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VIRULENCE OF GENUS *STAPHYLOCOCCUS*

III. Studies on the Pathogenicity to Mice

BY

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ABSTRACT

Six representative strains of staphylococci were selected on the basis of DNase activity and inoculated into the mice to study the correlation of the presence of this enzyme to the virulence of the staphylococci. Two strains of staphylococci among them produced DNase but not the free coagulase, and one of these two strains had an obvious virulence against the mice.

The results of experiments suggest that there are at least certain strains which may be designated as *Staphylococcus aureus* among these DNase-producing staphylococci producing no free coagulase. This is an additional evidence that this enzyme activity should be adopted as a criterion to distinguish from the other *S. aureus* which has lost the free coagulase activity.

In this sense, the staphylococci producing neither free coagulase nor DNase are after all *Staphylococcus epidermidis,* regardless of their ability to ferment mannitol or not.

INTRODUCTION

The tests for free coagulase activity and mannitol fermentation are almost universally accepted as the primary criteria for distinguishing *Staphylococcus aureus* and *Staphylococcus epidermidis*[1]. However, there are some strains of staphylococci, unable to produce free coagulase but able to ferment mannitol. These strains can be determined as neither *S. aureus* nor *S. epidermidis* by the above-mentioned criteria.

Miyoshi et al.[2] previously reported that about 30% of the 151 clinical isolates of staphylococci, which were classified into 16 biological types (type A to type P), could ferment mannitol but could not produce free coagulase, and that these strains could be divided into 2 groups by their ability to produce deoxyribonuclease (DNase). Taking into account the origins of these staphylococci, the proposal of adopting the DNase activity as a criterion of staphylococcal virulence seems to have some significance.

In the present study, 4 strains of mannitol-fermenting staphylococci producing no free coagulase were selected. For the purpose of confirming the relationship between the virulence and DNase activity by animal experiments, groups of mice were infected with these staphylococci and comparison was made of their mortality rates due to staphylococcal septicemia.

MATERIALS AND METHODS

Bacteria used

Among the staphylococci divided into 16 biological types in the previous reports[2], 4 strains, which were able to ferment mannitol but unable to produce free coagulase,

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Table 1. Patterns of biological characteristics of staphylococci used in the experiments on infection

Staphylococci		Free coagulase	Mannitol fermentation	DNase activity	Phosphatase activity	Gelatinase activity
Strain No.	Biological type[a]					
S-1	A[b]	+	+	+	+	+
S-17	F	−	+	+	+	+
S-19	H	−	+	+	−	+
S-21	I	−	+	−	+	+
S-30	K	−	+	−	−	+
S-35	P[c]	−	−	−	−	−

[a] See literature 2)
[b] *Staphylococcus aureus*
[c] *Staphylococcus epidermidis*

were selected (S-17, S-19, S-21 and S-30). In addition, a typical *S. aureus* (S-1) and a typical *S. epidermidis* (S-35) were used as controls. The patterns of the biological characteristics of these 6 strains are summarized in Table 1.

Markers of the staphylococci

To decide on the markers of these 6 strains in culturing the organisms from the infected mice, the minimum inhibitory concentration of the 5 antimicrobials against these strains were tested by the agar dilution method as a preliminary experiment. These antimicrobial drugs were dihydrostreptomycin (SM), chloramphenicol (CP), tetracycline (TC), nalidixic acid (NA) and polymixin B (PLB). When necessary, agar plates containing certain antimicrobials in suitable concentrations were heavily inoculated with one of these strains. The colonies growing after the incubation were reinoculated onto the fresh media containing the same drug but with a concentration twice as much as the first media. By repeating these procedures, a certain strain showing a high resistance to certain antimicrobials was obtained. The media used in these experiments were Antibiotic Medium No. 3 (Difco), and Sensitivity Test Agar (Eiken). Six strains of staphylococci thus obtained were again tested and confirmed for their biological characteristics and then used in the infection experiments.

For further reference, sheep red blood cell-agar plates were prepared and the types of hemolysis of these 6 strains of staphylococci were tested.

