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In Vitro and In Vivo Activity of Penicillinase Inhibitor KA-107 Against *Staphylococcus aureus* FS-1277

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The activities of penicillinase inhibitor, KA-107 extracted from culture filtrates of *Streptomyces gedanensis* ATCC 4880, were studied by using penicillin-resistant *S. aureus* FS-1277 and by means of in vitro and in vivo tests.

When the penicillin-resistant *Staphylococcus aureus* FS-1277 strain is cultured in vitro, production of penicillinase starts immediately, reaching a maximum after 1 h of cultivation, and then decreases rapidly. When penicillin was added as an inducer at zero time, the production of penicillinase in the culture was four to five times greater than in the case where penicillin was not added. When KA-107 and penicillin were added to the culture simultaneously, the growth of the penicillin-resistant strain was completely inhibited. After a certain period of time, however, growth of FS-1277 started suddenly. This phenomenon seems to indicate that the induced penicillinase (PCase) was inactivated by KA-107, and FS-1277 thus became vulnerable to the action of penicillin, but as soon as penicillin was exhausted, the surviving FS-1277 cells started to grow rapidly again. Therefore, KA-107 combined with penicillin was added repeatedly at certain time intervals for continuous inhibition of growth over a long period. This was also the case in the in vivo experiments. Mice were infected with 10^{-2} mg (wet weight) of *S. aureus* FS-1277 per kg, and KA-107 was administered intraperitoneally (IP). Then, either penicillin alone or KA-107 combined with penicillin was given repeatedly.

In our first report (3) it was stated that we established a screening method for PCase inhibitors, and succeeded in isolating KA-107, a PCase inhibitor, produced by *Streptomyces gedanensis*. By combined use of KA-107 together with PC-G, ampicillin, and D- or L-phenethicillin, which are all inactivated by the PCase derived from *S. aureus* FS-1277, the growth of this penicillin-resistant strain was inhibited.

In this investigation, the activity of KA-107 in vitro and in vivo is described in detail. In the first investigation, it was recognized that the growth of FS-1277 was inhibited when a certain amount of KA-107 and PC-G were used together, but after a certain period of time growth was resumed. The reason for the resumption was sought in this report. Next, mice were infected with FS-1277, and an examination was carried out to determine whether or not the combined use of KA-107 and PC-G could prevent the infection of mice caused by FS-1277.

MATERIALS AND METHODS

Test strain. As a PC-resistant strain, *S. aureus* FS-1277 was used for testing the penicillinase-inhibiting activity of KA-107, as in the previous investigation (5).

Preparation of PCase inhibitor KA-107. The preparation of PCase inhibitor KA-107 was carried out as in the previous investigation (5).

Estimation of penicillinase inhibitor. Activity of penicillinase inhibitor was measured by the modified bioassay method as in the first investigation (3).

In vitro growth experiment. A 0.1-ml amount of *S. aureus* FS-1277 culture in the stationary growth phase was added to 5 ml of the medium in a Monod tube and incubated with shaking at 37 C. The cell concentration in the liquid medium was adjusted to an optical density of 0.05 to 0.1 at 660 nm by a Coleman spectrophotometer. This value corresponds to 10^8 cells of FS-1277.

Experimental treatment in mice. FS-1277 was cultured in heart infusion broth while shaking for 18 to 20 h at 27 C. The cells were harvested by centrifugation (3,400 × *g*, 10 min) and washed three times with 0.85% saline. A dose of 10^{-2} mg (wet weight) of *S. aureus* FS-1277 per kg in 5% mucin (Wako Pure Chemical Industries, Ltd.) was injected IP into DD-S mice, about 20 g each, in groups of 10 mice. KA-107

combined with PC-G was injected IP at 15- and 30-min intervals, from one to three times. Survival and change in weight of the treated mice were observed for 7 days to estimate the effect.

