai Med J 2005 ; 52 : 21-9)

Key words : *Staphylococcus aureus*, lysostaphin, infectious foci, leukocyte, kidney cell

INTRODUCTION

Staphylococcus aureus is known to be a pathogen of dermatitis[1,2], endocarditis[3-5], and osteomyelitis[6,7] in human, and has been experimentally demonstrated to proliferate in various murine tissues[8-15]. Our previous reports stated[11,12] that about 2×10^4 CFU of *S. aureus* Cowan I adhered to one kidney of the mouse intravenously innoculated with 1×10^7 CFU. Kondo et al. reported[8] that a large number of the *S. aureus* organisms remained in the mouse kidney more than 7 days after their intravenous inoculation, although their numbers had decreased within a few days in other organs such as the liver, spleen, and lung. Why can *S. aureus* proliferate so strongly in the kidney ? One reason may be that there are only a little resident phagocytes in the kidney, and so bactericidal activity in the kidney might be low. On the other hand some organs have some specialized phagocytic cells such as the Kupffer cells in the liver, the alveolar macrophages in the lung, or the splenocytes in the spleen. Another reason for the ability of *S. aureus* to proliferate strongly in the kidney may be the involvement of staphylococcal cell surface-associated substances. The kidney is composed of various types of tissues, and the cells that form these tissues secrete some of their extracellular matrices. The extracellular matrix-binding proteins of *S. aureus* include laminin-binding protein[16,17] vitronectin-binding protein[18], fibrinogen-binding pro-

Received for publication, October 19, 2004
関　啓子，進士ひとみ，村井　美代，田嶌亜紀子，益田　昭吾
Mailing address : Keiko SEKI, Department of Microbiology (II), The Jikei University School of Medicine, 3-25-8, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan.
E-mail address : kseki@jikei.ac.jp

tein[19], and collagen-binding protein[17,20-22]. Other well-known attachment factors include staphylococcal protein A[23-25], clumping factor[26,27], and fibronectin-binding protein[28-31]. However, it has not yet been clarified why or how *S. aureus* can multiply in the kidney tissue or the renal cells of the mouse.

It has been reported[8] that abscesses were formed when a kidney was stabbed with a needle carrying only a few staphylococcal cells, although no abscess was observed in the kidney after the intravenous injection of the same amount of *S. aureus*, suggesting that a very small number of *S. aureus* organisms was sufficient to form an infectious focus. Most of the intravenously-inoculated bacterial cells may have been trapped by tissue-resident phagocytes such as splenocytes and Kupffer cells, or killed by circulating leukocytes and serum factors. This speculation seemed to have a relation to one of our findings[11]: *S. aureus* organisms disappeared in a linear fashion from the bloodstream during the first 30 min after the injection via intravenous routes, but the disappearance of the organisms followed a gentle slope in mice which had previously received a large number of heat-killed bacteria. That result suggested that the massive administration of killed organisms may have influenced the phagocytic function of the leukocytes.

In order to clarify the mechanisms through which *S. aureus* organisms adhere to and proliferate in the kidney cells, we designed an *in vivo* experiment using lysostaphin. Lysostaphin is a product of *Staphylococcus simulans*, and is known to enzymatically breakdown peptidoglycan, the major component of the bacterial cell wall. We demonstrate that staphylococcal proliferation in the mouse kidney was inhibited by lysostaphin challenge, and discuss the possibility that leukocytes may play an important role in helping *S. aureus* organisms survive and colonize by giving refuge to the bacteria.

MATERIALS AND METHODS

Bacteria

S. aureus Cowan I organisms were cultured overnight on heart infusion agar (HIA, Difco Co. Ltd., Detroit, Mich., USA) plates at 37°C. Bacteria were collected, washed twice and suspended in physiological saline. The bacterial concentration was estimated by turbidimetry at 660 nm using a Junior® III spectrophotometer Model 6/8 (Perkin-Elmer Instruments, Oak Brook, Ill., USA). Heat-killed bacteria (KB) were prepared by boiling the live ones for 30 min and washing with saline. The concentration of KB was then adjusted.

Mice

Female ICR mice (5 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan), and were allowed food *ad libitum*. Leukopenic mice were created by injecting 4 mg of cyclophosphamide (Endoxan™, Shionogi Co. Ltd., Osaka, Japan) every other day for 5 days, after which the average number of leukocytes per ml of the peripheral blood of the leukopenic mice was 3.7×10^5 cells.

