J Infect Chemother 23 (2017) 312–318

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Enhancement of bactericidal activity against group B streptococci with reduced penicillin susceptibility by uptake of gentamicin into cells resulting from combination with β-lactam antibiotics

Yoshifumi Ebara [a, b, 1], Miyuki Morozumi [b, 1], Mamiko Sato [c], Nobuko Moritoki [c], Meiwa Toyofuku [d], Misako Takata [b], Mitsuru Murata [a], Kimiko Ubukata [b], Satoshi Iwata [b, *]

[a] Department of Laboratory Medicine, Keio University, School of Medicine, Tokyo, Japan
[b] Department of Infectious Diseases, Keio University, School of Medicine, Tokyo, Japan
[c] Laboratory of Electron Microscopy, Japan Women's University, Tokyo, Japan
[d] Department of Pediatrics, Yokohama Rosai Hospital, Yokohama, Japan

ARTICLE INFO

Article history:
Received 21 November 2016
Received in revised form
21 February 2017
Accepted 23 February 2017
Available online 22 March 2017

Keywords:
Reduced penicillin susceptibility
Streptococcus agalactiae
Group B streptococci
Antibiotic combinations
Bactericidal effect
Synergistic effect

ABSTRACT

Combined effects of penicillin (PEN) and gentamicin (GM) against *Streptococcus agalactiae*, i.e. group B streptococci (GBS), are known to occur, but synergy has not been examined in strains with reduced PEN susceptibility, usually called PEN-resistant GBS (PRGBS). We therefore studied combined effects of β-lactam antibiotics and GM in cultures of 3 PRGBS strains belonging to serotype Ia or III that were isolated from Japanese adults with invasive infections. Killing kinetics were determined at 2-h intervals from 0 to 6 h after exposure to ampicillin (AMP) or cefotaxime (CTX) combined with GM. Concentrations of GM in bacterial cells were measured by liquid chromatography-tandem mass spectrometry. Morphologic changes after exposure to agents were observed by transmission electron microscopy.

Combining AMP or CTX with GM synergistically increased bactericidal activity against PRGBS beyond that of either β-lactam alone. GM concentrations in bacterial cells increased 5- to 8-fold when GM was combined with AMP or CTX. Electron microscopically, bacterial cells showed aggregates of strands and ribosomal damage most likely reflecting enhanced GM uptake into bacterial cells. This uptake appeared to result from cell wall damage caused by β-lactam antibiotics.

This study suggests that combining β-lactam antibiotics with GM might be useful against severe PRGBS infection.

1. Introduction

Streptococcus agalactiae, also referred to as group B streptococci (GBS), is a cause of serious invasive infections that occur in infants as 2 distinct clinical syndromes, early-onset disease (EOD) and late-onset disease (LOD) [1–7]. The most common invasive diseases caused by GBS in infancy, sepsis and meningitis, carry high likelihood of mortality or permanent sequelae [8–11]. In elderly persons, GBS can cause various severe infections that result in significant morbidity including sepsis, meningitis, and pneumonia, especially in the presence of underlying conditions such as diabetes, renal disorders, and immune compromise [12–17].

GBS with reduced penicillin (PEN) susceptibility, often termed PEN-resistant GBS (PRGBS), also show reduced susceptibility to cefotaxime (CTX). PRGBS was first reported from Japan in 2008 [18], subsequently emerging in the US [19] and Canada [20,21].

To date, PRGBS with amino acid substitution(s) in penicillin-binding protein (PBP2X) have been isolated infrequently from elderly patients, and are notable for having capsular type III or Ia [17,18]. Strains representing these capsular types are well known to cause severe infections in neonates, usually transmitted from their mothers at delivery [6,22]. For pregnant carriers of such PRGBS, risk of serious neonatal infection is high.

Conventionally, PEN antibiotics have been first-line agents for treatment of invasive GBS infections. In the 1980s, combined

* Corresponding author. Department of Infectious Diseases, Keio University, School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo, 160-8582, Japan. Fax: +81 3 3582 7068.
E-mail address: siwata@a8.keio.jp (S. Iwata).
[1] Ebara Y. and Morozumi M. contributed equally to this article.

http://dx.doi.org/10.1016/j.jiac.2017.02.010
1341-321X/

treatment of PEN-susceptible GBS (PSGBS) in culture with PEN and a low concentration of an aminoglycoside (AG) was found to show enhanced bactericidal activity [23–25], a finding also supported by results of animal experiments [26,27]. Combination therapy with an AG and a β-lactam agent has been found to produce a better clinical response than β-lactam antibiotics alone, but this conclusion was not based on a controlled clinical trial [28]. Presently, combination therapy with ampicillin (AMP) plus gentamicin (GM) is recommended for invasive GBS (iGBS) infections in neonates [29]. However, details of the mechanisms underlying this apparent synergy remain unclear.

In the present in vitro study, we aimed to clarify the putative synergistic effect of combinations of β-lactam antibiotics (AMP or CTX) with GM against PRGBS.

