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Effects of Intrarenal Inoculation of *Staphylococcus aureus* on Mice

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The extreme susceptibility of the mouse kidney to infection with *Staphylococcus aureus* was confirmed by direct intrarenal (i.r.) inoculation with this organism. By applying Poisson's distribution formula to the results from infection of mice with small inocula (as few as one coccus or less per kidney), it was estimated that even three organisms could multiply in situ to produce abscess lesions, if the organisms were inoculated directly into the kidneys. This susceptibility of the mouse kidney for staphylococcal infection was not uniformly manifested with every strain, but correlated well with the virulence found through intravenous infection of the strains tested. The i.r. inoculation method by which infection of mice was easily established, using a small inoculum of *S. aureus*, similar to the amount suspected to occur in natural infections of man, is applicable for the analysis of the mechanisms of staphylococcal infections and any resulting immunity in man. The present paper describes the details and some experimental results obtained by our method.

One of the most serious obstacles encountered in the experimental studies on the mechanism of infection of and immunity to *Staphylococcus aureus* is that we have no suitable experimental model to reproduce accurately the human disease caused by this organism. Further, the rather large inocula experimentally required for most strains is far removed from those presumed to occur in natural infections in animals or in man.

Many techniques have been devised to enhance the pathogenicity of this organism in experimental infections. Intraperitoneal inoculation with gastric mucin (3), infection during acute starvation of the animal (14), and inoculation of the organisms impregnated on sutures (6) or mixed with foreign materials (1, 12) have been employed. However, none of these methods has been entirely satisfactory.

Recently, in the course of an experiment for another purpose, the authors found that virulent strains of *S. aureus* could readily multiply and produce abscesses in mouse kidneys when inoculated directly into this organ. Infections with some strains were usually possible even with extremely small inocula in the range of 10 to 100 organisms.

Freedman et al. (4, 5, 13) have reported on a series of experiments in which intrarenal (i.r.) inoculation of mice, guinea pigs, or rabbits with some species of bacteria, including staphylococci and *Escherichia coli*, could be used to elucidate the mechanisms involved in the establishment of bacterial nephritis or pyelonephritis.

We have developed a method of i.r. infection in mice with *S. aureus* to study the mechanism of staphylococcal infection of mice and its resulting immunity.

MATERIALS AND METHODS

Bacterial strains. The following *S. aureus* organisms were used. *S. aureus* 248 was isolated from a surgical source in our laboratory and the original strain and its various mutants were employed in our studies (8-10). *S. aureus* 248 (original) did not produce any toxins or hemolysins, but produced coagulase (bound and free), fibrinolysin, deoxyribonuclease, and penicillinase. It fermented mannitol and grew on 6% NaCl nutrient agar. A prominent feature of this strain was to produce a very small colony (1 mm or less in diameter) on nutrient agar medium. *S. aureus* 248 αH, mutant isolated from 248 (original), produced an alpha hemolysin. *S. aureus* 248 βH, another mutant isolated from 248 (original), produced beta hemolysin. *S. aureus* 248 Nβ was a mutant isolated from 248 βH which did not produce hemolysin but was similar to its parent in all other respects. *S. aureus* CN-15 was a coagulase-negative mutant isolated from 248 βH. All of these mutants were similar to the original strain 248 except for their respective mutant characteristics and their larger colonies on nutrient agar medium. They all had the same phage pattern (80/81/847B).

Other bacteria used were: *S. epidermidis* 73W, *Escherichia coli* C50, and *Streptococcus pyogenes* (group A) Saito.

Mice. The ICR strain of mice (4-week-old females, 13 to 15 g) was used.

Methods of i.r. inoculation: exposure of the kidney. Under slight narcotization with ether, mice were fixed prone, the skin of the back was sterilized with alcohol, an incision was made along the right paravertebral line, and the right kidney was approached via lumbar route. The exposed kidney was fixed with forceps and directly inoculated with the bacterial suspension under investigation.