Estimation of the number of intrarenal bacteria

Each mouse received 0.1 ml of bacterial suspension via the tail vein. The inoculation level of live bacteria was 1×10^7 CFU per mouse. One hour before receiving the live bacteria, the mouse was injected with 1×10^9 cells of KB, if necessary. Mouse was also intravenously given lysostaphin derived from *S. simulans* (Sigma-Aldrich Co., St. Louis, Mo., USA) 1 hr after receiving the live bacteria, if necessary. Phosphate buffered saline (PBS) was used instead of either KB or lysostaphin.

The estimation of the bacterial numbers in the kidney was carried out in almost the same way described previously[11,12]. In short, 2 hr after the injection of live bacteria, both kidneys were aseptically removed from each mouse under ether anesthesia, and homogenized with a sterile mortar and pestle in 2 ml of nutrient broth. Appropriately diluted homogenate was spread on a nutrient agar plate (Difco), and incubated at 37°C overnight for colony counting. The number of bacteria per kidney was calculated.

Monitoring bacteria in kidney homogenate mixed with lysostaphin

Both kidneys of the mouse given live bacteria 1 hr earlier were homogenized in 2 ml of nutrient broth (Difco). The homogenate was mixed with 1.1 units/ml of lysostaphin in a test tube, and incubated at 37°C. The bacterial numbers were counted after 0, 1 and 3 hr of the incubation time by colony counting method.

Phagocytic plaque method

Changes in the phagocytic activities of leukocytes after the injection of KB were examined using the phagocytic plaque method developed by Seki et al.[32], with slight modification. In short, a 0.8-ml heparinized blood sample was put into a plastic dish covered with an *S. aureus*-thin layer. After 1 hr of incubation at 37°C, the dish was washed twice with physiological saline, dried, fixed with methanol and stained with Giemsa's solution. Phagocytic function was expressed in terms of the number of phagocytic plaques (plaques) which were empty spaces formed by leukocytes after ingesting the bacterial layer. Image analyses were carried out using the FRM Tool-Kit computer program (ver 2.1, Photoron Co., Tokyo, Japan) with a light microscope under a 10× objective lens. In this method, the size was represented as the numbers of pixels. The sum of the areas of plaques within a certain microscopic field was expressed as the "total area", and the mean was based on the values obtained from five locations per dish. The size of plaque was separately measured about 100 plaques, each of which represented the area covered by the movement of one leukocyte during phagocytosis.

Determination of the types of neutrophils

A smear was prepared from the blood of each mouse, fixed with methanol, and stained with Giemsa's solution. The percentages of both segmented and stab cells were calculated; and the number of leukocytes was counted with a hemocytometer. Then, both segmented and stab cell numbers per ml of blood were determined.

Statistics

Data were analyzed for statistical significance by Student's t test for each paired sample. P values less than 0.05 were regarded as significant.

Results

Inhibition of staphylococcal adhesion to the kidney by lysostaphin

At first, the appropriate concentration of lysostaphin to administer to mice for the following experiments was determined. Mice received various concentrations of lysostaphin 1 hr after the injection of live bacteria, and another hour later, both kidneys were removed and the number of bacteria was counted to evaluate the action of lysostaphin. As shown in Fig. 1, the number of *S. aureus* organisms in the kidney decreased with the injection of lysostaphin. The degree of inhibition depended on the dose of lysostaphin within the range of 0 to 0.88 units per mouse. There was not a significant difference between the number of bacteria recovered from mice injected with 0.88 and 1.76 units of lysostaphin. According to this result, 1.76 units of lysostaphin per mouse were used about the following experiments.

A large amount of live bacteria were recovered from the kidneys of mice which had received KB in advance, and this result was independent of lyso-

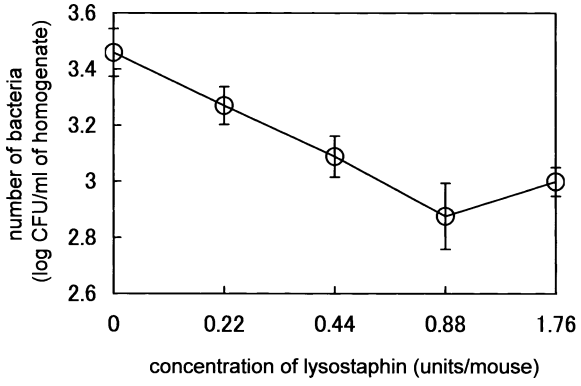

Fig. 1. Effect of lysostaphin on the staphylococcal proliferation in the kidney. One hour after the injection of live *S. aureus* organisms, various concentrations of lysostaphin were intravenously injected into mice. Details are described in Materials and Methods. Values are mean±SD (n=5).

