RESTORATION OF ANTIBACTERIAL ACTIVITY OF β-LACTAMS BY EGCG DEPENDING ON LOCATIONS OF β-LACTAMASES

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Summary

The broth microdilution checkerboard and time-kill techniques were used to investigate the combination effects of (-)-epigallocatechin gallate (EGCg) and β-lactams against various β-lactamase-producing clinical isolates. Penicillin in combination with 12.5 μg/ml EGCg showed the potent synergy against the penicillinase-producing *S. aureus* (21 strains) and restored the antibacterial activity of penicillin. However, cefotaxime or imipenem in combinations with EGCg (100 μg/ml) only showed slight synergy against 2 of 17 β-lactamase-producing gram-negative rods. EGCg directly inhibited the activity of the various β-lactamases, thereby protecting β-lactams from β-lactamases. The different combination effects on the different β-lactamase-producing species were confirmed to be related to the cellular locations of β-lactamases, since the extracellular fractions of β-lactamase showed a correlation with the restoration of the antibacterial activities of β-lactams by EGCg.

Keywords

(-)-epigallocatechin gallate (EGCg), β-lactams, β-lactamases, synergy

Introduction

The production of β-lactamases is a critical mechanism for the bacterial resistance to β-lactams. The recent emergence of bacterial strains producing inhibitor-resistant enzymes, for example IRT, could be related to the frequent use of clavulanate. Extended-spectrum β-lactamases (ESBLs), the mutant enzymes mostly derived from TEM or SHV and often found in *Escherichia coli* and *Klebsiella pneumoniae*, are threatening the value of expanded-spectrum cephalosporin and monobactams against enterobacteria. Carbapenems are stable to ESBLs, and imipenem has been used successfully in vivo against many enzyme producers. Unfortunately, a new metallo-β-lactamase, IMP-1, poses a serious emerging threat to the use of carbapenems against *Serratia marcescens* and other gram-negative rods. The use and overuse of new antibiotics in the clinical practice have selected and expanded such new variants of β-lactamases. Therefore, any effort to prevent β-lactams from inactivation by β-lactamases is of particular significance. We have previously reported the combination effects between various antibiotics and (-)-epigallocatechin gallate (EGCg, a main constituent of tea catechins) against MRSA. In the study, we observed the combination effects between β-lactams and EGCg against β-lactamase-producing clinical isolates.

Materials and Methods

Susceptibility test, β-Lactamase assay and *bla* gene detection. Minimum

inhibitory concentrations (MICs) were determined by a broth microdilution method. The effects of combinations were confirmed by the checkerboard method. The results were evaluated by a fractional inhibitory concentration (FIC) index. All the strains were identified by nitrocefin assay for β-lactamase expression and by PCR analysis for *bla* gene presences.

Protection of β-lactams from β-lactamases by EGCg. The β-lactam-susceptible *S. aureus* or *E. coli* was inoculated in MHB containing β-Lactam and EGCg as well as the purified penicillinase from *Bacillus cereus* or crude extract of β-lactamase from resistant strain. The changes of β-lactam MICs were observed as the evidence of EGCg-induced protection of β-lactam from β-lactamases.

Inhibition of penicillinase activity by EGCg. The β-lactamases were incubated with EGCg. Nitrocefin was added as the substrate. The color changes were recorded by detecting OD 492 nm. The concentration of EGCg required for 50% inhibition (IC_{50}) of the enzyme activity was determined.

Preparation and location of β-lactamases. Penicillinase-producing *S. aureus* #226, TEM-derived ESBL-producing *E. coli* #5, inhibitor-resistant SHV- and TEM-β-lactamse-producing *K. pneumoniae* #1 and IMP-1-producing *S. marcescens* #8 were cultured and collected. After sterilization via filtration, the part of β-lactamase in the supernatants was considered as the extracellular enzyme. The pellets were washed and resuspended in MHB followed by sonication. The enzyme in the solution was considered as intracellular enzyme, including that in the periplasmic space.