RESULTS

In vitro experiments: (i) effect of inoculum size. Various sizes of inoculum (10^{10}, 10^{8}, and 10^{6} cells) of *S. aureus* FS-1277 were inoculated into tubes to which 1.25 U of KA-107 per ml and 400 U of PC-G per ml was added simultaneously. After shake culturing at 37 C, the increase in bacterial cells was determined by optical density in time sequence. The growth started within 2 h in the case of 10^{10} cells; in the case of 10^{8} cells, it was a little delayed, starting after 3 h (Fig. 1). In both cases, growth reached a maximum 6 h later. However, in the case of 10^{6} cells, even after 14 h, growth was still completely inhibited. Thus, inhibition of growth of FS-1277 depended upon the number of the cells added, and in the case of 10^{6} cells, a single addition of KA-107 combined with PC-G completely inhibited the growth.

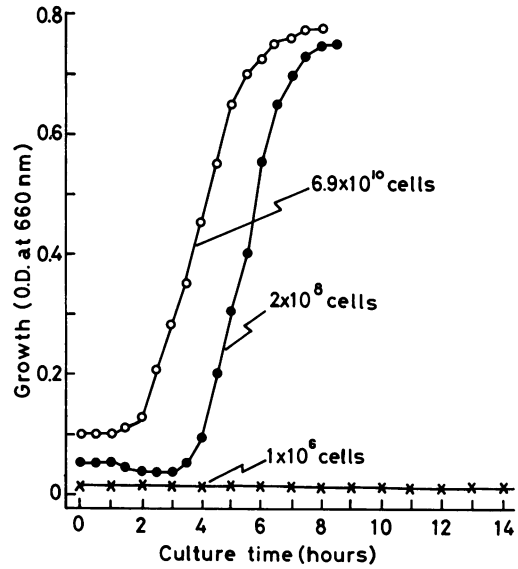

FIG. 1. *Effect of inoculum size on the growth of S. aureus FS-1277 with both KA-107 and PC-G. Both KA-107 (1.25 U/ml) and PC-G (400 U/ml) were added at 0 h of incubation at 37 C.*

(ii) Relationship between PCase production and cell growth. When 10^{8} cells of FS-1277 were inoculated at 37 C, a maximum of 2.5 U of PCase per ml was produced (Fig. 2). However, when 100 U of PC-G per ml was added to the culture at the beginning, induction of PCase occurred, and in 1 h, a PCase production peak of 12 U/ml was observed. On the other hand, as shown in Fig. 3, when 400 U of PC-G per ml and 1.25 U of KA-107 were added at the same time, both growth of FS-1277 and PCase production was inhibited. However, about 4 h afterwards, the growth of the cells started, accompanied by penicillinase production.

From this finding, it appears that in the presence of PC-G, PCase is induced in a short time, so that it is necessary to add KA-107 within 30 to 60 min after culture incubation to inhibit cell growth.

(iii) Relationship between number of cells and their growth. Growth of FS-1277 was inhibited by the combined use of PC-G and KA-107, but there was rapid growth after a certain period of time. To find out the reason for the occurrence of such growth, the number of live cells in the culture during the stationary phase was counted. When a mixture of PC-G and KA-107 was added to a culture containing 10^{8} cells of FS-1277, the number of live cells decreased rapidly within 2 h to less than 10^{5} cells per ml, and this number was maintained for about 3 h (Fig. 4). However, even during this period the cells did survive (the so-called static phase) and after about 5 h the number of cells increased rapidly, reaching 10^{11} cells per ml.