2. Materials and methods

2.1. Bacterial strains

As shown in Table 1, GBS strains used in this study were selected from GBS isolates (infants, n = 139; adults, n = 443) collected in the course of large-scale surveillance of invasive streptococcal and pneumococcal infections performed throughout Japan between April 2010 and March 2013 [17,22].

Nine PRGBS isolates, all obtained from adult patients with either pneumonia or bacteremia of unknown primary focus, were identified genetically [17]. Capsular type and sequence type (ST) of these PRGBS were identified by the methods described below: capsular type III [ST10 (n = 1), ST24 (n = 3), and ST464 (n = 2)]; capsular type Ia [ST1 (n = 1) and ST10 (n = 1)]; and capsular type Ib [ST358 (n = 1)]. Three of these PRGBS strains showing an MIC of 1 μg/mL to CTX were used in the study.

PSGBS strains used as controls were 1 strain from an elderly patient with pneumonia [17] and 2 strains from neonates, 1 with meningitis and 1 with bacteremia [22].

Antimicrobial susceptibility to PEN, AMP, CTX, and GM was measured according to the standards of the Clinical and Laboratory Standards Institute (www.clsi.org).

Capsular types of all strains were identified by our real-time PCR method [30], which is a modification of the procedure described by Poyart et al. [31]. Multilocus sequence typing (MLST) was performed with reference to the MLST website (http//pubmlst.org/sagalactiae/). Amino acid substitutions near the conserved amino acid motif Ser-Ser-Asn (SSN) in PBP2X encoded by the *pbp2x* gene were identified by sequencing.

Isolates were grown by routine methods on sheep blood agar II plates (Nippon Becton Dickinson, Tokyo, Japan) at 37 °C in a 5% CO_2 atmosphere.

2.2. Killing kinetics

Time-kill curves of PRGBS strains were measured for AMP, CTX, and GM individually, and for combinations of AMP or CTX with GM. Concentrations of these antibiotics were based on the minimum inhibitory concentrations (MICs) shown in Table 1: 1 μg/mL of AMP (4 × MIC) or of CTX (MIC) for each strain, respectively, and 0.5 or 2 μg/mL of GM. Killing curves of PSGBS strains were measured for combinations of 0.25 μg/mL of AMP (2 × MIC) or 0.125 μg/mL of CTX (2 × MIC) with GM (0.5 and 2 μg/mL), amikacin (AMK, 0.5 and 2 μg/mL), or arbekacin (ABK, 0.5 and 2 μg/mL).

After overnight culture on sheep blood agar II plates, bacterial cells were inoculated into tubes containing 10 mL of Müller-Hinton (MH) broth supplemented with 5% defibrinated sheep blood to attain a final concentration of 10^6 CFU/mL. After incubation at 37 °C for 1 h, 500 μL of bacterial suspension was inoculated into tubes containing 9.5 mL of fresh MH broth similarly supplemented with sheep blood and containing each antibiotic to be tested. All tubes were incubated continuously with gentle shaking, and samples were taken at 2-h intervals. These samples were serially diluted 10-fold with MH broth, after which 100 μL of each diluted sample was plated on 3 blood agar plates. Numbers of colonies grown on the blood agar plates after overnight incubation at 37 °C in a 5% CO_2 atmosphere were recorded. These experiments were repeated 3 times.

2.3. Assay of concentrations of GM

Concentrations of GM in bacterial cells (PRGBS: KK1949) were measured by liquid chromatography-tandem mass spectroscopy (LC/MS/MS).

Before LC/MS/MS, bacterial cells were cultured in 400 mL of Todd-Hewitt (TH) broth containing antimicrobial agents as outlined above. Cells were collected after 1, 2, or 4 h by centrifugation at 2200 ×*g* for 10 min. Subsequently, cells were washed once with TES buffer. Bacterial wet weight (mg) was recorded after complete removal of buffer. Cells then were mixed with 4 mL of TES buffer and 100 μL of mutanolysin (10 units/μL; Sigma, Aldrich, Germany) and kept in a water bath at 35 °C for 15 min. Finally, cells were disrupted by ultrasonication (Insonator 201 M, Kubota, Tokyo, Japan) at 200 W for 15 min while maintaining the temperature below 4 °C. After 2 serial centrifugations of the sonicated cells at 12 000 × g for 10 min, the supernatant was subjected to measurement of the GM concentration as described below.

GM was measured using an LC/MS/MS system (high-performance LC, or HPLC, LC-30AD; Shimadzu, Kyoto, Japan; MS/MS, Qtrap 6500; AB SCIEX, Framingham, MA). ABK was used as the internal standard (IS). Each 100 μL sample was transferred to a tube. The

Table 1
Streptococcus agalactiae strains used in this study.