Inoculation of bacteria. Ten milliliters of bacterial suspension of the desired concentration was placed in a petri dish (9 cm diameter). A sterilized sewing needle was immersed vertically to a depth of 1.5 mm into this bacterial suspension and immediately stabbed into the cortical layer of the above mentioned exposed kidney. The stabbing inoculation was repeated five times to effect an even and average inoculula distribution per kidney. With this method, approximately 10^{-4} of the cells contained in 1 ml of the suspension would be inoculated into the kidney. In some cases, when small inocula of one or two cocci (on the average) per kidney were required, 0.2 μliter of the suspension was injected by using a microsyringe.

Postinoculation. After inoculation, the open operation wound was clamped and pulled straight by its ends with forceps, and the wound edges were closed together with Aronalpha, a surgical bonding agent. The use of Aronalpha facilitated wound closure by shortening the inoculation process to 2 min at the longest.

RESULTS

i.r. Inoculation of some other bacterial strains. Figure 1 shows the results of the i.r. inoculation of ICR mice with three different doses of *S. aureus* 248 αH, *E. coli* C-50, *Salmonella choleraesuis* Hokkaido, *S. epidermidis* 73W, and *Streptococcus pyogenes* Saito.

Approximately 10^6, 10^4, and 10^2 organisms of the above five strains were inoculated into mouse kidneys. Also, five kidneys taken from other mice were stab-inoculated with the same size inocula of these bacterial strains and then immediately emulsified with a mortar and pestle. The resulting emulsions were then diluted, and portions were plated on nutrient agar plates followed by overnight culture at 37 C. The number of bacteria contained in the respective inocula were thus estimated by counting the colonies recovered.

Six days after inoculation, all mice were sacrificed, and the number of the living bacteria in the kidneys inoculated were similarly estimated.

All plots in Fig. 1 indicate the logarithmic average of the number of the living bacteria obtained from five mice

Remarkable multiplication of the inoculated bacteria was observed with *S. aureus* and *S. pyogenes*, but was not found with the other strains used.

i.r. Inoculation of various strains of S. aureus 248. Figure 2 shows the results of similar experiments with four strains of *S. aureus* 248: 248 αH, 248 βH, CN-15, and 248 (original). The LD_{50} values of these strains, estimated through intravenous infection of ICR mice, were 0.1, 0.06, 2.3, and more than 2.5 mg (wet weight), respectively.

Different patterns of the fate of the living cocci inoculated were observed with these four strains. In general, with the virulent strains, a remarkable multiplication of the inoculated cocci was seen. With the weakly virulent strains, a marked decrease in the numbers of surviving cocci or at least only slight increase was observed. However, the differences observed between the pattern of 248 αH and that of 248 βH is of interest. With 248 αH, a large inoculum resulted in an increase of the living cocci; however, when the initial inoculum was small (10^2 to 10^3 organisms per kidney), the average logarithmic number of living cocci recovered from the inoculated kidney after 6 days was not remarkably changed. In contrast to the former, the organisms of 248 βH did not increase from the large initial inoculum but could remarkably multiply to a population of 10^5 organisms from the initial inoculum of $10^{1.26}$ organisms. The pattern indicated by CN-15 was of a type intermediate between that of 248 βH and 248 (original). The most weakly virulent strain, 248 (original), showed the most rapid decrease in number of living organisms from the initial level of any inoculum size.

Fate of organisms of 248 βH strain inoculated i.r. into mice. The distribution and subsequent fate in various organs of the organisms of strain 248 βH inoculated i.r. into mice were examined with three kinds of inoculum doses. The living organisms in the kidney inoculated, the opposite kidney, blood, spleen, and liver were traced at intervals of 3 days.

Figures 3 to 5 show the results of the experiments with inoculum doses of $10^{6.08}$, $10^{3.4}$, and $10^{2.28}$ cocci, respectively, per kidney. The following three points were noticed from these figures. (i) The most rapid and marked multiplication of organisms was during the first day in the kidney inoculated. The number of living cocci recovered from the inoculated kidney on the first day was almost 10^4 times that recovered at 1 hr after the inoculation, and this increase was independent of the size of the initial inoculation. (ii) The distribution of the organisms inoculated in the kidney, the opposite kidney, the blood, and the reticuloendothelial system (RES) organs (such as the spleen and liver) was extremely poor when contrasted to those organisms inoculated intravenously (Fig. 6). (iii) The period during which the inoculated organisms survived in the host was