Bacterial suspensions for inoculation

Six strains of staphylococci were preincubated on sheep blood agar plates (basal medium was the Heart Infusion Agar, Eiken) at 37°C overnight. Being subcultured once more on the same media, each strain was inoculated in 5 m*l* of Trypto-soy Broth (Eiken) and cultured at 37°C for 24 hours by shaking. The cultures were centrifuged at 8,000 × g for 15 minutes, and the sediments were resuspended in a suitable volume of physiological saline. Concentration of each bacterial suspension was determined by either optical density or colony counting so as to contain about 10^9 colony-forming units (CFU) per m*l*.

Animals and staphylococcal infections

White, 4 weeks of age, 18 to 20g male mice, strain DD were used.

Mice were divided into 7 groups, each

consisting of 30 animals, and the groups were designated as A_1, A_2, F, H, I, K and P. These letters represent the types of staphylococcal biological patterns previously described. Mice of groups A_1 and A_2 were respectively infected with 10^8 and 10^7 CFU of washed type A staphylococci, suspended in 0.1 m*l* of physiological saline, by injecting the organism into the tail vein. Mice of 5 groups except groups A_1 and A_2 were inoculated with 10^8 cells of the representative 5 types of staphylococci by the same route. Then the mice were observed for 2 weeks after the infection. When the mice died from staphylococcal septicemia during the period of observation, they were autopsied immediately and bacterial cultures were made from their lungs, kidneys and heart blood in which the drug resistance and type of hemolysis of each bacterial strain were used as markers to distinguish from each other.

For culturing the marked staphylococci, the Sensitivity Test Agar (Eiken) plates containing the appropriate antimicrobials and Mannitol-salt Agar (Eiken) plates without drugs were used.

All mice surviving for 2 weeks after the infection were sacrificed on the 15th day and the organs and blood were cultured similarly.

Results

Susceptibilities to antimicrobials and resistance markers of staphylococci

Susceptibilities to SM, CP, TC, NA and PLB of the 6 strains of staphylococci tested by the agar dilution method are summarized in Table 2.

From these results, it was decided to use the following media to isolate selectively the strains from the cultures of the organs and blood of the mice. These were the Sensitivity Test Agar containing 10 μg of BLB per m*l* for the selection of the strains S-1 and S-17, 50 μg of NA for S-30, and 10 μg of TC for S-35, respectively.

As to the strain S-21, this strain was first inoculated on the media containing 50 μg of SM per m*l*, and then the colonies obtained were transferred onto the media with 100 μg/m*l* of the same antibiotics. Colonies appearing on this media were subcultured several times, thus the strains of S-21 resistant to more than 100 μg/m*l* of SM were obtained. These strains were tested for their biological characteristics, and after confirming that all the characteristics were not altered, the bacterial suspension for infecting mice was made from one of these strains. For culturing this strain from the mice, 50 μg/m*l* of SM

Table 2. Minimum inhibitory concentrations of antimicrobial agents and types of hemolysis of staphylococci

Staphylococci		Antimicrobial agents					Type of hemolysis
Strain No.	Biological type	Dihydrostreptomycin	Chloramphenicol	Tetracycline	Nalidixic acid	Polymixin B	
S-1	A	6.3[a]	3.1	<0.39	50	25	$\alpha\beta$
S-17	F	3.1	1.6	<0.39	50	25	β
S-19	H	>100	1.6	<0.39	>100	3.1	δ
S-21	I	25	0.78	<0.39	25	12.5	δ
S-30	K	>100	3.1	<0.39	>100	3.1	δ
S-35	P	<0.39	25	25	25	3.1	δ

[a] μg/m*l*

Table 3. Virulence of staphylococci in mice

Staphylococci (Type)			1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S-1 (A)	Inoculated 10^8 CFU	Mice died	0	2	6	7	5	3	5	2							
		Culture		+[c]	+	+	+	+	+	+							
	10^7 CFU	Mice died	0	0	0	0	2	1	2	4	2	1	3	1	3	1	10[b]
		Culture					+	+	+	+	+	+	+	+	+2 −1	+	+3 −7
S-17 (F)	10^8 CFU	Mice died	0	1	0	1	0	1	1	5	2	0	2	1	1	1	14
		Culture		+		+		+	+	+3 −2	+		−	−	−	−	−
S-19 (H)	10^8 CFU	Mice died	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30
		Culture															−
S-21 (I)	10^8 CFU	Mice died	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30
		Culture															−
S-30 (K)	10^8 CFU	Mice died	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30
		Culture															−
S-35 (P)	10^8 CFU	Mice died	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30
		Culture															−

[a] Days after infection, [b] Sacrificed mice, [c] +: Culture positive, −: Culture negative

were added to the media.