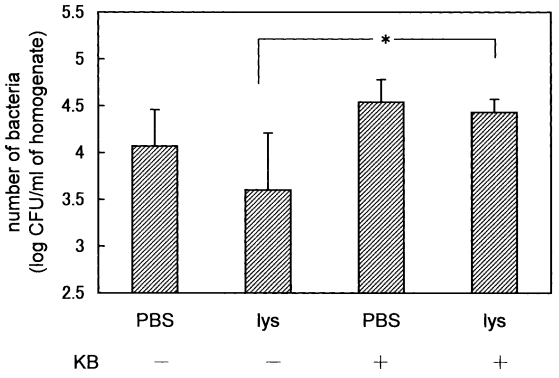

Fig. 2. Decline in lysostaphin action resulting from injection of KB. One hour before the injection of live bacteria, 1×10^9 cells of heat-killed counterparts were intravenously injected into mice. Lysostaphin (1.76 units) was injected, if necessary. Details are described in MATERIALS AND METHODS. Values are mean±SD ($n=5$). The difference between lysostaphin-treated mice with and without pre-injection of KB was statistically significant (*, $p<0.05$).

staphin treatment (Fig. 2). Without the pre-injection of KB, smaller number of bacteria was found in the kidneys of lysostaphin-injected mice than those in the control mice. The level in lysostaphin-injected mice was about one-third of that in the control.

Action of lysostaphin on the bacteria in kidney homogenate

This lysostaphin concentration used in this experiment, 1.1 units/ml, was determined on the basis of the quantity in the bloodstream of a mouse intravenously given 1.76 units. The bacterial numbers in kidney homogenate were about 1.5×10^4 CFU/ml for live bacteria-injected mouse, and 6.3×10^4 CFU/ml for KB- and live bacteria-injected mouse, respectively. With the addition of lysostaphin to the homogenate, the numbers decreased during the first hour and remained steady from 1 to 3 hr, although bacteria were proliferating throughout the incubation time without lysostaphin. When the kidney homogenate of an uninfected mouse was mixed with 2.7×10^4 CFU/ml of bacteria and 1.1 units/ml of lysostaphin, almost all the bacteria disappeared dramatically during the first hour and keep the same level for another 2 hr. Table 1 shows the bacterial numbers at 0 and 3 hr after the incubation and the survival rate of the bacteria during the incubation. The differences of the survival rate between three groups were significant ($p<0.05$). In case 1×10^8 CFU/ml of bacteria were mixed with 1.1 units/ml of lysostaphin, bacteria quickly disappeared to be undetectable (data not shown).

Table 1. Changes in the bacterial numbers during the incubation with lysostaphin

	Live[a]	KB & Live[a]	None[b]
bacteria (CFU/ml of homogenate)[c]	0 h $(1.5\pm1.3)\times10^4$	$(6.3\pm1.2)\times10^4$	$(2.7\pm1.1)\times10^4$
	3 h $(6.7\pm1.3)\times10^2$	$(1.3\pm1.3)\times10^4$	$(1.4\pm1.5)\times10^2$
survival rate (%)[d]	4.55±1.63	20.98±2.42	0.55±0.21

Values are expressed as mean±SD of three experiments under each condition.
a) Mice were intravenously injected with either live, or KB and live bacteria. The kidney homogenate of each mouse prepared as written in MATERIALS AND METHODS, was mixed with 1.1 units/ml of lysostaphin.
b) The kidney homogenate of uninfected mouse was mixed with bacteria and 1.1 units/ml of lysostaphin.
c) The bacterial numbers in each homogenate were checked at 0 and 3 hr after incubation.
d) The survival rate was calculated as: $\frac{\text{bacterial numbers (3 h)}\times100}{\text{bacterial numbers (0 h)}}$

Phagocytic ability of leukocytes

Figure 3 shows the micrographs of plaques during the time after the injection of KB. Leukocytes in a normal condition (at 0 hr) formed only very few, small, and round plaques, and the numbers and sizes of the plaques increased with time. Figure 4 shows the total area of the plaques within a certain microscopic field. Before the injection of KB (0 hr), the value was 300.5±135.6 pixels. A 220-fold increase was observed during the first two hours, and then the level remained constant for the next hour. Significant difference in values between 2 and 3 hr was not recognized. The size frequency distributions of plaques at each time after the injection of KB were investigated (Fig. 5). The distribution patterns were different from each other, and the pattern 2 hr after the injection of KB shifted to the right. Some larger plaques, whose area was greater than a relative area of 600, were recognized 2 hr after the injection of KB.