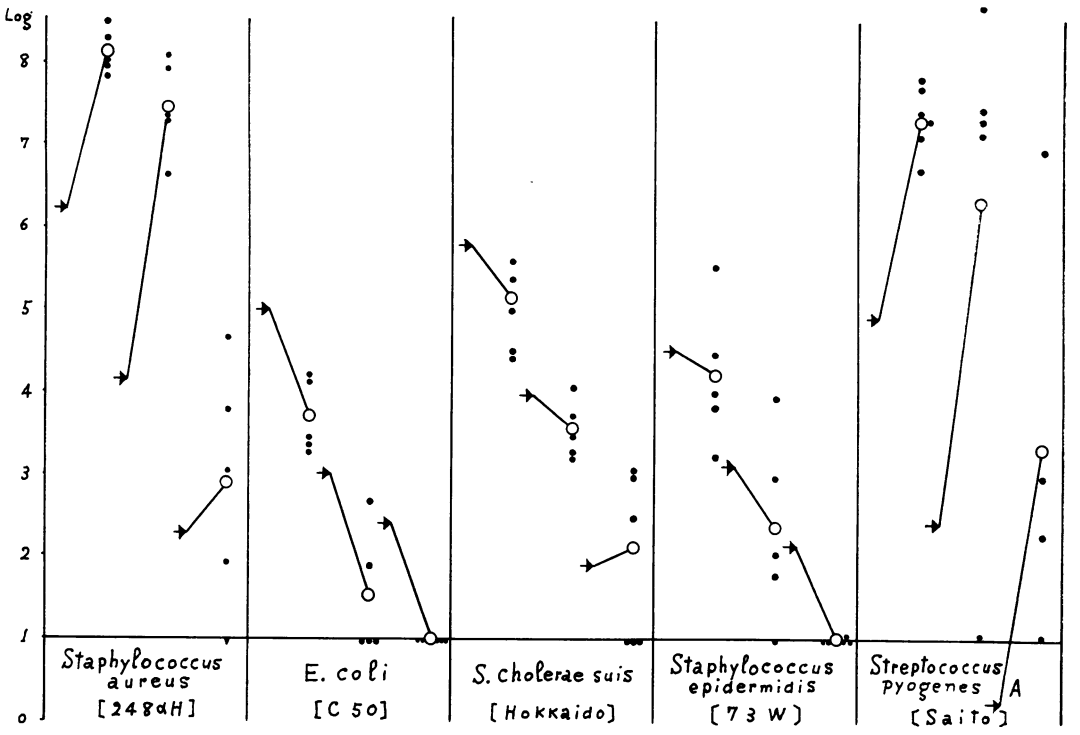

FIG. 1. *Growth of various bacterial strains in the kidney directly inoculated with three different doses. Arrow indicates the level of the initial inoculum. Symbols:* (●) *individual and* (○) *average log numbers of the colony-forming units recovered from the inoculated kidney after 6 days.*

not as long as for those inoculated intravenously, which persisted for 4 to 6 weeks according to results from our previous experiments.

ID_{50} in the i.r. infection. The dose to establish infection in 50% of the mice inoculated i.r. with the organisms (i.e., ID_{50}) was estimated with the same strains of *S. aureus* 248. Serial fivefold dilutions were made from the desired concentration of the bacterial suspension of the strain to be tested. Portions of each dilution were inoculated into the kidneys of five mice as a group. Six days after inoculation all mice were sacrificed, and the number of living cocci from the kidney inoculated was estimated. The mice in which the number of bacteria recovered was more than one hundred times the inoculated dose were regarded as infected.

The ID_{50} was calculated from the proportion of the infected mice in each tested group using the Behrens-Kärber method (7). Figure 7 indicates the results of experiments with 248 αH and 248 βH. Kidney-shaped figures show the inoculated kidney, and black spots indicate the population of the living cocci recovered from the kidney after 6 days. Two spots correspond to a 1 log count of living cocci in which fractions of 0.5 and above are counted as another unit; a measurement less than 0.5 unit was discounted. The ID_{50} of 248 βH and 248 αH were calculated to be 19 and 247 cocci. Interpreting these numbers of cocci required for ID_{50}, the former was about 10 times more virulent than the latter. On the other hand, there can be seen a tendency similar to those noticed in the results of i.r. inoculation of various strains of *S. aureus* 248. The 248 αH strain inoculated with larger doses can multiply at a higher rate than those inoculated with smaller doses, whereas the situation with the 248 βH strain is almost reversed.