Infections and mortality rates of mice

Seven groups of mice were infected by injecting intravenously 0.1 m*l* of the staphylococcal suspensions of strains S-1 (type A), S-17 (type F), S-19 (type H), S-21 (type I), S-30 (type K) and S-35 (type P), as described under Materials and Methods. The prognoses of the mice till 2 weeks after the infection are shown in Table 3, and the cumulative mortality rates (CMR) taken from Table 3 are illustrated in Figure 1.

The first two mice of group A_1, which were infected with 10^8 CFU of strain S-1, died on the 2nd day of infection. Then, CMR of the mice of this group rose rapidly and straight, and all mice died by the 8th day from staphylococcal septicemia. In this group, the CMR of 50% was obtained on the 4th day. However, in the animals of group A_2, infected with 10^7 CFU of the

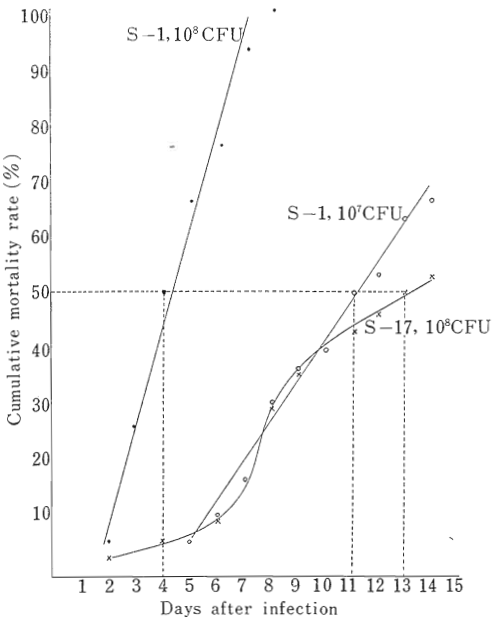

Fig. 1. Cumulative mortality rate of mice infected with each type of staphylococci

same strain; the first death occurred on the 5th day of infection, and the CMR of the mice rose straight but not so rapidly as that of group A_1. The fifty percent CMR of this group was attained on the 11th day, and on the 15th day of the experiment 10 mice were still alive.

Strain S-1 staphylococci were cultured from all mice of group A_1 which died. However, among the mice of group A_2, the bacteria could not be cultured from 1 of 3 mice dying on the 13th day and from 7 of 10 mice which were sacrificed on the 15th day.

On the other hand, the CMR of group F, inoculated with 10^8 CFU of strain S-17, rose in a characteristic sigmoid curve. Thirteen days were required for this group to attain the CMR of 50%. Fourteen mice survived on the last day of the experiment.

Results of staphylococcal culture of this group were also rather specific. S-17 staphylococci could neither be isolated from 2 of 5 mice dying on the 8th day nor from the 5 other mice dying on the 11th to the 14th day of infection. Bacterial cultures from the surviving 14 mice on the 15th day of the experiment were also all negative.

As to the groups H, I, K and P infected respectively with strains S-19, S-21, S-30 and S-35, no death occurred throughout the entire period of the observation. All cultures made on the last day of the experiment were shown to be negative also.

It was found from these experiments that the mice of group F, inoculated with type F staphylococci, died from type F staphylococcal septicemia in considerable population, but the mortality rate of the mice of this group was fairly low in comparison with that of groups A_1 and A_2, suffered from the infections of type A staphylococci. That is to say, there are certain strains of DNase-producing staphylococci, which are designated as neither *S. aureus* nor *S. epidermidis* from the criteria of mannitol fermentation and free coagulase activity, showing an evident virulence against the mice. Additionally, it was recognized that the strains of staphylococci, lacking the ability to produce both free coagulase and DNase, show no virulence against the mice regardless of their mannitol fermentation.

Discussion

Free coagulase test is the most widely used criterion for identifying the pathogenic staphylococci (*S. aureus*), and many clinical laboratories still rely solely on this test to distinguish *S. aureus* from the closely related saprophytic organisms. However, it has been recognized by many investigators[2,3] that on rare occasions the staphylococci thought to be undoubtedly pathogenic may lose this characteristic, so some new criteria available for such staphylococci become necessary to estimate their virulence.

