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Molecular Characterization of a Cephamycin-Hydrolyzing and Inhibitor-Resistant Class A β-Lactamase, GES-4, Possessing a Single G170S Substitution in the Ω-Loop

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The nosocomial spread of six genetically related *Klebsiella pneumoniae* strains producing GES-type β-lactamases was found in a neonatal intensive care unit, and we previously reported that one of the six strains, strain KG525, produced a new β-lactamase, GES-3. In the present study, the molecular mechanism of cephamycin resistance observed in strain KG502, one of the six strains described above, was investigated. This strain was found to produce a variant of GES-3, namely, GES-4, which was responsible for resistance to both cephamycins (cefoxitin MIC, >128 μg/ml) and β-lactamase inhibitors (50% inhibitory concentration of clavulanic acid, 15.2 ± 1.7 μM). The GES-4 enzyme had a single G170S substitution in the Ω-loop region compared with the GES-3 sequence. This single amino acid substitution was closely involved with the augmented hydrolysis of cephamycins and carbapenems and the decreased affinities of β-lactamase inhibitors to GES-4. A cloning experiment and sequencing analysis revealed that strain KG502 possesses duplicate bla_{GES-4} genes mediated by two distinct class 1 integrons with similar gene cassette configurations. Moreover, the genetic environments of the bla_{GES-4} genes found in strain KG502 were almost identical to that of bla_{GES-3} in strain KG525. From these findings, these two phenotypically different strains were suggested to belong to a clonal lineage. The bla_{GES-4} gene found in strain KG502 might well emerge from a point mutation in the bla_{GES-3} gene harbored by its ancestor strains, such as strain KG525, under heavy antibiotic stress in order to acquire extended properties of resistance to cephamycins and carbapenems.

Over the past decade, a number of new plasmid-mediated β-lactamases with wide substrate specificities have appeared mainly in gram-negative bacilli belonging to the family *Enterobacteriaceae* (11, 19, 20, 27). In particular, the emergence of bacteria producing TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs) has made chemotherapy for bacterial infections more complex than ever (13). Furthermore, non-TEM- and non-SHV-type ESBLs, such as the CTX-M-type (23, 31), GES-type (8, 21, 25, 28, 29), and VEB-type (3, 22) β-lactamases, have also been identified in these gram-negative bacilli. Generally, the β-lactamases described above are often plasmid encoded and can hydrolyze oximino-cephalosporins and monobactams as well as penicillins but not 7-α-methoxy-cephalosporins, the so-called cephamycins. Carbapenems are also very stable against these enzymes. Therefore, at present cephamycins and carbapenems are potent agents for the treatment of infections caused by the gram-negative bacilli that produce these new class A β-lactamases with wide substrate specificities. Among the various β-lactamase genes described above, the genes encoding the GES-type β-lactamases as well as the VEB-type β-lactamases are often located in integrons as gene cassettes (8, 17, 21, 22, 25, 29). Integrons have been described to play a sophisticated role in the accumulation and expression of genes responsible for antibiotic resistance as well as their dissemination among gram-negative bacilli (9, 10).

Among the GES-type β-lactamases, GES-1 was first reported from a *Klebsiella pneumoniae* clinical isolate in France in 1998 (21); and then two other GES-type β-lactamases, IBC-1 and GES-2, were found in *Enterobacter cloacae* and *Pseudomonas aeruginosa*, respectively (8, 25). GES-2 has an amino acid substitution (glycine to asparagine at position 170) compared to the sequence of GES-1 and shows a higher imipenem-hydrolyzing activity than GES-1.