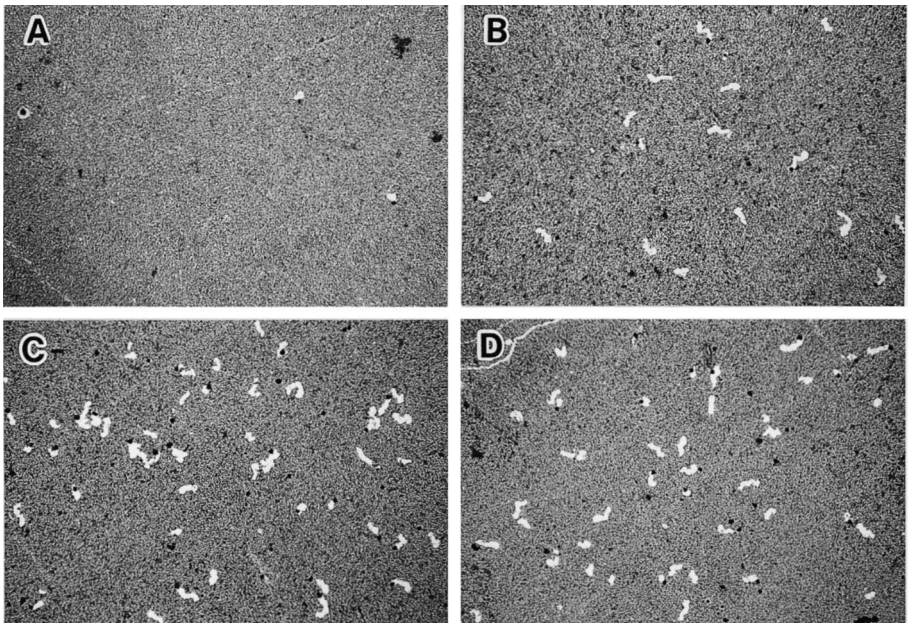

Fig. 3. Micrographs of phagocytic plaques obtained from mice injected with heat-killed bacteria. The phagocytic plaque method was carried out each time. A to D correspond with 0, 1, 2, and 3 hr after the injection of KB, respectively. The numbers and sizes of plaque became larger with time.

Fig. 4. Effect of heat-killed bacteria on the phagocytic activity of leukocytes. The phagocytic plaque method was carried out at 0, 1, 2, and 3 hr after the injection of KB, and the area of all plaques within a certain microscopic field was summed up. Details are described in MATERIALS AND METHODS. Each value is expressed as a mean from five spots per dish. Error bars show the SD.

Mean sizes of plaques about each time were calculated (Table 2). As for the mean area, statistically significant differences between 0 and 3 hr, and 1 and 2 hr were not.

Figure 6 shows the transition of the numbers and types of neutrophils after the KB-injection. The maximum total of neutrophils was seen 2 hr later. As for individual cell types, the segmented cells decreased dramatically with time, while the stab cells increased in number, peaking during the same period.

Bacterial growth in kidney of leukopenic mice

The intrarenal bacterial growth of normal and leukopenic mice was compared. As Fig. 7A indicates, more bacteria were recovered from leukopenic mice than from normal mice. On the other hand, when mice received lysostaphin (1.76 units) at 1 hr after the injection with live bacteria, the bacterial growth in both leukopenic and normal mice was nearly equal, and there were fewer bacteria than were obtained from mice receiving no lysostaphin (Fig. 7B).

DISCUSSION

Although leukocytes are generally considered to constitute a part of the host defense mechanisms, the data presented here indicate the possibilities that leukocytes played an important role in protecting the *S. aureus* organisms ingested by the leukocytes, and that several kinds of cells in kidney also played as the protector.