Strain no.	Age of patient[a]	Site of isolation	Capsular type	Sequence type	AA substitutions in PBP2X				MIC (μg/mL)			
					Gly398	Val405	Gly429	Gln557	PEN[d]	AMP	CTX	GM
PSGBS												
KK0132	71 y	Blood	III	1	Gly	Val	Gly	Gln	0.063	0.125	0.063	64
KK1104	10 d	CSF[b]	III	17	Gly	Val	Gly	Gln	0.063	0.125	0.063	64
KK0220	36 d	Blood	III	17	Gly	Val	Gly	Gln	0.063	0.125	0.063	>64
PRGBS												
KK1419	29 y	PE[c]	Ia	10	Ala	Ala	Gly	Glu	0.25	0.25	1	64
KK1949	73 y	Blood	III	10	Ala	Ala	Gly	Glu	0.25	0.25	1	64
KK2145	85 y	Blood	Ia	1	Ala	Ala	Asp	Gln	0.25	0.25	1	>64

[a] y: years old, d: days after birth.
[b] CSF: cerebrospinal fluid.
[c] PE: pleural effusion.
[d] PEN, penicillin G; AMP, ampicillin; CTX, cefotaxime; GM, gentamicin.

standard solution (20 μL), IS solution consisting of ABK solution (20 μL), and 30% trichloroacetic acid (TCA, 50 μL) were added and mixed with the sample using a vortex agitator. The mixture was centrifuged at 9100 × *g* for 10 min at 4 °C, after which the supernatant was filtered through a membrane with a 0.2-μm pore size. The resulting samples were used for measurement.

GM was separated chromatographically on an analytical column (Capcell Pak ADME, 2.1 × 50 mm, 5 μm, Shiseido) using a gradient of 5 mM heptafluorobutyric acid (HFBA) in water with 5 mM HFBA in acetonitrile as the mobile phase at 40 °C. The flow rate was set at 0.5 mL/min.

The tandem mass spectrometer was operated in positive-ion mode. GM C1 major component was used for quantitation in this study. GM was monitored as the precursor ion at 478 *m/z* and as the product ion at 322 *m/z*. ABK as IS was monitored as the precursor ion at 553 *m/z* and as the product ion at 425 *m/z*. The assay was linear over an interval from 0.025 to 1 μg/mL for GM. The experiment measuring uptake of GM into bacterial cells, including the assay, was repeated 3 times.

2.4. Observation by transmission electron microscopy (TEM)

PRGBS strain KK1949 was grown in TH broth, thus avoiding contamination of TEM samples by eliminating any need for sheep blood cells, at 37 °C in a 5% CO_2 atmosphere for 2 h. Ten milliliters of the culture was inoculated into 100 mL of fresh TH broth containing