We found that the high-level ceftazidime resistance of six genetically related *K. pneumoniae* clinical strains, which had been isolated from a neonatal intensive care unit (NICU) over a 1-year period, depended on the production of GES-type β-lactamases, and one of the six isolates was found to produce the GES-3 β-lactamase (30). The bla_{GES-3} gene encoding GES-3 was located as a gene cassette in a class 1 integron, as has been observed for the GES-type β-lactamase genes found in Europe. GES-3 production does not affect the level of cephamycin resistance in the *Escherichia coli* host, as has been reported for the other Ambler class A β-lactamases, including ESBLs. However, the levels of resistance to cephamycins varied widely among the six GES-type β-lactamase-producing strains. Among these, the highest MICs of the carbapenems as well as the cephamycins were seen for strain KG502 (30), which also showed an inhibitor resistance phenotype. The goal of this study was to elucidate the molecular mechanism responsible for resistance to cephamycins and carbapenems in strain KG502, as well as its inhibitor-resistant nature.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
K. pneumoniae KG502	Clinical isolate, resistant to cephamycins, carrying bla_{GES-4}	This study
K. pneumoniae KG525	Clinical isolate, susceptible to cephamycins, carrying bla_{GES-3}	30
E. coli CSH-2	*metB* F^- nalidixic acid^r rifampin^r	T. Sawai, Chiba University
E. coli XL1-Blue	*supE44 recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 lac* [F' *proAB*$^+$ *lacIqZ*ΔM15::Tn*10* (Tet^r)]	Stratagene
E. coli BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
Plasmids		
pKGL502	Recombinant plasmid carrying a 6.6-kb BamHI fragment containing bla_{GES-4} of *K. pneumoniae* KG502	This study
pKGS502	Recombinant plasmid carrying a 6.0-kb BamHI fragment containing bla_{GES-4} of *K. pneumoniae* KG502	This study
pKGB525	Recombinant plasmid carrying a 6.7-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	30
pET-GES4	Recombinant plasmid containing PCR-amplified bla_{GES-4} ligated to pET29a(+)	This study
pET-GES3	Recombinant plasmid containing PCR-amplified bla_{GES-3} ligated to pET29a(+)	This study
pBCSK+	Cloning vector, chloramphenicol^r	Stratagene
pCR2.1	Cloning vector, ampicillin^r kanamycin^r	Invitrogen
pET29a(+)	Expression vector; kanamycin^r	Novagen

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* strain KG502 was isolated in May 2002 from the pus of a neonate under treatment in the NICU of a general hospital in Japan. This strain was resistant to oximino-cephalosporins and cephamycins. GES-3-producing strain KG525 was isolated in the same NICU where strain KG502 was isolated.

Susceptibility testing. MICs were determined by the agar dilution method with Mueller-Hinton agar (Becton Dickinson, Cockeysville, Md.), according to the guidelines in National Committee for Clinical Laboratory Standards document M7-A5 (18). *E. coli* ATCC 25922 and ATCC 35218 were purchased from the American Type Culture Collection (ATCC) and served as control strains for MIC determinations. The double-disk synergy test for the detection of ESBL production and an inhibitory test with thiol compounds for the detection of metallo-β-lactamase producers were carried out by the methods described elsewhere (1, 6, 12), with the modification that 2-mercaptopropionic acid was replaced with sodium mercaptoacetic acid.

Transfer of β-lactam resistance genes. Conjugation experiments were performed by the filter mating method with rifampin- and nalidixic acid-resistant *E. coli* CSH-2 as the recipient. Transconjugants were detected on Luria-Bertani (LB) agar supplemented with rifampin (100 μg/ml), nalidixic acid (100 μg/ml), and either ceftazidime (4 μg/ml) or cefminox (2 μg/ml). Transformation of *E. coli* XL1-Blue with the large plasmids of the parental strain *K. pneumoniae* KG502 was performed by electroporation. Transformants were selected on LB agar containing ceftazidime (4 μg/ml) or cefminox (2 μg/ml).

PCR amplification, cloning, and sequencing of β-lactamase gene. To amplify the bla_{GES} gene, PCR was performed with the primers under the conditions described elsewhere (30). The cloning experiment was carried out as follows: total DNA prepared from strain *K. pneumoniae* KG502 was digested with BamHI, and the resultant fragments were ligated to vector pBCSK+ (Stratagene, La Jolla, Calif.), which had been digested with the same enzyme. Transformants carrying recombinant plasmids were selected on LB agar plates containing chloramphenicol (30 μg/ml) and either ceftazidime (4 μg/ml) or cefminox (2 μg/ml). Both strands of the DNA fragments inserted into the recombinant plasmids (pKGL502 and pKGS502) were sequenced.

Purification of β-lactamase. β-Lactamases were purified by exactly the same protocol described elsewhere (30). In brief, β-lactamases were overproduced with the pET system, extracted by use of a French press, and cleared by ultracentrifugation. After ultracentrifugation the supernatant was loaded onto a Hi-Load 16/60 Superdex 200 and anion-exchange Hitrap Q HP column (Pharmacia Biotech, Uppsala, Sweden). The purity of the enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