Minimal infectious dose calculated by Poisson's formula. The minimal dose to establish infection via i.r. inoculation was estimated with the 248 βH strain, applying Poisson's distribution formula to the result from infection of mice with inocula as small as one coccus or less per kidney. The results for one of the experiments are mentioned in Table 1. By using a 10-μliter microsyringe for gas chromatography, 0.2 μliter of cell suspension (an average of 0.77 organisms)of strain 248 βH was inoculated into the kidneys of 27 mice. Six days after the inoculation, all of the mice were sacrificed, and their kidneys were examined for

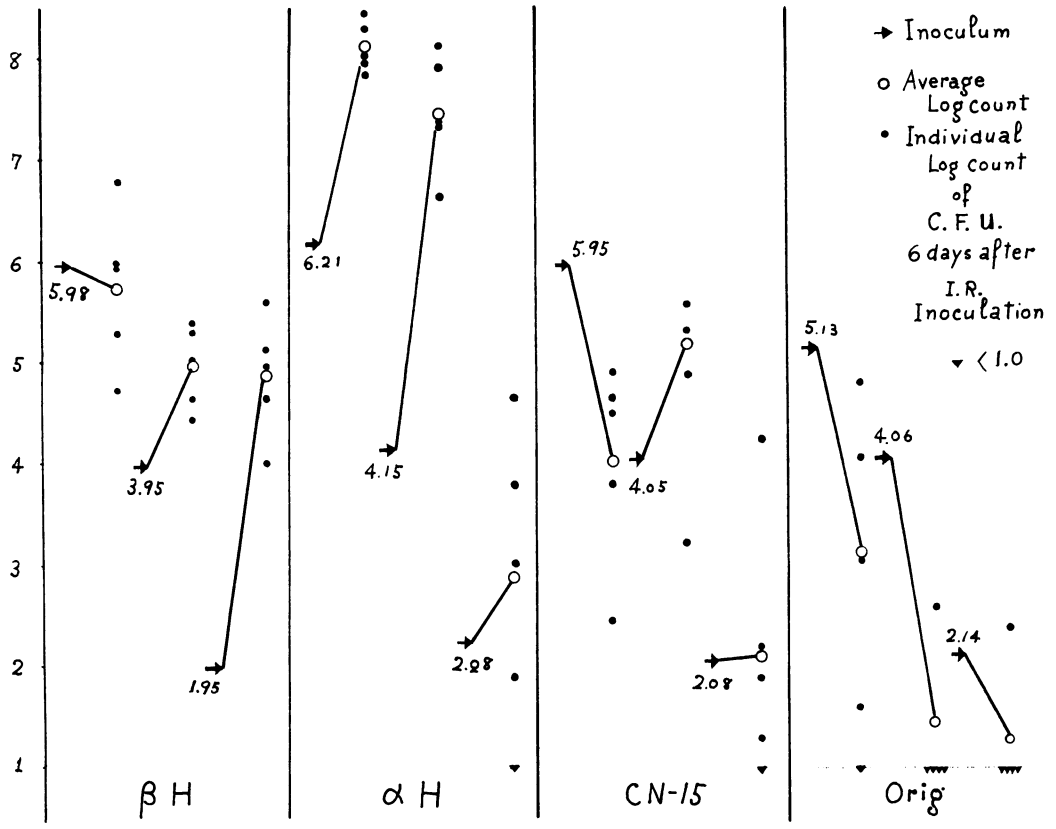

FIG. 2. *Growth of four strains of S. aureus no. 248 in the kidney directly inoculated with three different doses. Arrow indicates the level of the initial inoculum. Symbols: (●) individual and (○) average log numbers of the colony-forming units recovered from the inoculated kidney after 6 days.*