Results

Combination effects of β-lactams and EGCg against β-lactamase-producing strains

All the clinical isolates (21 *S. aureus*, 6 *E. coli*, 3 *K. pneumoniae* and 8 *S. marcescens*) were β-lactamase producers confirmed by nitrocefin test and *bla* gene analysis. Synergy occurred against 48%, 81% and 100% strains of the penicillinase-producing *S. aureus* in combination of penicillin with EGCg 3.125, 6.25 and 12.5 μg/ml, respectively. However, the combinations of cefotaxime or imipenem with the higher concentrations of EGCg (100 μg/ml) showed slight synergy against 2 and addition against 5 of 17 strains of *E. coli*, *K. pneumoniae* and *S. marcescens*. The remaining strains showed indifferent effect.

Protection of β-lactams from β-lactamase by EGCg

The penicillin-susceptible *S. aureus* ATCC 25923 cells were inoculated in the cell-free supernatant containing about 0.015 U/ml of penicillinase (PCase) in the presence of the two-fold serial dilutions of penicillin and EGCg. The penicillinase resulted in the rise of penicillin MIC from 0.125 μg/ml to 512 μg/ml. EGCg dose-dependently blocked the penicillinase activity and thus restored the MICs of penicillin from 512 μg/ml to 256, 64, 8 and 0.125 μg/ml at the concentrations of 3.125, 6.25, 12.5 and 25 μg/ml, respectively (Fig. 1A). Similarly, the MIC of imipenem rose from 0.125 to 8 μg/ml in the presence of IMP-1 prepared from 3.6×10^6 cells of *S. marcescens* #8. EGCg blocked the IMP-1 activity and restored the MICs of imipenem from 8 μg/ml to 4, 1 and 0.25 μg/ml at concentrations of 6.25, 12.5 and 25 μg/ml, respectively (Fig 2B).

Inhibition of β-lactamase activity by EGCg.

EGCg directly inhibited the activity of β-lactamase in a dose-dependent manner. The concentration of EGCg required for 50% inhibition (IC_{50}) of penicillinase activity (10U/ml) was 10 μg/ml. Similarly, The concentration of EGCg was 10.5 μg/ml for IC_{50} of IMP-1 activity prepared from 1.8×10^6 cells of *S. marcescens* #8.

The cellular location of β-lactamases

Extracellular and intracellular β-lactamases were collected separately from the cultures of the strains and their activities were then detected. In contrast to 32.7% extracellular fraction of β-lactamase in the penicillinase-producing *S. aureus* #226, the fractions were 0.6%, 0.6% and 1.2% in the TEM-derived ESBL-producing *E. coli* #5, the inhibitor-resistant β-lactamase-producing *K. pneumoniae* #1 and the IMP-producing *S. marcescens* #8, respectively (Table 1).

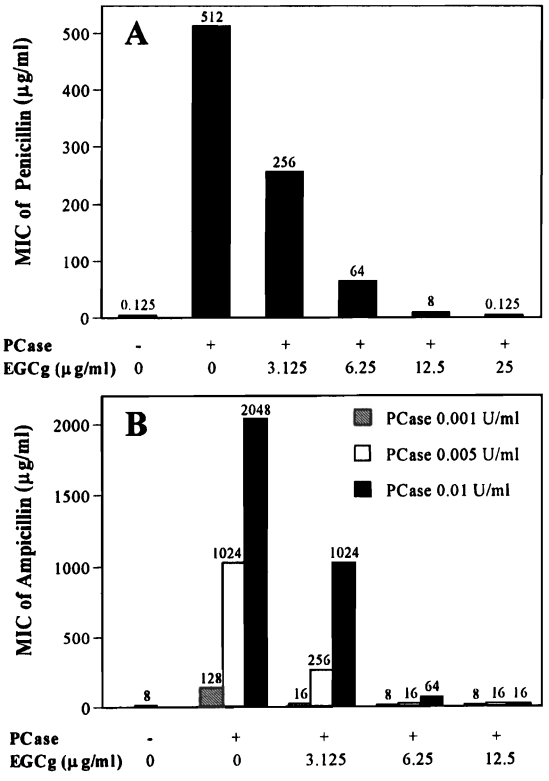

Figure 1. Protection of penicillin (A) and ampicillin (B) from penicillinase by EGCg. In A, the penicillin-susceptible *S. aureus* ATCC 25923 cells were inoculated in the cell-free supernatant containing about 0.015 U/ml of penicillinase (PCase) in the presence of the two-fold serial dilutions of penicillin and EGCg. In B, the ampicillin-susceptible *E. coli* ATCC 25922 cells were inoculated in the MHB containing the purified penicillinase at 0.001, 0.005 and 0.01 U/ml in the presence of the two-fold serial dilutions of ampicillin and EGCg. After incubated at 35℃ for 24 h (*S. aureus*) and for 18 h (*E. coli*), the MICs were then determined.