β-Lactamase assay. Substrate hydrolyses by GES-4 and GES-3 were assayed at 30°C in phosphate buffer (50 mM; pH 7.0) by use of an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The molar extinction coefficients (Δε values) used were as follows: for benzylpenicillin (232 nm), 1.077 mM^{-1} cm^{-1}; for ampicillin (235 nm), 1.121 mM^{-1} cm^{-1}; for cephaloridine (300 nm), 0.384 mM^{-1} cm^{-1}; for cefotaxime (264 nm), 5.725 mM^{-1} cm^{-1}; for ceftazidime (274 nm), 6.123 mM^{-1} cm^{-1}; for cefpirome (290 nm), 4.057 mM^{-1} cm^{-1}; for cefoxitin (293 nm), 0.325 mM^{-1} cm^{-1}; for cefminox (298 nm), 1.878 mM^{-1} cm^{-1}; for imipenem (297 nm), 8.061 mM^{-1} cm^{-1}; and for aztreonam (315 nm), 0.68 mM^{-1} cm^{-1}. Fifty percent inhibitory concentrations (IC_{50}s) were determined with benzylpenicillin as the substrate and the inhibitors clavulanic acid, sulbactam, tazobactam, and imipenem. Purified enzyme and various concentrations of these inhibitors were preincubated in 50 mM phosphate buffer (pH 7.0) at 30°C for 5 min. Purified GES-4 and GES-3 β-lactamases and nonpurified extracts of 50-ml cultures of strain KG502 were subjected to isoelectric focusing (IEF) analysis with an Immmobiline Drystrip (pH 3 to 10; Pharmacia Biotech) and an IPGphor electrophoresis system (Pharmacia Biotech).

Nucleotide sequence accession numbers. The nucleotide sequences described in this work appear in the GenBank nucleotide database under accession numbers AB116260 and AB116723.

RESULTS

Properties of *K. pneumoniae* isolate KG502. *K. pneumoniae* KG502 was isolated from the pus of a neonate in May 2002. This strain was one of the six GES-type β-lactamase-producing strains that we reported previously (30). Strain KG502 exhibited resistance to oximino-cephalosporins and the cephamycins and intermediate susceptibility to carbapenems. No synergy between an amoxicillin-clavulanic acid disk and a ceftazidime and/or a cefotaxime disk was detectable against this strain. The lack of production of metallo-β-lactamases was also suggested by the results of inhibition testing with sodium mercaptoacetic acid. Preliminary PCR detection of some class A β-lactamase and metallo-β-lactamase genes was performed as we reported in our previous study (30), and all PCRs gave negative results.

Transfer of β-lactam resistance by conjugation and transformation. Our previous Southern hybridization experiment with a digoxigenin-labeled bla_{GES}-specific probe demonstrated that the GES-type β-lactamase genes of strain KG502 are located on two distinct plasmids. Therefore, we performed conjugation by filter mating in an attempt to transfer these plasmids to *E. coli* CSH-2, as well as electroporation to introduce them directly into *E. coli* XL1-Blue. However, the transfer of these plasmids into *E. coli* was unsuccessful, despite repeated attempts.

Cloning and sequencing of the β-lactamase gene. Sequencing of the DNAs of the PCR products obtained with the primers specific for the GES-type β-lactamase gene revealed the presence of a variant of the bla_{GES-3} gene in strain KG502. Cloning was performed by standard procedures to determine the entire nucleotide sequences of this new gene. Two distinct recombinant plasmids, one of which carried a 6.6-kb BamHI

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                    20        30        40        50        62
GES-4  MRFIHALLLAGIAHSAYASEKLTFKTDLEKLEREKAAQIGVAIVDPQGEIVAGHRTAQRF
GES-3  ----------------------------------------------------T----
GES-2  ----------------------------------------------------M----
GES-1  ----------------------------------------------------M----

          70        80        90       100 104   110       120
GES-4  AMCSTFKFPLAALVFERIDSGTERGDRKLSYGPDMIVKWSPATERFLASGHMTVLEAAQA
GES-3  -------------------------------------K----------------------
GES-2  -------------------------------------E----------------------
GES-1  -------------------------------------E----------------------

          130       140       150       160 164   170       179
GES-4  AVQLSDNGATNLLLREIGGPAAMTQYFRKIGDSVSRLDRKEPEMSDNTPGDLRDTTTPIA
GES-3  ----------------------------------------G--------------------
GES-2  ----------------------------------------N--------------------
GES-1  ----------------------------------------G--------------------

          190       200       210       220       230       240
GES-4  MARTVAKVLYGGALTSTSTHTIERWLIGNQTGDATLRAGFPKDWVVGEKTGTCANGGRND
GES-3  ------------------------------------------------------------
GES-2  ------------------------------------------------------------
GES-1  ------------------------------------------------------------