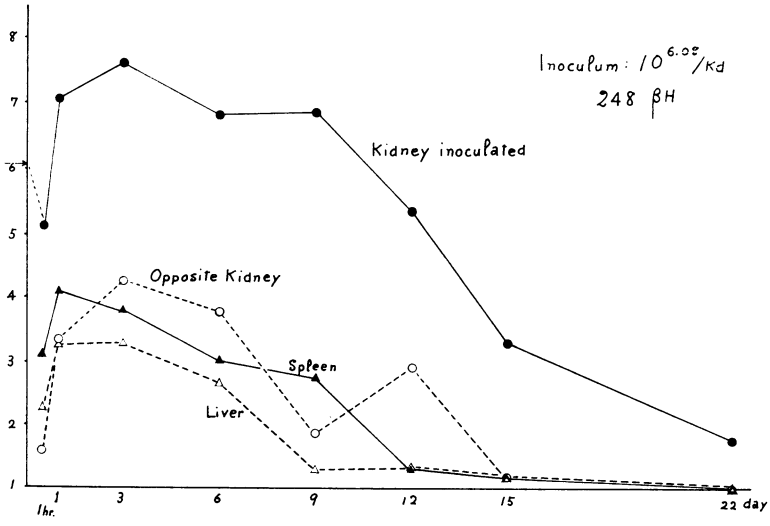

FIG. 3. *Distribution and subsequent fate of the intrarenally inoculated ($10^{6.08}$ colony-forming units per kidney) organisms of S. aureus 248 βH in various organs of mice.*

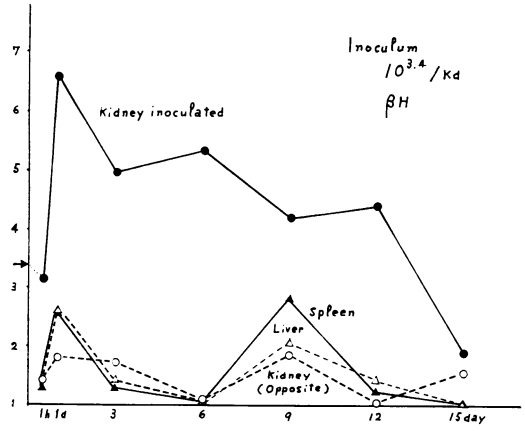

FIG. 4. *Distribution and subsequent fate of the intrarenally inoculated ($10^{3.4}$ colony-forming units per kidney) organisms of S. aureus 248 βH in various organs.*

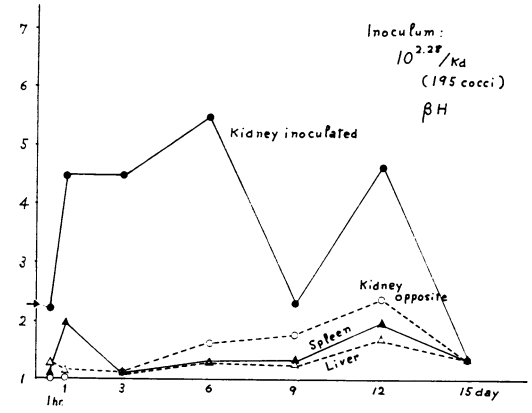

FIG. 5. *Distribution and subsequent fate of the intrarenally inoculated ($10^{2.28}$ colony-forming units per kidney) organisms of S. aureus 248 βH in various organs of mice.*

multiplication of organisms and formation of abscess lesions. Only one of the mice showed an apparent formation of a kidney abscess, and more than 10^6 cocci were recovered from the inoculated kidneys. However, the remainder of the mice showed neither multiplication of the organisms nor abscess formation. The proportion of the infected mice was thus estimated as 0.038. On the other hand, if 0.77 (the average number of the inoculated organisms) is substituted for m in the Poisson's formula, $P_r = e^{-m}m^r/r!$, the probability with which various number of organisms were randomly distributed in the inoculated kidney could be calculated (Table 1). The second and third columns, respectively, indicate the progressive cumulations from the