The DNase activity of the strains of *S. aureus* has been studied extensively since its discovery by Cunningham *et al.*[4] The remarkable thermostability, as well as the role of the calcium ions, and the characteristics of the dinucleotides obtained from the hydrolyzed nucleic acids make this enzyme a subject of interest chemically and bacteriologically to many investigatiors[5,6]. The marked relation of DNase to the virulence of the staphylococci was first pointed out by Weckman and Catlin[7] in their observation on the high DNase activity in the clinical isolates of *S. aureus*. Since then, the correlation between this enzyme and the staphylococcal virulence confirmed by the succeeding several works[2,8–13]. To detect the staphylococcal DNase activities, Jeffries *et al.*[14] incorporated the deoxyribonucleic acid (DNA) in the agar medium

and streaked the medium with a test organism. By flooding the medium with 1N HCl after the incubation, the clear zone appearing around the colonies indicated the presence of DNase. Smith et al.[15] modified this method by adding 0.005% methyl green to the medium. Recent assays for the staphylococcal DNase were done by the metachromatic agar diffusion method using toluidine blue by Lachica et al.[16,17] and the turbidimetric assay method of Erickson and Deibel[18]. With these various techniques, Lachica et al.[5,19] and Barry et al.[20] detected the DNase from some species of gram-positive cocci, compared their characteristics and concluded that the thermostable DNase is specific to *S. aureus*. The DNase discussed in the present study were also thermostable (Miyohsi, unpublished data), but the data regarding its correlation with the animal experiments are not yet available.

For this reason, animal experiments using mice were carried out with special reference to the virulence of the DNase-producing staphylococci. The results obtained showed that the strain S-17 of the staphylococcus, without the free coagulase activity, killed the mice by staphylococcal septicemia. The virulence of this type F staphylococcus suggested evidently that this strain was pathogenic. In other words, this strain was close to *S. aureus* which had lost the ability to produce free coagulase. However, the virulence of this strain was fairly low compared with the typical *S. aureus* strain S-1. The difference in the period of days required to attain the 50% CMR of mice infected with these 2 strains clearly reflected the discrepancy in their virulence. Strain S-17 was subcultured on the blood agar plates for 10 further generations, but the ability to produce free coagulase never appeared.

Prognosis of the mice infected with the S-17 staphylococcus showed several differences from that of the S-1 infected animals. In both group A_1 and group F, the first death occurred on the 2nd day of the experiment, but the mice of group A_1 died totally by the 8th day, and the 14 mice of group F were still alive on the 15th day. The CMR of group F mice rose abruptly on the 7th to the 9th day of infection and then became slow again. Furthermore, from the mice of group F which died on and after the 11th day of infection, no colony of the S-17 staphylococcus was cultured. It seems from this observation that the resistance of the group F mice to the infection of the S-17 staphylococcus reached the minimum on the 8th day, and the mice born during this period might gradually recover from the infection. However, no mouse of any group infected with staphylococci other than strains S-1 and S-17 died at all during the period of observation. Accordingly, the cause of the death of the group F mice, from which no positive culture of S-17 could be obtained, should not be unrelated to the S-17 infection.

Though the S-17 staphylococci appear to be eliminated from the infected mice by the 10th day of infection, the organisms may either be the primary cause of death by septicemia or the secondary cause by prostration after the elimination of the staphylococci. This will offer, hereafter, several interesting subjects concerning the specific or nonspecific resistance to the staphylococcal infection.

Mice of group H, infected with another DNase-producing staphylococcus S-19, did not die throughout the observation period. As compared with the biological characteristics of strains S-17 and S-19, the latter was lacking in phosphatase activity. However, correlation between the phosphatase

activity and free coagulase was rather low[2,17], and the estimation of this enzyme in staphylococcal virulence was somewhat questionable. As yet notable was the results of the hemolysis tests on these 6 strains of staphylococci that strains S-1 and S-17 had ß-hemolysin but all the others did not. Though some reports[21-23] concerning the relationship between the DNase and staphylococcal hemolysin are available, more precise studies should be expected on this subject.

S-21 and S-30, both mannitol-fermenting strains of staphylococci producing neither free coagulase nor DNase, had no virulence against the mice. Therefore, it may be considered that the staphylococci without these 2 enzyme activities are *S. epidermidis,* though they may have the ability to ferment mannitol.

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