          250       260       270       280       290
GES-4  IGFFKAQERDYAVAVYTTAPKLSAVERDELVASVGQVITQLILSTDK
GES-3  ---------------------------------------------
GES-2  ---------------------------------------------
GES-1  ---------------------------------------------
```

FIG. 1. Amino acid alignments of GES-4, GES-3, GES-2, and GES-1 β-lactamases. Hyphens indicate identical amino acids, and the Ω-loop region of β-lactamase is underlined.

fragment and the other of which carried a 6.0-kb BamHI fragment, were obtained independently. The nucleotide sequences of both genetic determinants for β-lactam resistance were the same and differed by a glycine (G)-to-alanine (A) mutation at position 509 compared with the sequence of bla_{GES-3}, so they were designated bla_{GES-4}. The deduced amino acid sequence of GES-4 had an amino acid substitution of G to serine (S) at position 170 (G170S) within the Ω-loop region of the Ambler class A β-lactamase compared with the sequence of GES-3 (Fig. 1). Among the GES-type β-lactamases, a similar amino acid substitution was reported at position 170, G to asparagine (N), leading to the conversion from GES-1 to GES-2 (25).

Sequencing of bla_{GES-4} flanking region. The inserts in recombinant plasmids pKGL502 and pKGS502 were sequenced, which revealed that both fragments commonly contained bla_{GES-4} gene in the class 1 integron separately, followed by an *aacA1-orfG* fused gene, as the first and second gene cassettes, respectively. The integron in pKGL502 differed from that in pKGS502, in that a third gene cassette, *orfA*, was present (Fig. 2). The product encoded by *orfA* had no significant homology with any other known protein at the amino acid sequence level, so the function of the product could not be presumed. Moreover, the backbone genetic structure surrounding the integron containing the bla_{GES-4} gene from strain KG502 was otherwise identical to that surrounding the integron containing the bla_{GES-3} gene from strain KG525, except that it lacked the outer 128-bp nucleotide sequences, including the 25-bp terminal repeat (IRt) at the left end of IS*6100*, as shown in Fig. 2.

Susceptibilities to various β-lactams. The MICs of β-lactams for *K. pneumoniae* strain KG502 and *E. coli* XL1-Blue harboring recombinant plasmid pKGL502 are listed in Table 2. GES-4 β-lactamase-producing strain KG502 exhibited resistance to cefminox, moxalactam, and cefmetazole and intermediate susceptibility to imipenem and meropenem, whereas GES-3-producing strain KG525 was susceptible to all these agents. These resistance trends were also observed in each of the *E. coli* clones harboring pKGL502 or pKGB525, but the overall resistance levels of the clones were lower than those of the parent strains.

Two notable differences were detected between the MICs for a GES-4-producing *E. coli* clone (pKGL502) and those for a GES-3-producing *E. coli* clone (pKGB525), expressed under the same promoters located within *intI1*. One was a difference in the levels of resistance to cephamycins. The MICs of cephamycins, such as cefminox, cefoxitin, moxalactam, and cefmetazole, were much higher for the GES-4-producing clone than the GES-3-producing clone. In addition, the meropenem MIC for the GES-4 producer was 16-fold higher than that for the GES-3 producer.

The other major differences were the inhibition profiles obtained when the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam were added. The MICs of ampicillin, amoxicillin, piperacillin, ceftazidime, and cefotaxime for the GES-4-producing *E. coli* clone were decreased a maximum of only 8-fold in the presence of β-lactamase inhibitors, whereas those for the GES-3-producing *E. coli* clone decreased at least 32- to 512-fold.

IEF analysis. IEF analysis of the crude extract of parent strain KG502 revealed the presence of two major bands with β-lactamase activities corresponding to pIs of 6.9 and 7.6, respectively. The band with a pI of 7.6 was likely the chromosomally encoded

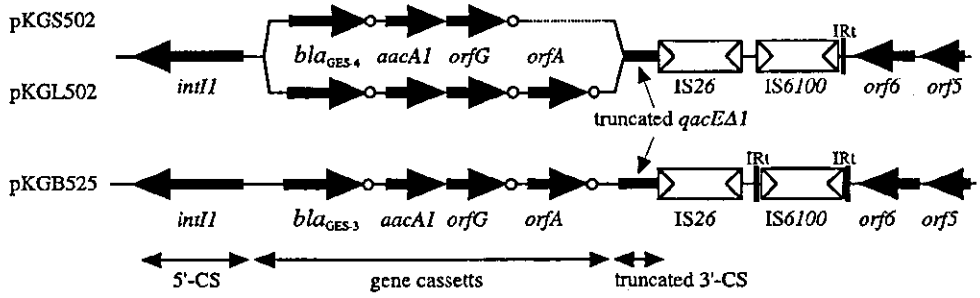

FIG. 2. Schematic comparison of the genetic environments of three class 1 integrons mediating bla_{GES-4} on pKGL502 and pKGS502 and bla_{GES-3} on pKGB525. Open circles represent the positions of the 59-base element. CS, conserved segment.

TABLE 2. MICs of β-lactams

β-Lactam[a]	MIC (μg/ml)				
	K. pneumoniae KG502(GES-4)	*K. pneumoniae* KG525(GES-3)	*E. coli* XL1-Blue pKGL502(GES-4)	*E. coli* XL1-Blue pKGB525(GES-3)	*E. coli* XL1-Blue pBCSK+
Ampicillin	>128	>128	>128	>128	4
Ampicillin + sulbactam	>128	>128	>128	2	2