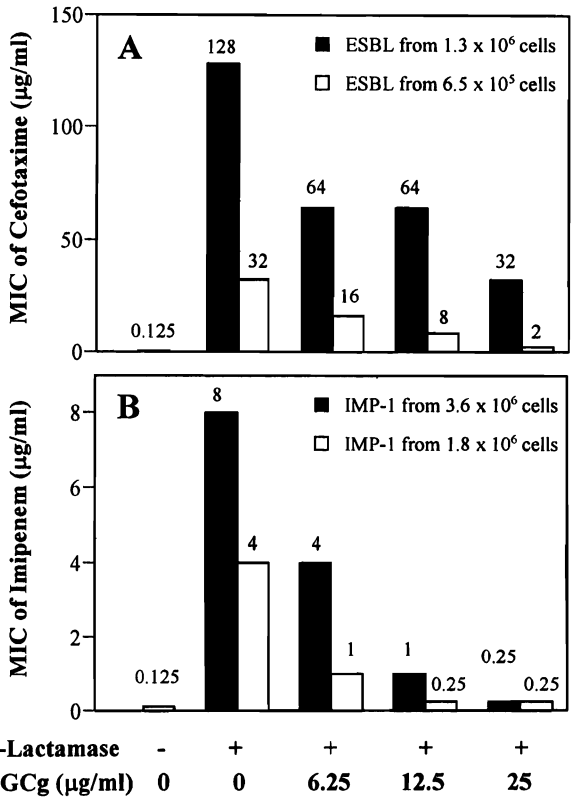

Figure 2. Protection of β-lactams by EGCg. The susceptible *E. coli* ATCC 25922 was used as target cell (5×10^5 cells/ml). In A, cells were inoculated in MHB containing TEM-derived ESBL prepared from *E. coli* #5 in the presence of the two-fold serial dilutions of cefotaxime and EGCg. In B, the cells were inoculated in the MHB containing IMP-1 prepared from *S. marcescens* #8 in the presence of the two-fold serial dilutions of imipenem and EGCg. After incubated at 35℃ for 18 h, the MICs were determined.

Table 1. Characteristics of β-lactamases

strain	*bla* gene	type	extracellular fraction (percent of total)
S. aureus #226	bla_Z	PCase	32.7 ± 1.5*
E. coli #5	bla_{TEM}	TEM-derived ESBL	0.6 ± 0.1
K. pneumoniae #1	$bla_{SHV+TEM}$	Inhibitor-resistant β-lactamase	0.6 ± 0.3
S. marcescens #8	$bla_{IMP+TEM}$	IMP-1and TEM	1.2 ± 0.5

● $P < 0.01$ compared to the data from *E. coli, K. pneumoniae or S. marcescens* (Student's *t* test).

Discussion

Inhibition of penicillinase by EGCg results in restoration of antibacterial activity of penicillin against penicillinase-producing *S. aureus in vitro*. Disappointingly, no obvious restoration was observed by the combinations of β-lactams and EGCg against gram-negative rods, even though EGCg directly inhibited the extracted β-lactamase from the rods and protected β-lactams from inactivation. Contrary to Staphylococcal β-lactamase that is extracellular, the β-lactamases of gram-negative rods are regularly periplasmic, although some extracellular release may occur due to leakage rather than secretion. We compared the extracellular and intracellular enzyme activities between *S. aureus* and various gram-negative rods. The extracellular fractions of total β-lactamase activity clearly showed a correlation with the restoration of the antibacterial activities of β-lactams by EGCg against β-lactamase-producing strains. Penicillin in combination with EGCg at a possibly bioavailable concentration showed potent synergy against penicillinase-producing *S. aureus* in vitro. However, the combinations of β-lactams and EGCg against β-lactamase-producing gram-negative rods do indicate a limitation owing to the cellular location of β-lactamase.

References

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