The renal bacterial numbers in mice given with

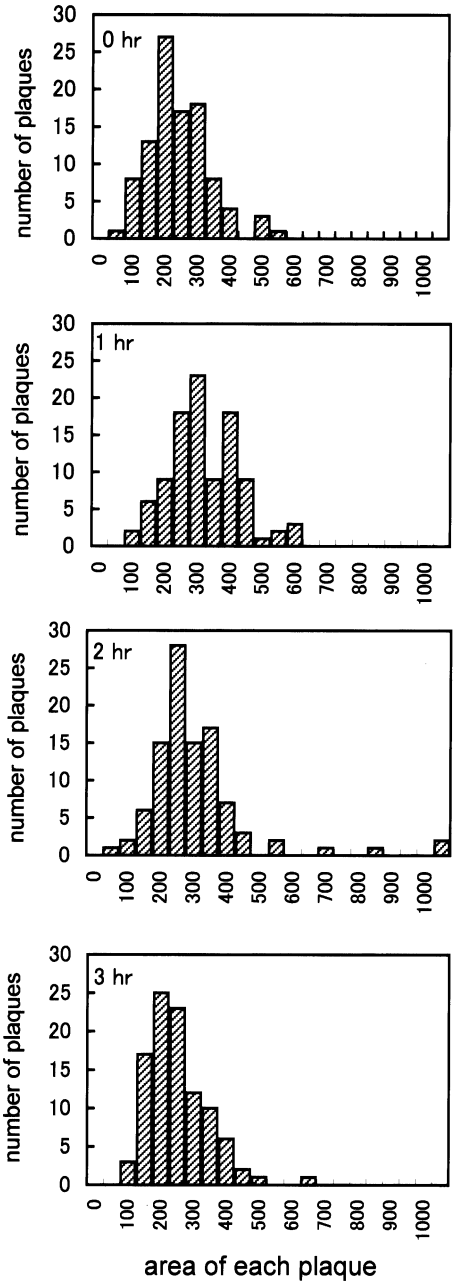

Fig. 5. Phagocytic activity of each leukocyte. The relative area of each plaque after the KB-injection was estimated about 100 plaques. Size frequency distributions are shown. Details are described in MATERIALS AND METHODS.

lysostaphin were lower than those in mice without lysostaphin (Figs. 1 and 2), suggesting that, at most, one-third of the bacteria that had adhered to the kidney were killed by lysostaphin while the remainder were not, one reason being that some of the injected lysostaphin may have been inactivated by substance(s) in the bloodstream. Some reports suggested[33-35] that lysostaphin given to the animal was able to reach infectious foci without alteration and with bactericidal effectiveness. Another reason was that *S. aureus* organisms administered to the mice may have been in a location where they were protected from the action of lysostaphin; for example, in leukocytes, in renal cells, or in interstitial spaces between renal tissues. Another experiment revealed that the intravenous injection of either live or heat killed *S. aureus* cells could induce neutrophil-dominant leukocytosis (Fig. 6). The number of the stab cells reached a maximum at 2 hr after the injection of KB, suggesting that inflammation was induced by the injection of KB and that a large quantity of stab cells were supplied in a short time from bone marrow. According to results obtained using a phagocytic plaque method (Figs. 3-5 and Table 2), the phagocytic level of each neutrophil was highest at 1 hr after the injection of KB, while the number of neutrophils was maximal at 2 hr. The phagocytic function of mouse blood was considered to be fully activated 2 hr or more after the injection of KB.

Incidentally, in an *in vitro* experiment (Table 1) the live bacteria in the kidney homogenate of a mouse given both killed and live bacteria were not lysed by lysostaphin as much as in the other two conditions. The live bacteria injected later might be ingested but probably not killed by the leukocytes, whose phagocytic activity had been strongly boosted by the injection of KB. Such live bacteria might be difficult to be lysed by lysostaphin because of being protected within the leukocytes. When no pre-injection of KB was given, the number of live bacteria ingested by leukocytes was low, because the phagocytic activities of normal mouse leukocytes were not raised as shown in Fig. 3A. Furthermore, the reason why the bacteria mixed with the kidney homogenate of uninfected mice were immediately lysed by lysostaphin might be that the bacteria were outside the host cells.

The result previously obtained in our laboratory[12] was that *S. aureus* organisms have been more frequently phagocytosed by leukocytes than coagulase-negative staphylococci of low pathogenicity. Another work of our laboratory stated[36] that about

Table 2. Changes in size of individual plaque after injection of heat-killed *S. aureus*

	time after KB-injection (h)			
	0	1	2	3
relative area	221.8±99.1	290.3±107.2	279.4±156.0	231.5±92.4

The size of each plaque was estimated. Values are mean±SD of 100 plaques. Details are described in MATERIALS AND METHODS.
Significant differences were confirmed between 0 and 1 hr, 0 and 2 hr, 1 and 3 hr, and 2 and 3 hr, respectively ($p<0.005$).