Amoxicillin	>128	>128	>128	>128	4
Amoxicillin + clavulanate	>128	>128	>128	32	4
Piperacillin	128	128	64	16	1
Piperacillin + tazobactam	64	128	16	0.5	1
Cefotaxime	16	64	1	2	0.13
Cefotaxime + clavulanate	16	8	0.25	0.06	0.06
Cefotaxime + sulbactam	16	32	1	0.06	0.06
Cefotaxime + tazobactam	16	64	1	0.06	0.06
Ceftazidime	1,024	>1,024	64	128	0.13
Ceftazidime + clavulanate	512	256	8	4	0.06
Ceftazidime + sulbactam	>128	>128	32	0.25	0.13
Ceftazidime + tazobactam	>128	>128	32	0.5	0.13
Cephaloridine	>128	>128	64	16	2
Cefminox	>128	8	16	1	0.5
Cefoxitin	>128	128	>128	8	4
Moxalactam	128	4	16	0.5	0.13
Cefmetazole	>128	16	128	2	0.5
Cefpirome	128	>128	1	2	0.06
Cefepime	8	16	0.25	0.25	0.06
Aztreonam	32	64	2	4	0.06
Imipenem	8	0.25	0.25	0.13	0.13
Meropenem	8	0.5	0.25	0.015	0.015

[a] Clavulanate, tazobactam, and sulbactam were each used at a fixed concentration of 4 μg/ml.

SHV-type β-lactamase of *K. pneumoniae*. The estimated pI of 6.9 was identical to those of the purified GES-3 and GES-4 enzymes.

Kinetic studies. The kinetic parameters of the GES-4 and GES-3 β-lactamases for representative β-lactams are given in Table 3. The hydrolyzing efficiencies (k_{cat}/K_m) of GES-4 for the penicillins were about twice as high as those of GES-3, although GES-2 showed less efficient hydrolysis but lower K_m values for cephaloridine and penicillin. On the other hand, GES-3 hydrolyzed ceftazidime and cefotaxime more efficiently than GES-4 did. GES-4 measurably hydrolyzed cefoxitin, cefminox, and imipenem, which accounted for the increases in the MICs of these agents for the clone harboring pKGL502, but no measurable hydrolysis of these agents as substrates by GES-3 was observed under the same experimental conditions used in the present study. No measurable hydrolysis was observed for aztreonam as the substrate with each type of β-lactamase. The IC_{50}s measured with benzylpenicillin as the substrate are listed in Table 4. GES-2 was reported to be inhibited by lower concentrations of clavulanic acid and tazobactam; but GES-4 was inhibited 10-fold less by clavulanic acid, 16-fold less by sulbactam, 8-fold less by tazobactam, and 21-fold less by imipenem than GES-3 was. These results corroborate the inhibitor-resistant nature of GES-4.

DISCUSSION

GES-4 had a single G170S substitution within the Ω-loop region of class A β-lactamases compared with the sequence of

TABLE 3. Kinetic parameters of GES-4, GES-3, and GES-2

Substrate	GES-4			GES-3			GES-2[c]		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Benzylpenicillin	160 ± 10	130 ± 10	7.8×10^5	33 ± 7	15 ± 1	4.5×10^5	4	0.4	9.6×10^4
Ampicillin	62 ± 4	19 ± 11	3.1×10^5	120 ± 30	23 ± 4	1.9×10^5	NP[c]	NP	NP
Cephaloridine	2,200 ± 400	490 ± 30	2.3×10^5	2,300 ± 100	270 ± 10	1.2×10^5	7.7	0.5	6.5×10^4
Cefotaxime	700 ± 200	17 ± 6	2.4×10^4	1,100 ± 100	120 ± 10	1.1×10^5	890	2.2	2.5×10^3
Ceftazidime	1,500 ± 400	2.5 ± 0.5	1.7×10^3	990 ± 130	23 ± 2	2.3×10^4	>3,000	ND[d]	ND
Cefpirome	340 ± 70	3.6 ± 0.4	1.1×10^4	550 ± 30	36 ± 2	6.6×10^4	NP	NP	NP
Cefoxitin	810 ± 70	85 ± 3	1.1×10^5	NH	NC	NC	—[f]	—	—
Cefminox	370 ± 70	7.7 ± 2.0	2.0×10^4	NH	NC	NC	NP	NP	NP
Imipenem	4.7 ± 0.7	0.38 ± 0.07	8.1×10^4	NH	NC	NC	0.45	0.004	9.0×10^3
Aztreonam	NH[a]	NC[b]	NC	NH	NC	NC	—	—	—

[a] NH, no measurable hydrolysis was detected after 2 h of incubation.
[b] NC, not calculated.
[c] Data for GES-2 were reported in reference 25.
[d] ND, not determined due to very high K_m values.
[e] NP, not provided in reference 25.
[f] —, not hydrolyzed (the initial rate of hydrolysis was less than 0.001 μM^{-1} s^{-1}).

TABLE 4. Inhibition profiles of GES-type β-lactamases

Inhibitor	IC_{50} (μM)				
	GES-4	GES-3	GES-2[a]	GES-1[a,b]	IBC-1[c]
Clavulanic acid	15 ± 1.7	1.5 ± 0.15	1.0 ± 0.5	5.0	1.1
Sulbactam	15 ± 0.9	0.91 ± 0.10	—[d]	—	—
Tazobactam	1.4 ± 0.31	0.19 ± 0.03	0.5 ± 0.2	2.5	0.12
Imipenem	2.1 ± 0.16	0.10 ± 0.01	8 ± 2	0.1	0.06

[a] Data were reported in reference 25.
[b] Data were reported in reference 21.
[c] Data were reported in reference 8.
[d] —, data not provided in the references.