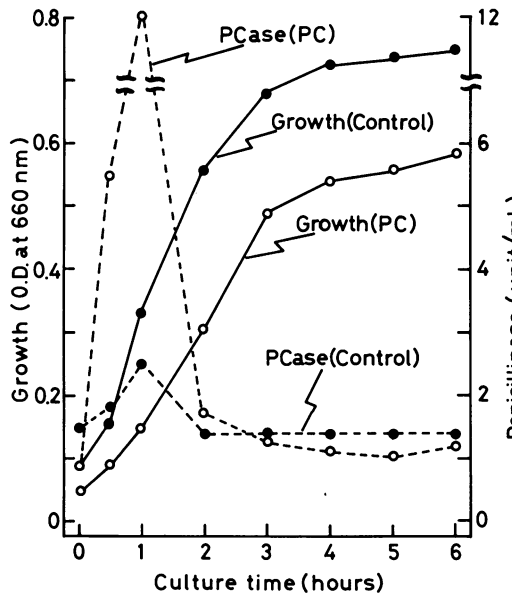

FIG. 2. *Relationship between growth and PCase production. PC:PC-G (100 U/ml) was added at 0 h of incubation at 37 C.*

(iv) Continuous inhibition of cell growth. (a) When 400 U of PC-G per ml was added to FS-1277 culture and 1.25 U of KA-107 per ml was

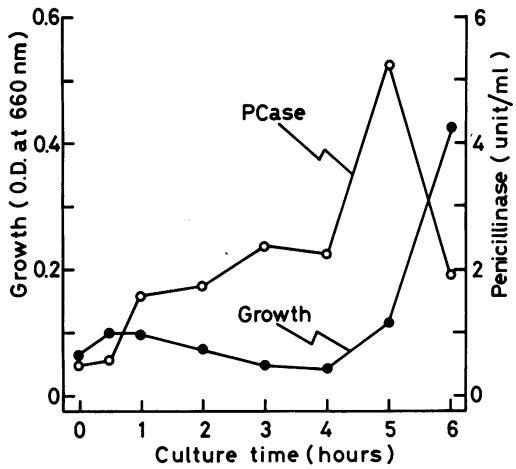

FIG. 3. *Relationship between growth and PCase production. Both KA-107 (1.25 U/ml) and PC-G (400 U/ml) were added at 0 h of incubation at 37 C.*

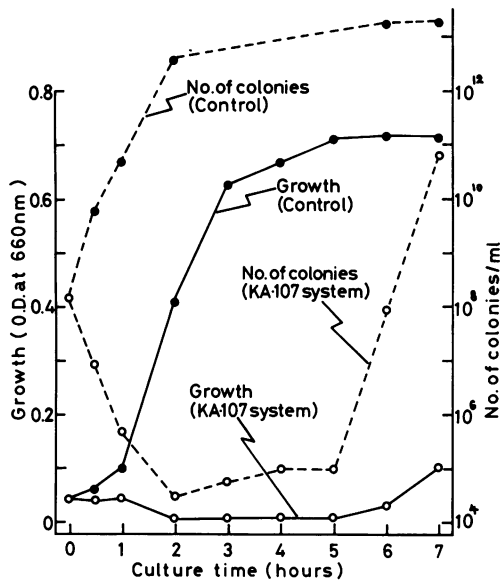

FIG. 4. *Relationship between growth and viable cells. KA-107 system: both KA-107 (1.25 U/ml) and PC-G (400 U/ml) were added at 0 h and 30 min after incubation at 37 C.*

then added at 15 or 30 min after incubation, growth of FS-1277 in each case was inhibited until 17 h as shown in Fig. 5.

(b) When 1.25 U of KA-107 per ml was added to the FS-1277 culture and 400 U of PC-G per ml was later added at 30, 60, or 90 min after incubation, growth started after 15 h when PC-G was added 30 min later. When it was added 60 min later, it started after 13 h, and when it was added 90 min later, it started after 11 h as shown in Fig. 6.

(c) Simultaneously, with the inoculation of FS-1277, either 400 U of PC-G per ml or 400 U of PC-G per ml combined with 1.25 U of KA-107 per ml was added. Addition of these compounds was repeated every 4 h. When PC-G alone was added, there was a slight delay in the growth (2 to 4 h), compared with the control group, but there was no significant killing effect (Fig. 7). However, when PC-G was combined with KA-107, inhibition of growth was maintained over the 28-h test period.