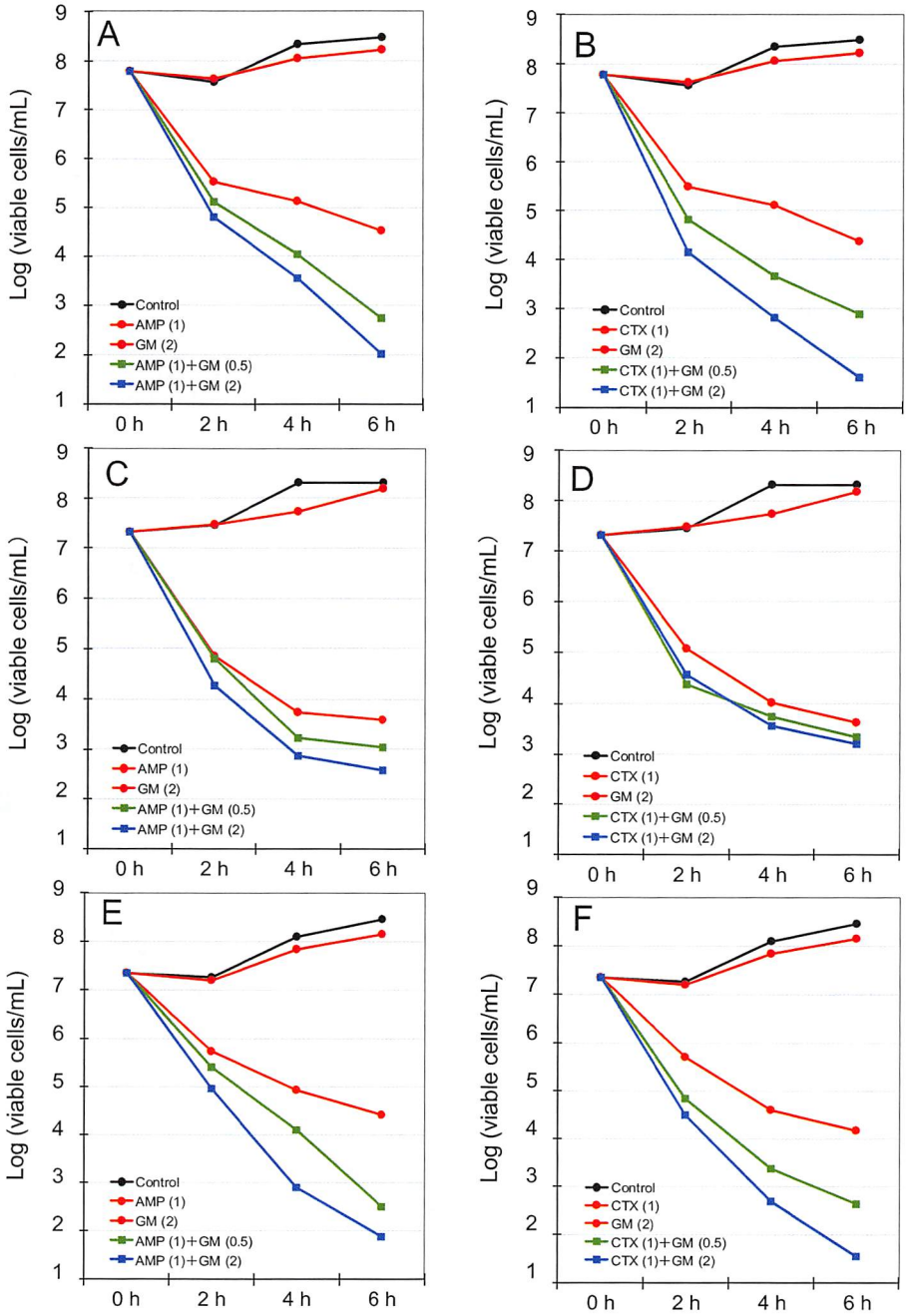

Fig. 1. Time-kill curves against PRGBS for AMP, CTX, and GM alone, and for combination of AMP or CTX with GM. Each plot shown in the killing curves presents the median for results of experiments repeated 3 times. A and B, strain KK1949; C and D, PRGBS KK1419; E and F, KK2145. Time-kill curves were measured from time zero until 6 h later. The Concentrations of agents used are 1 μg/mL for AMP, 1 μg/mL for CTX, and 2 μg/mL for GM. Combinations of AMP or CTX with GM involved 0.5 μg/mL or 2 μg/mL of GM.

each antimicrobial agent, followed by culture at 37 °C for 2 h. Concentrations of antibiotics were as follows: AMP (1 μg/mL, 4 × MIC), CTX (1 μg/mL, MIC), and GM (2 μg/mL, 1/32 of MIC). Antibiotic concentrations in cultures with combinations of AMP or CTX plus GM were the same as above.

For pre-fixation of bacterial cells, glutaraldehyde (25% solution, Wako Pure Chemical Industries, Osaka, Japan) was added to the cultures (final concentration, 0.5%), which were fixed at room temperature for 30 min. Cells then were harvested by centrifugation at 2200 ×*g* for 5 min and fixed with 2.5% glutaraldehyde at 4 °C for 2 h. After washing twice with 0.1 M phosphate buffer (pH 6.8), cells were post-fixed with 1% osmium tetroxide (2% w/v OsO4, TAAB, Reading, UK) at 4 °C overnight.

Samples were embedded in 1.5% low-melting agar (Agarose LE, Wako Pure Chemical Industries, Osaka, Japan) and cut into 1-mm cubes, which were dehydrated by serial passage from 50% to 70%, 80%, 90%, 95%, and 100% ethanol, with changes at 10-min intervals. After a final change from ethanol to methyl glycidyl ether (QY2, Nisshin EM, Tokyo, Japan), the cubes were embedded in resin [methyl nadic anhydride (MNA), 3.49 mL: quetol 812 (Q-812), 5.98 mL: nonenyl succinic anhydride (NSA), 2.30 mL: 2, 4, 6, Tris-dimethylaminomethyl phenol (DMP-30), 0.2 mL; Nisshin EM] to solidify gradually over several days at 37 °C, 42 °C, and finally 45 °C.

An ultra-thin section of each sample was cut with an ultramicrotome (Reichert Ultracut S, Leichert Technologies, Depew, NY). Each section was then stained with 4% uranyl acetate solution for 15 min followed by lead citrate solution for 7 min. These ultra-thin sections were observed and photographed using TEM (JEM-1400 type, JEOL Ltd, Tokyo, Japan) at 120 kV.

2.5. Ethics

The study was performed with the approval of the Ethical Review Board of the Keio University School of Medicine (approval no., 20140130; approval date, July 25, 2014).

3. Results

3.1. Characteristics and antibiotic susceptibility of PRGBS

Characteristics and antibiotic susceptibilities of PRGBS and PSGBS strains are compared in Table 1. The 3 PRGBS strains all were isolated from samples from normally sterile sites in adults with GBS infection.

MICs (0.25 μg/mL) of PEN and AMP for these PRGBS strains were 2 or 4 times greater than those for PSGBS strains (PEN, 0.063 μg/mL; AMP, 0.125 μg/mL), while those for CTX (1.0 μg/mL) were 16 times greater than for PSGBS strains (0.063 μg/mL). Four amino acid substitutions affecting resistance, including Val405Ala and Gln557Glu, were identified near the conserved amino acid motif Ser-Ser-Asn in PBP2X in these PRGBS strains.