GES-3. Replacement of the side chain (—H) of the glycine residue with that (—CH_2—OH) of the serine residue may indeed contribute to the acceleration of cephamycin hydrolysis as well as the inhibitor resistance profile. The GES-2 β-lactamase, identified as a variant of GES-1, had a substitution from glycine to asparagine at position 170, which is the same position leading to the conversion from GES-3 to GES-4. In comparison with GES-1, GES-2 showed an extended substrate specificity for imipenem and a lower affinity for β-lactamase inhibitors (25), as was seen with GES-4. However, the obvious increases in the MICs of cephamycins and meropenem seen for GES-4 were not detected for GES-2. These findings suggest that a single amino acid substitution at position 170, the center of the Ω-loop region, would play a key role in the expansion of the substrate specificities among GES-type β-lactamases. To elucidate the nature of GES-4, molecular modeling analysis as well as X-ray crystallographic analysis will be undertaken in the next study.

Although amino acid substitutions in the Ω-loop region, which influence hydrolyzing activities against oximino-cephalosporins and carbapenems, have also been observed in several class A β-lactamases, such as those of the TEM type (5, 16), SHV type (2, 15), CTX-M type (23), and GES type (25). Disruption of the salt bridge between R164 and D179 was suggested to be mainly involved in the expansion of substrate specificity for oxyimino-cephalosporins in these enzymes. However, substitutions resulting in increased cephamycin resistance have not been reported in class A β-lactamases so far. To our knowledge, this is the first report of a class A β-lactamase with cephamycin-hydrolyzing ability as a result of a single amino acid substitution in the center of the Ω-loop region. Poyart et al. (26) also reported a similar phenomenon in a TEM-type β-lactamase (TEM-52), in which significant decreases in vitro susceptibilities to some cephamycins were not due to an amino acid substitution in the Ω-loop region. The same investigators reported, however, that the combination of three amino acid substitutions E104K, M182Y, and G238S (on the basis of the sequence of TEM-1) in TEM-25 was responsible for the elevated MICs of moxalactam and cefotetan.

The G170S substitution found in the GES-4 β-lactamase affected not only cephamycin and carbapenem resistance but also inhibitor resistance. The IC_{50}s of clavulanic acid, sulbactam, tazobactam, and imipenem for GES-4 were considerably higher than those of GES-3. Since 1990 IRT β-lactamases derived from TEM-type β-lactamases have been reported to be inhibitor resistance class A β-lactamases. The IRT β-lactamases differ from parental enzyme TEM-1 or TEM-2 by several amino acid substitutions at different locations. The IC_{50}s of clavulanic acid and tazobactam for GES-4 (15.2 and 1.43 μM, respectively) were similar to those of some IRT β-lactamases, including IRT-7 (23 and 0.9 μM, respectively), IRT-8 (25 and 1 μM, respectively), and IRT-14 (22.5 and 1.48 μM, respectively), while the IC_{50} of sulbactam for GES-4 was much lower than those for IRTs (4). GES-4 seems to be a very characteristic enzyme, because it has a strong inhibitor-resistant nature like IRT enzymes, while it maintains the capacity to hydrolyze cephamycins and carbapenems.

As with the other bla_{GES} genes, the bla_{GES-4} gene was located in the class 1 integron as a gene cassette. Strain KG502 was unique, in that it possessed two distinct class 1 integrons which carried similar gene cassette configurations, including the bla_{GES-4} gene cassette. The coexistence of class 1 integrons with similar gene cassette arrays might result from the duplication of a region containing one original class 1 integron by mobile elements, such as transposons in strain KG502. It was speculated that in this strain a region containing one original class 1 integron with the bla_{GES-4}, *aacA1-orfG*, and *orfA* gene cassettes was first duplicated in the bacterium. Next, one of the class 1 integrons might have excised the *orfA* gene cassette by a site-specific recombination mechanism catalyzed by some recombinases, including integrases or transposases. Consequently, strain KG502 might have come to have two class 1 integrons with very similar backbone structures.

The entire genetic structure of the flanking region containing bla_{GES-4} on pKGL502 was almost identical to that containing bla_{GES-3} on pKGB525. Taken together with the facts that both the bla_{GES-3} and the bla_{GES-4} genes were found in genetically related *K. pneumoniae* strains and that the genetic environments of these two genes are almost the same, it is probable that the bla_{GES-4} gene emerged from the point mutation in the bla_{GES-3} gene under conditions of antibiotic stress in order to acquire resistance to additional groups of drugs, i.e., the cephamycins and carbapenems.