Fig. 6. Appearance of stab cells after the injection of KB. Segmented and stab cells in peripheral blood were counted after KB were injected. Details are described in MATERIALS AND METHODS. Open bar indicates segmented cells, and shaded bar indicates stab cells.

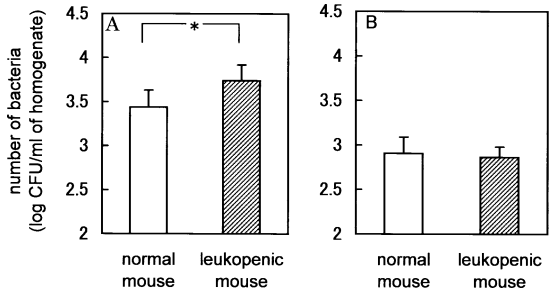

Fig. 7. Effect of lysostaphin on bacterial proliferation in leukopenic mice. Both normal and leukopenic mice received *S. aureus* organisms. One hour after the bacterial injection, mice were given PBS (A) or lysostaphin (B), respectively. Details are described in MATERIALS AND METHODS. Values are mean±SD ($n=5$). The difference between normal and leukopenic mice without lysostaphin-injection was statistically significant (*, $p<0.05$).

20% of *S. aureus* organisms were phagocytosed by leukocytes when mixed in the ratio of 1 : 1, and that over 90% of the phagocytosed bacteria were killed for the first 30 min in an *in vitro* experiment. Furthermore, the result was shown that 30-fold growth was observed after the following 21 hr-incubation, while *Staphylococcus saprophyticus* of low pathogenicity were killed and could not proliferate any more. These phenomena may be hard to account for on the basis of the common view that a man avoids sickness as a result of ingestion and destruction of bacteria by his leukocytes. Thus, it is interesting to consider what kind of relationship there is between the *S. aureus* characteristics of being easy to phagocytose and having a high pathogenicity. These two reports suggested that not every bacterium phagocytosed was killed by the leukocytes, even if most of the bacteria given to the mouse were ingested. This may be explained by the various resistance factors possessed by a bacterium against the sterilizing action of the leukocyte, and by the inability of a leukocyte to sterilize effectively under a heavy load such as hyperphagocytosis. *S. aureus* within a leukocyte might conceivably be protected from attack by bactericidal or toxic substances in serum such as lysozyme, some interferons, or others, and can proliferate in the human body afterwards to make the infectious foci. From the standpoint of the leukocytic role, a leukocyte appeared to have two opposite roles, those of sterilization and the protection, against bacteria present in a host.

There is other evidence indicating that peripheral neutrophils influence the ciruculating bacteria intravenously injected to a mouse. The previous report, that even an extremely small quantity of *S. aureus* organisms could establish infectious foci after direct inoculation to the kidney, suggested[8] that almost *S. aureus* organisms were killed by circulating leukocytes before they reached the kidney. More-

over, as mentioned in "RESULTS", more bacteria were seen in leukopenic mice than normal mice (Fig. 7A), although, regarding lysostaphin-injected mice, the numbers of bacteria in leukopenic mice were equal to those in their normal counterparts (Fig. 7B). In leukopenic mouse which has only a few leukocytes, it might be considered that lysostaphin sterilization was effective because the bacteria were able to escape phagocytosis by leukocytes and to multiply efficiently outside the leukocytes. Furthermore, there was a possibility that the bacteria were also ingested by the cells other than leukocytes in the kidney tissue. Some evidence exists that *S. aureus* may be internalized into, and be able to multiply within, cells including renal cells[37,38], epithelial cells[39,40], osteoblasts[41], endothelial cells[42], and fibroblasts[43], although *S. aureus* has been classically described as an exclusively extracellular pathogen. The bacteria inside these cells would probably be hardly affected at all by lysostaphin or by neutrophils. Because the number of bacteria did not decrease very much under such conditions, and because they were ingested by neutrophils, it was concluded that lysostaphin exclusively killed extracellular bacteria, while intracellular bacteria could escape its action. Therefore, from the viewpoint of bacteria, there are perhaps obvious advantages to the maintenance of an intracellular location, because the bacteria would be able to shelter themselves from many host defense mechanisms such as antibodies and serum, or from antibiotics.

Further experiments are in progress to define the fate of both leukocytes and, for example, renal cells that have ingested many *S. aureus* organisms. We are also attempting to examine how the bacteria inside a cell are able to expand infectious foci.

Acknowledgements: The authors thank Ms. Yoko Kobayashi and Ms. Atsuko Uchida for their technical assistances.

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