3.2. Killing kinetics of β-lactam antibiotics and GM for PRGBS

Fig. 1 shows time-kill curves against three PRGBS strains for AMP, CTX, and GM alone as well as curves for combinations of AMP or CTX with GM. PRGBS strains studied were KK1949 (Fig. 1A and B), KK1419 (Fig. 1C and D), and KK2145 (Fig. 1E and F). MICs of AMP, CTX, and GM for these isolates were 0.25 μg/mL, 1.0 μg/mL, and ≥64 μg/mL, respectively.

Bactericidal activity against PRGBS was shown by 1 μg/mL of either AMP or CTX alone, but not by GM alone. Bactericidal activity of AMP and CTX clearly increased when GM was included at a low concentration (0.5 μg/mL or 2 μg/mL). Viable PRGBS cells decreased with time, from 10^7 cells/mL initially to 10^4 to 10^5 cells/mL at 2 h and to 10^2 to 10^4 cells/mL at 4 h when AMP or CTX was combined with GM. Enhancement of bactericidal effect by a combination of CTX at 1 μg/mL with GM was weaker against KK1419 than against the other strains, but clear enhancement in that strain with the combination was evident for CTX at 2 μg/mL.

As shown in Supplement 1, bactericidal activity of AMP and CTX against PSGBS KK1104, a hypervirulent strain with capsular type III and ST17, also increased in time-kill curves obtained in the presence of GM, AMK, or ABK at low concentrations.

3.3. Uptake of GM into GBS cells

Intracellular GM concentrations after treatment of PRGBS KK1949 strain with GM alone and in combination with AMP or CTX against were measured using LC/MS/MS at 1, 2, and 4 h after exposure to the antibiotic(s).

As shown in Fig. 2, the concentration of GM in cells after combined exposure with AMP or CTX increased markedly over time compared with GM treatment alone (5- to 8-fold at 1, 2, and 4 h, respectively). Although some differences were found among individual GM concentrations, concentrations increased 5 times or more in all strains upon combined exposure with AMP or CTX.

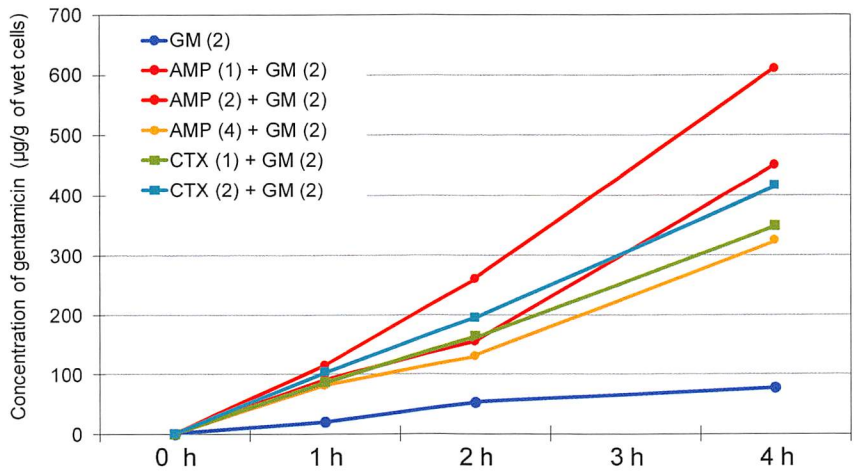

Fig. 2. Uptake of GM into cells of the KK1949 PRGBS strain. Concentrations of GM in cells after exposure in combination with AMP or CTX were measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Each plot of the GM uptake shows the median for results of experiments repeated 3 times.