We characterized here for the first time a novel class A β-lactamase, GES-4, which acquired extended substrate specificity for the cephamycins through a single amino acid substitution within the Ω-loop region. This finding indicates that β-lactamases which are capable of hydrolyzing cephamycins are not limited to the Ambler class B and class C β-lactamases. The emergence of a cephamycin-hydrolyzing class A β-lactamase might complicate treatment in clinical settings, because cephamycins have generally been considered stable to class A β-lactamases and to retain good efficacies for the treatment of infectious diseases caused by organisms producing class A β-lactamases. Moreover, the inhibitor resistance of the GES-4 β-lactamase may introduce confusion during the routine laboratory detection of class A β-lactamase-producing strains, including ESBL producers. The much higher IC_{50} of clavulanic acid for GES-4 hampered the detection of GES-4-producing clinical isolates by conventional double-disk synergy testing.

Since GES-type β-lactamase-producing gram-negative bacteria have been identified worldwide and nosocomial outbreaks caused by these microorganisms have been reported worldwide (7, 14, 24), due consideration must be given to the possible emergence of variants of GES-type β-lactamases like GES-4 which have acquired several amino acid substitutions to

expand their substrate specificities to cope with the extensive use of broad-spectrum β-lactams in clinical settings.

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Study on Body Temperature Monitoring During Brain Hypothermia in Newborn Infants with Severe Hypoxic-Ischemic Encephalopathy

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Summary. In neonates, a definite method of monitoring brain temperature during brain hypothermia has not been established. To estimate the optimal body temperature monitoring during brain hypothermia, we investigated the changes in cephalic vein blood temperature (CVBT), and statistically compared CVBT with the other temperatures such as the tympanic membrane (TMT), nasopharyngeal (NPT), esophageal (EPT) and rectal temperatures (RT) in severe asphyxiated infants with hypoxic-ischemic encephalopathy (HIE). We performed brain hypothermia in four severely asphyxiated infants with HIE. Selective head cooling was performed in two patients, and general body cooling using extracorporeal membrane oxygenation (ECMO) in the other two. In all cases, CVBT, TMT, NPT, EPT, and RT were continuously monitored. Cephalic vein blood temperature during brain hypothermia was 35.0° ± 0.7°C (mean ± SD), TMT 33.9° ± 0.7°C, NPT 34.4° ± 0.7°C, EPT 34.6° ± 0.6°C, and RT 34.5° ± 0.6°C. Cephalic vein blood temperature was significantly higher than the other temperatures ($P < 0.0001$). The differences of CVBT from TMT, NPT, EPT and RT during brain hypothermia were 1.1° ± 0.5°C, 0.6° ± 0.4°C, 0.5° ± 0.5°C, and 0.4° ± 0.4°C, respectively. This study suggested that during brain hypothermia, CVBT could be regarded as the optimal body temperature, since brain temperatures seemed to be underestimated when TMT, NPT, EPT, and RT were the markers of body temperatures.

Key words. Brain hypothermia, Infant, Hypoxic-ischemic encephalopathy, Cephalic vein blood temperature, Extracorporeal membrane oxygenation

Introduction

Recent studies have revealed that brain hypothermia has neuroprotective effects on asphyxiated infants with severe hypoxic-ischemic encephalopathy (HIE) [1,2]. As described in these reports, selective head cooling using a cooling cap is a popular technique for inducing brain hypothermia, and the rectal and nasopharyngeal temperatures are regarded to be the markers of brain temperatures. However, Hayashi reports that brain temperature is cor-

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related to the changes of the internal jugular vein blood temperatures more closely than to those of other body temperatures such as tympanic membrane temperatures [3]. Therefore, we speculated that this would be a good way to measure the cephalic vein blood temperature (CVBT) from the internal jugular vein to evaluate brain temperatures because compared with CVBT, nasopharyngeal or rectal temperatures might be influenced by several factors including room temperature, the power of the radiant heater, locations of sensors, and conditions of infants.

In the present study, we report on four severely asphyxiated infants treated with brain hypothermia. The changes in CVBT, tympanic membrane (TMT), nasopharyngeal (NPT), esophageal (EPT), and rectal temperatures (RT) were monitored during brain hypothermia. To estimate the optimal body temperature monitoring during brain hypothermia, we compared the changes of CVBT with those of the other temperatures.