In vivo experiments. Based upon the results obtained in in vitro experiments, mice were infected with FS-1277, and experimental therapy was given, using KA-107 or PC-G, or both.

Firstly, 10^{-2} mg (wet weight) of FS-1277 cells

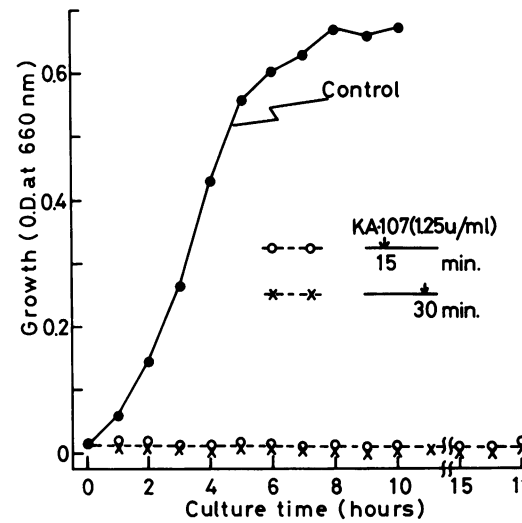

FIG. 5. *Effect of addition time of KA-107 on the growth of S. aureus FS-1277. PC-G (400 U/ml) was added at 0 h of incubation at 37 C.*

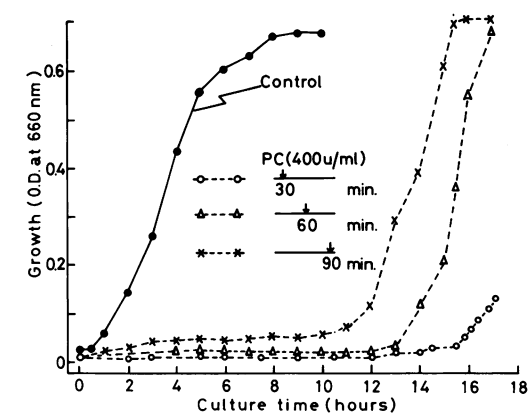

FIG. 6. *Effect of addition time of PC-G on the growth of S. aureus FS-1277. KA-107 (1.25 U/ml) was added at 0 h of incubation at 37 C.*

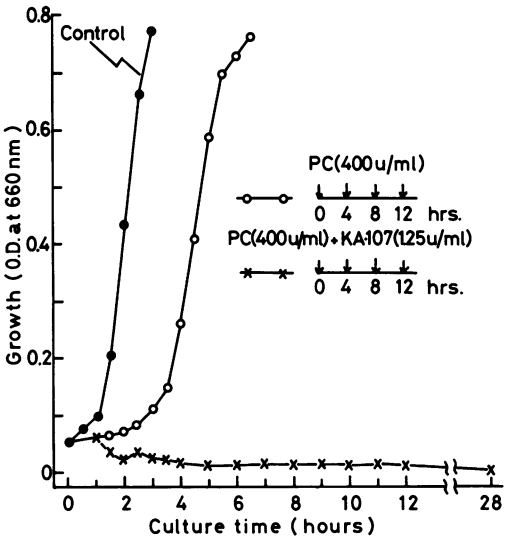

FIG. 7. *Effect of addition time of both PC-G and KA-107 on the growth of S. aureus FS-1277.*

per kg was suspended in 5% mucin solution, and injected into DD-S mice IP. Then 500 U of PC-G per kg and 25 U of KA-107 per kg was injected IP either separately or together, and survival of the mice was observed for 7 days. The control group animals, which were given each saline solution and 25 U of KA-107 per kg, and 400 U of PC-G per kg alone, died of infection within 2 days, at the rate of 80, 100, and 100 to 75%, respectively (Table 1).