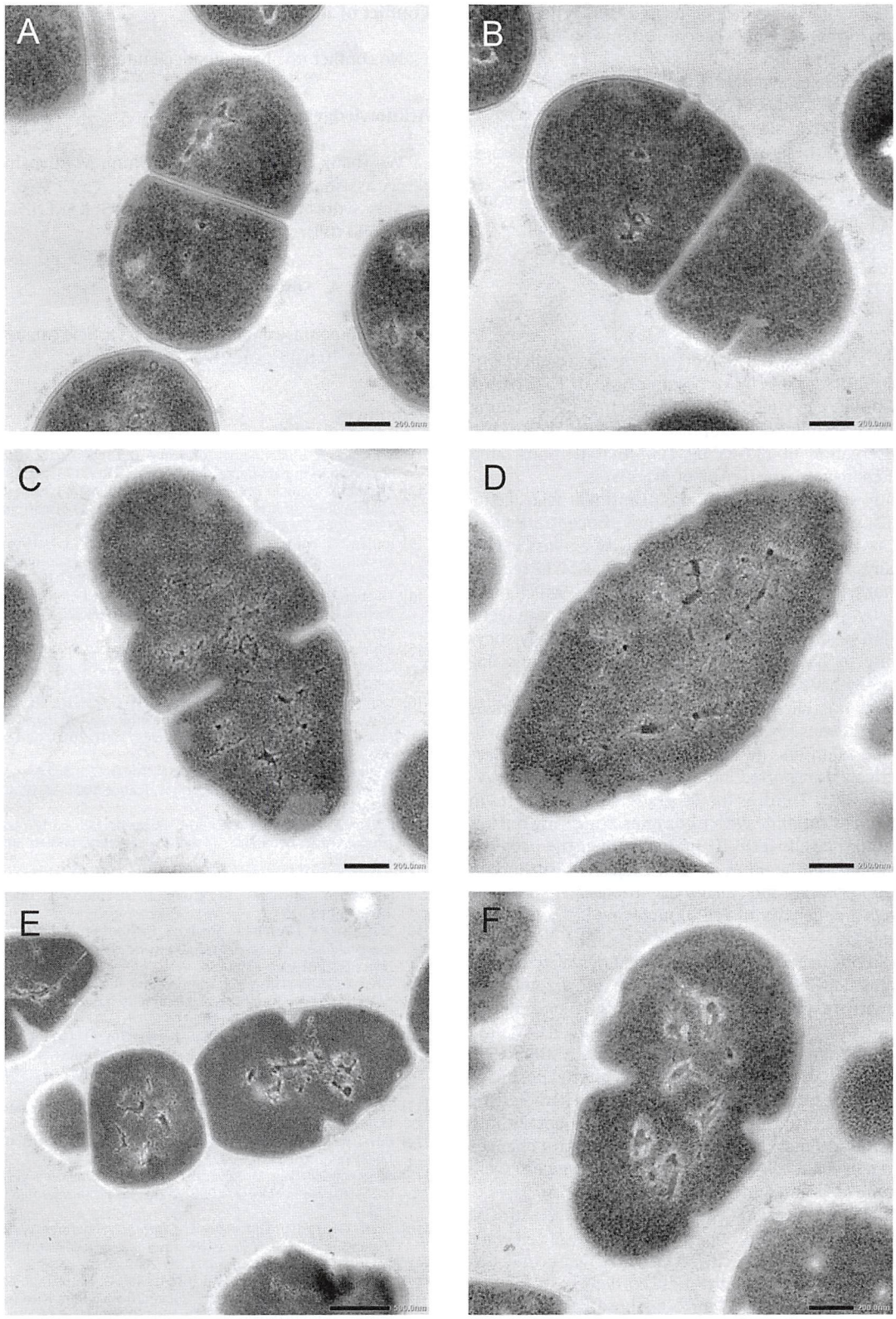

Fig. 3. Morphologic changes of the KK1949 PRGBS strain after exposure to antimicrobial agents at 2 h. Observations were performed by TEM (JEM-1400 type) at 120 kV. A, Control without any agents; B, 2 μg/mL of GM; C, 1 μg/mL of AMP; D, 1 μg/mL of CTX; E, 1 μg/mL of AMP plus 2 μg/mL of GM; F, 1 μg/mL of CTX plus 2 μg/mL of GM. The black bar shows 200 nm.

3.4. Morphologic changes

Morphologic changes of PRGBS KK1949 bacterial cells are shown in Fig. 3, as demonstrated by TEM after 2-h exposures to AMP (1 μg/mL), CTX (1 μg/mL), and GM (2 μg/mL) alone, as well as to combinations of AMP or CTX with GM.

Compared to bacterial cells grown without antibiotic (control, Fig. 3A), no morphologic changes were observed in cells exposed to

GM alone (Fig. 3B), while abnormal, elongated cells reflecting inhibition of septum formation were observed after exposure to AMP (Fig. 3C) or CTX (Fig. 3D) alone.

More dramatic changes resulted from combinations of AMP (Fig. 3E) or CTX (Fig. 3F) with GM. Changes particularly typical of AGs were abnormal nucleic acid strand aggregates resulting from inhibition of protein synthesis as well as degradation of ribosomes. Elongated cells reflected the action of the β-lactam antibiotic.

Low-magnification images of KK1949 after exposure to a combination of AMP (1 μg/mL) or CTX (2 μg/mL) with GM (2 μg/mL) are shown in Supplement 2.

4. Discussion

In the present study, we elucidated the synergistic effect obtained against 3 PRGBS strains by combining AMP or CTX with a small amount of GM by carrying out in vitro experiments including killing kinetics, measurement of GM uptake into bacterial cells, and morphologic observation of PRGBS by TEM that demonstrated enhancement of changes characteristic of AG effects.

Previous in vitro studies of combinations of antimicrobial agents reported that PEN or AMP combined with low concentrations of GM resulted in an increase in bactericidal activity against PSGBS [23–25]. Clinically, the combination of PEN and GM is recommended widely for treatment of infective endocarditis, which most often is caused by streptococcal and staphylococcal pathogens [32,33]. Such Gram-positive streptococcal pathogens usually show little susceptibility to GM alone. Nevertheless, mechanisms underlying this synergy have been incompletely understood.