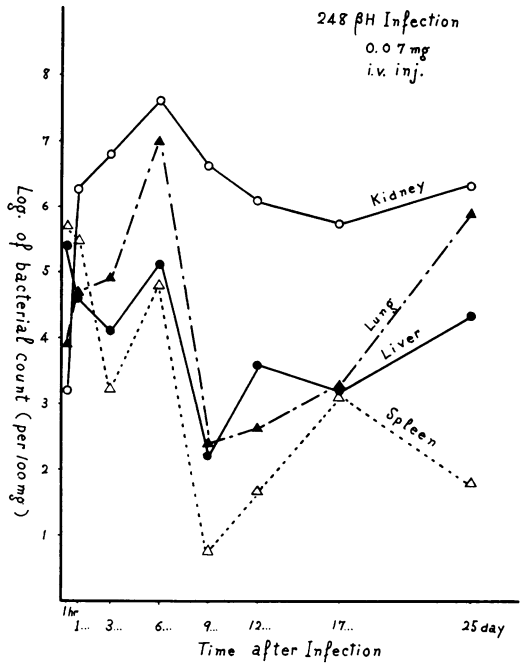

FIG. 6. *Distribution and subsequent fate of the intravenously inoculated organisms of S. aureus 248 βH in various organs of mice.*

values of P_0 to those of P_1, P_2 $\cdots$ P_6 and in reverse from P_6 to P_0. The value calculated for P_7 is practically negligible.

The number 0.038 is closest to the value of P_3 and the cumulation of the values of P_6 through P_3 and is furthest from the values of P_2 and P_4.

Subsequently, it is a reasonable presumption that three cocci at most were distributed in the kidneys where infection was observed in this experiment.

DISCUSSION

In several papers, Freedman and others previously reported data on experimental nephritis or pyelonephritis with several species of bacteria (including *S. aureus* and *E. coli*) in rabbits, guinea pigs, and mice when these pathogens were directly inoculated into kidneys.

The authors have improved on their technique and elaborated on the i.r. inoculation method to provide a routine experimental approach to the staphylococcal infection of mice.

Lumbar approach of the kidney and the use of Aronalpha facilitated this method. Not more than 2 min per animal is needed to complete an inoculation. Repeated (five times) stab-inoculation was sufficient for effecting an even and