Treatment with the penicillinase inhibitor was conducted in three ways. Among the treated mice given PC-G combined with KA-107 three times after an injection of FS-1277, at 15- or 30-min intervals, 80% survived (those administered two administrations showed a 40 to 60% survival rate).

Among those receiving with KA-107 at the time FS-1277 was injected and then treated with PC-G alone injected three times at either 15 or 30 min intervals, 60 to 80% survived (those injected twice showed a 60 to 75% survival rate).

Among those which were injected with FS-1277 and 200 U of PC-G per kg initially and then treated with PC-G combined with KA-107 three times, the survival rate was 50 to 80%.

In all of these treatments, better results were obtained after two injections than after only one injection. The survival figures obtained after two injections do not differ from those obtained after three injections.

DISCUSSION

It is known that penicillin-resistant organisms, especially gram-positive bacteria, produce PCase and that in the presence of PC, these strains induce PCase (1, 2, 4). It is also known that inactivation of penicillin results from hydrolysis of its β-lactam ring by penicillinase. On the supposition that there are substances which could inactivate the action of PCase and thus allow PC to exert its activity against PC resistant strains, screening for PCase inhibitors was conducted by using fermented culture broths of microorganisms. An effective, high molecular weight substance named KA-107 was extracted from the culture filtrate of *S. gedanensis*. KA-107 had no inhibitory action itself on *S. aureus* FS-1277, a PC resistant strain, but when used together with PC-G, it was found to inhibit the growth of FS-1277. It was also found that the combined use of a semi-synthetic PC such as ampicillin, or L- or D-phenethicillin with KA-107, resulted in growth inhibition of the penicillin resistant strain just as occurs with penicillin sensitive ones.

When FS-1277 is cultured, growth starts immediately reaching a maximum in 3 to 5 h. However, production of PCase precedes it reaching a maximum in 1 h. When 100 U of PC-G per ml is added from the start as PCase inducer, PCase production is about five times higher than that found in the case where penicillin was not added. Therefore, when 400 U of PC-G per ml combined with 1.25 U of KA-107 per ml was added as soon as FS-1277 was inoculated, the growth was inhibited for a certain period of time, but after this period, the

TABLE 1. *Effect of KA-107 on S. aureus FS-1277 in mice*[a]

Challenge	Administration	Rate of survival (%)					
		Frequency					
		1	2	3[b]	1	2	3[c]
FS-1277 (10^{-2} mg/kg)	None			20			20
	PC-G			0	20	25	20
	KA-107			0	0		0
	PC-G + KA-107	0	40	80	40	60	80
FS-1277 + KA-107	PC-G	75	75	80	40	60	60
FS-1277 + PC-G (200 U/kg)	PC-G + KA-107	0	50	50	50	80	80

[a] KA-107 and PC-G were administered in amounts of 25 U/kg and500 U/kg, respectively.

[b] Interval of 15 min; 15 min starting time after inoculation.

[c] Interval of 30 min; 30-min starting time after inoculation.

cells resumed growth. During the 2- to 5-h period where growth was stationary the 10^8 cells originally inoculated decreased to less than 10^5 cells upon addition of PC-G combined with KA-107, although sterilization of the culture did not occur; thus, the surviving cells ultimately resumed rapid growth possibly because the added PC-G had lost its activity. Therefore, to maintain inhibition of growth for longer periods, it was necessary to add both PC-G and KA-107 at 4-h intervals. Actually, when either one was added three times in succession, growth was inhibited for more than 28 h. Based on these in vitro experiments, in vivo experiments were conducted. Mice were infected with FS-1277, and at the same time, KA-107 and PC were injected. As a result, deaths due to infection caused by the PC-resistant strain could be significantly reduced. It will be necessary to continue the experiments using other PC-resistant strains, and to establish the optimal dosage of KA-107 and penicillin and their time of application.

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