The antimicrobial activity of AGs on gram-negative bacilli is thought to involve 3 steps [34,35]. First, the AG binds ionically to the cell membrane in an energy-independent manner (EIP). Second, the AG is actively transported into bacterial cells by an energy-dependent phase (EDP-I). Third, as the AG gradually accumulates in the cells, AG molecules combine with ribosomes, triggering further transport of AG into the cells (EDP-II). In this manner the bacterial cells are rapidly killed. On the other hand, the natural resistance to AGs among streptococcal and anaerobic bacterial pathogens apparently reflects a deficiency of EDP-II involving lack of aerobic energy production [35].

As described in our results, the time-kill bactericidal activity of AMP or CTX against PRGBS and PSGBS was increased significantly by combination with an amount of GM as small as 0.5 or 2 μg/mL. GM concentrations in PRGBS cells measured by LC/MS/MS increased 5- to 8-fold when GM was combined with AMP or CTX. Electron microscopic examination of PRGBS cells after exposure to GM with a β-lactam antibiotic showed abnormalities characteristic of AG-induced damage resulting from enhanced GM uptake into the cells. Such intracellular changes were similar to changes observed when *Escherichia coli* and *Pseudomonas aeruginosa* were exposed to 1 μg/mL of GM in a previous study [36].

We believe that the synergistic effect of AMP or CTX with GM against PRGBS progresses through 3 steps. First, bacterial cell walls are damaged by β-lactam agents such as AMP and CTX. Second, GM enters the cells through the β-lactam-induced defects in the cell walls. Third, GM rapidly binds to 30S and 50S ribosomes, disrupting protein synthesis.

Even though the first-line agent of choice for invasive PRGBS infections most likely would be VCM, combination therapy using a β-lactam agent with GM may well see increasing use against PRGBS treatment in the future, especially in neonatal infections. Such combination therapy also may be helpful in avoiding selection of highly resistant PRGBS with amino acid substitutions in PBP1A or PBP2B, thus maintaining effectiveness of β-lactam agents.

Conflict of interest

No conflict involving financial interests exists.

Acknowledgements

We thank Madoka Naito and Shinji Masuyoshi for their laboratory assistance.

This work was supported by JSPS KAKENHI Grant Number 15K19590 to Dr. M. Morozumi.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jiac.2017.02.010.

References

[1] Schuchat A. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin Microbiol Rev 1998;11:497–513.
[2] Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, et al. Epidemiology of invasive group B streptococcal disease in the United States, 1999–2005. JAMA 2008;299:2056–65.
[3] Jordan HT, Farley MM, Craig A, Mohle-Boetani J, Harrison LH, Petit S, et al. Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease. Pediatr Infect Dis J 2008;27:1057.
[4] Thigpen MC, Whitney CG, Messonnier NE, Zell ER, Lynfield R, Hadler JL, et al. Bacterial meningitis in the United States, 1998–2007. N Engl J Med 2011;364:2016–25.
[5] Edmond KM, Kortsalioudaki C, Scott S, Schrag S, Zaidi AKM, Cousens S, et al. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet 2012;379:547–56.
[6] Lamagni LT, Keshishian C, Efstratiou A, Guy R, Henderson LK, Broughton K, et al. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis 2013;57:682–8.
[7] Joubrel C, Tazi A, Six A, Dmytruk N, Touak G, Bidet P, et al. Group B streptococcus neonatal invasive infections, France 2007–2012. Clin Microbiol Infect 2015;21:910–6.
[8] Levent F, Baker CJ, Rench MA, Edwards MS. Early outcomes of group B streptococcal meningitis in the 21st century. Pediatr Infect Dis J 2010;29:1009–12.
[9] Libster R, Edwards KM, Levent F, Edwards MS, Rench MA, Castagnini LA, et al. Long-term outcomes of group B streptococcal meningitis. Pediatrics 2012;130:e8–15.
[10] Matsubara K, Hoshina K, Suzuki Y. Early-onset and late-onset group B streptococcal disease in Japan: a nationwide surveillance study, 2004–2010. Int J Infect Dis 2013;17. e379–84.
[11] Tibussek D, Sinclair A, Yau I, Fittipaldi N, Richards SE, Mayatepek E, et al. Late-onset group B streptococcal meningitis has cerebrovascular complications. J Pediatr 2015;166:1187–92.
[12] Tyrrell GJ, Senzilet LD, Spika JS, Kertesz DA, Alagaratnam M, Lovgren M, et al. Invasive disease due to group B streptococcal infection in adults:results from a Canadian, population-based, active laboratory surveillance study-1996. J Infect Dis 2000;182:168–73.
[13] Farley MM. Group B streptococcal disease in nonpregnant adults. Clin Infect Dis 2001;33:556–61.
[14] Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis 2005;41:839–47.
[15] Bergseng H, Rygg M, Bevanger L, Bergh K. Invasive group B streptococcus (GBS) disease in Norway 1996–2006. Eur J Clin Microbiol Infect Dis 2008;27:1193–9.
[16] Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, et al. Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990–2007. Clin Infect Dis 2009;49:85–92.
[17] Morozumi M, Wajima T, Takata M, Iwata S, Ubukata K. Molecular characteristics of Group B streptococci isolated from adults with invasive infections in Japan. J Clin Microbiol 2016;54:2695–700.
[18] Kimura K, Suzuki S, Wachino J, Kurokawa H, Yamane K, Shibata N, et al. First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrob Agents Chemother 2008;52:2890–7.
[19] Dahesh S, Hensler ME, Van Sorge NM, Gertz Jr RE, Schrag S, Nizet V, et al. Point mutation in the group B streptococcal *pbp2x* gene conferring decreased susceptibility to beta-lactam antibiotics. Antimicrob Agents Chemother 2008;52:2915–8.
[20] Gaudreau C, Lecours R, Ismail J, Gagnon S, Jette L, Roger M. Prosthetic hip joint infection with a *Streptococcus agalactiae* isolate not susceptible to penicillin G and ceftriaxone. J Antimicrob Chemother 2010;65:594–5.