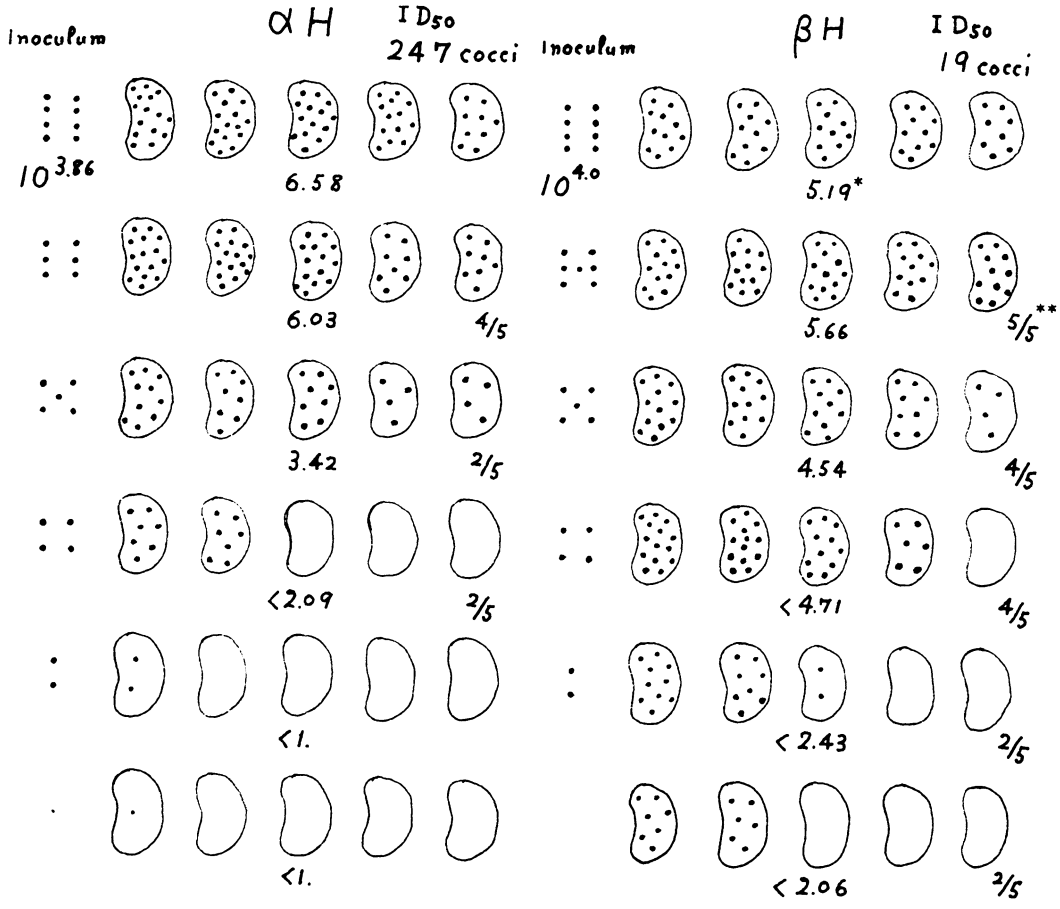

FIG. 7. *Comparison of the growth of 248αH and 248βH in the kidney directly inoculated. Black spots in the kidney-shaped figures show the log numbers of the living cocci recovered from the kidney inoculated after 6 days, each spot corresponding to 0.5 log. Symbols: *, average of the log numbers of colony-forming units recovered from the kidney of five mice. **, proportion of the mice showing the recognizable growth of the inoculated organisms to the mice tested. The inocula were serially fivefold decreased, and the effective 50% infectious dose, ID_{50}, was estimated from experimental results after Behrens-Kärber's method.*

average inoculum distribution per kidney and could be used for the quantitative estimation of virulence of this organism. By stab-inoculation, we can also minimize the volume of the liquid contained in an inoculum, localize the inoculated organisms in situ, and facilitate the contact of organisms with target cells or loci distributed in the cortical region of mice kidneys. Furthermore, the use of the microsyringe made it possible to inoculate accurately extremely small volumes (such as 0.2 μliter) of bacterial suspensions.

One of the merits of this method is that the inoculated organisms do not distribute into the main RES organs (liver or spleen) until they have large populations in the inoculated kidney. In contrast, intravenous or the intraperitoneal infection requires an inoculum of more than 10^6 or 10^7 cocci, and almost 90% of the inoculated organisms are immediately distributed and incorporated in RES organs or phagocytized. Thus, we may not remove the unnatural influence due to the RES, which is activated from the very beginning stages of infection on the response of the infected animal, especially when such large numbers of organisms are incorporated in the RES organs.

Although no histological examination was accomplished, it appeared that as few as three cocci could form an abscess in the kidney inoculated from which millions of living cocci were later recovered.

Because of the small size of inoculum used, we

TABLE 1. *Minimum infectious dose estimated from Poisson's formula*[a]

$m = 0.77$	Progressive cumulations	
	From P_0 to P_6	From P_6 to P_0
$P_0 = 0.4649$	0.4649 ↓	1.0010
$P_1 = 0.3566$	0.8215	0.5361
$P_2 = 0.1368$	0.9583	0.1795
$P_3 = 0.0349$	0.9932	0.0427[b]
$P_4 = 0.0067$	0.9999	0.0078
$P_5 = 0.0010$	1.0009	0.0011
$P_6 = 0.0001$	1.0010	0.0001 ↑
$P_7 = 0.0000$		

[a] Intrarenal infection of 248 βH with an inoculum size of as small as 0.77 cocci (on an average) per kidney caused abscess formation in the kidney inoculated in proportion of 1/27 (0.037). Poisson's formula: $P_r = e^{-m} m^r/r!$.

[b] The nearest number to the rate of abscess formation.

feel that this experimental model reproduces the conditions of natural infection in man with this organism.

It should be noticed that there was observed some interesting differences between the virulence of 248 αH and 248 βH (Fig. 2 and 6). We cannot explain these different manifestations of virulence at present, but further study is in progress to analyze the quantitative and qualitative differences in their virulence and among other strains of *S. aureus* using this i.r. inoculation method.

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