[21] Longtin J, Vermeiren C, Shahinas D, Tamber GS, McGeer A, Low DE, et al. Novel mutations in a patient isolate of *Streptococcus agalactiae* with reduced penicillin susceptibility emerging after long-term oral suppressive therapy. Antimicrob Agents Chemother 2011;55:2983–5.
[22] Morozumi M, Wajima T, Kuwata Y, Chiba N, Sunaoshi K, Sugita K, et al. Associations between capsular serotype, multilocus sequence type, and macrolide resistance in *Streptococcus agalactiae* isolates from Japanese infants with invasive infections. Epidemiol Infect 2014;142:812–9.
[23] Schauf V, Deveikis A, Riff L, Serota A. Antibiotic-killing kinetics of group B streptococci. J Pediatr 1976;89:194–8.
[24] Cooper MD, Keeney RE, Lyons SF, Cheatle EL. Synergistic effects of ampicillin-aminoglyciside combinations on group B streptococci. Antimicrob Agents Chemother 1979;15:484–6.
[25] Swingle HM, Bucciarelli RL, Ayoub EM. Synergy between penicillins and low concentrations of gentamicin in the killing of group B streptococci. J Infect Dis 1985;152:515–20.
[26] Develkis A, Schauf V, Misen M, Riff L. Antimicrobial therapy of experimental group B streptococcal infection in mice. Antimicrob Agents Chemother 1977;11:817–20.
[27] Kim KS. Effect of antimicrobial therapy for experimental infections due to group B streptococcus on mortality and clearance of bacteria. J Infect Dis 1987;156:1233–41.
[28] Tessin I, Trollfors B, Thiringer K, Larsson P. Ampicillin-aminoglycoside combinations as initial treatment for neonatal septicaemia or meningitis. Acta Padiatr Scand 1991;80:911–6.
[29] Edwards MS, Nizet V, Baker CJ. Group B streptococcal infections. In: Wilson CB, Nizet V, Maldonado Y.Remington JS, Klein JO, editors. Remington and Klein's infectious diseases of the fetus and newborn infant. 8th ed. Amsterdam: Elsevier Inc; 2016. p. 411–56.
[30] Morozumi M, Chiba N, Igarashi Y, Mitsuhashi N, Wajima T, Iwata S, et al. Direct identification of *Streptococcus agalactiae* and capsular type by real-time PCR in vaginal swabs from pregnant women. J Infect Chemother 2015;21:34–8.
[31] Poyart C, Tazi A, Reglier-Poupet H, Billoet A, Tavares N, Raymond J, et al. Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. J Clin Microbiol 2007;45:1985–8.
[32] Le T, Bayer AS. Combination antibiotic therapy for infective endocarditis. Clin Infect Dis 2003;36:615–21.
[33] Habib G, Lancellotti P, Antunes MJ, Bongiorni MG, Casalta JP, Del Zotti F, et al. ESC guidelines for the management of infective endocarditis. Eur Heart J 2015;36:3075–128.
[34] Hancock REW. Aminoglycoside uptake and mode of action-with special reference to streptomycin and gentamicin. II. Effect of aminoglycosides on cells. J Antimicrob Chemother 1981;8:429–45.
[35] Taber HW, Mueller JP, Miller PF, Arrow AS. Bacterial uptake of aminoglycoside antibiotics. Microbiol Rev 1987;51:439–57.
[36] Konno M, Ubukata K. Observation of morphological changes in gram-negative bacilli by electron microscopy. In: Ueda Y, editor. The aminoglicosides. Tokyo: Nankodo Press; 1985. p. 087–94.