Patients and Methods

The subjects were infants who were admitted to the neonatal intensive care unit of Kagoshima City Hospital due to neonatal asphyxia, and met the following criteria to receive the brain hypothermia therapy. The infants should (1) have more than 36 weeks of gestation and 2000 g body weight, (2) have Apgar scores of 0–5 beyond 10 min, (3) require positive pressure ventilation 10 min or longer, (4) show evidence of metabolic acidosis in the umbilical cord or very early neonatal blood sample (pH < 7.00), (5) show early onset of severe or moderate neonatal encephalopathy, and (6) show severe or moderate depression and abnormal spikes on EEG.

We performed two methods of hypothermia therapy for these infants. In those with relatively stable respiration and circulation, selective head cooling was performed with a therm-exchanger blanket (Medi-Therm II Hyper/Hypothermia Machine MTA5900 Series; Gaymar Industries, Orchard Park, NY, USA) being put under their head. An internal jugular vein (IJV) catheter, 5.5F-OPTICATH (Abbott Laboratories, North Chicago, IL, USA) was placed at the beginning of selective head cooling, and the blood temperature from the IJV (i.e., CVBT) was monitored using an Oximetrix SO2 System (Abbott Laboratories).

If asphyxiated infants had multiple organ failures such as cardiopulmonary dysfunctions, they were treated with extracorporeal membrane oxygenation (ECMO) and underwent hypothermia of general body cooling. Extracorporeal membrane oxygenation was performed utilizing a venovenous (VV) bypass via a double-lumen catheter (Blood Access UK-Catheter Kit, Argyle) placed through the right IJV to the right atrium. Another catheter, A 8-F cannula (Fem-Flex II Femoral Venous Cannula, Baxter Healthcare, Irvine, CA, USA), was cannulated through the IJV to the cephalic vein, and CVBT was monitored. Brain hypothermia was performed by regulating temperatures of inflow to the right atrium using a heat exchanger. All affected infants were monitored and cooled within 6 h after birth if parental consent had been obtained. Cephalic vein blood temperature was maintained at 35°C for 72 h.

Cephalic vein blood temperature and the other temperatures such as TMT, NPT, EPT, and RT were monitored continuously during cooling. Mon-a-Therm Temperature Probes were used for probes of TMT, NPT, and EPT (Mallinckrodt, St. Louis, MO, USA).

Data are expressed as the mean ± standard deviation. Statistical analysis was performed using the Mann-Whitney *U*-test to determine the significance of differences.

Results

The characteristics of the four infants enrolled in this study are summarized in Table 1. General body cooling using ECMO was performed in two patients. They were found to have cardiopulmonary dysfunctions on admission, and were treated with ECMO. Selective head cooling was accomplished in two patients. Since the degrees of brain edema and cardiopulmonary functions in patient 1 were improved 48 h after the beginning of general body cooling, he was weaned from general body cooling using ECMO. At that point, the method of cooling was changed to selective head cooling.

During hypothermia, no distinct adverse effects such as cardiac arrhythmia, hypotension, and coagulation disorder were found in any patient. The circulatory status of factors such as blood pressure and heart rate, and the respiratory status including the ventilator settings, were stable.

Comparison Between CVBT and Other Temperatures During Hypothermia

The changes in temperatures that occurred during brain hypothermia are analyzed and demonstrated in Table 2. During hypothermia, CVBT was the highest, and TMT was the lowest among all observed temperatures. The difference between CVBT and TMT was 1.1° ± 0.5°C (mean ± SD). The differences of CVBT from NPT, EPT, and RT were 0.6° ± 0.4°C, 0.5° ± 0.5°C, and 0.4° ± 0.4°C, respectively. Nasopharyngeal temperature and RT, which were regarded as the markers of body temperatures during brain hypothermia, were lower by about 0.5°C than CVBT.

TABLE 1. Characteristics of asphyxiated infants treated with brain hypothermia in the present study

Case	GW (weeks)	BW (g)	Obstetric history	Seizure	Apgar score	ECMO	Head cooling
1	39	2382	Placental dysfunction	●	1–3	●	●
2	38	2718	Cord prolapse	●	0–4	●	×
3	39	3460	Placental abruption	●	2–4	×	●
4	Unknown	2810	Delivery at home	●	Unknown	×	●

GW, gestational weeks; BW, body weight; ECMO, extracorporeal membrane oxygenation

TABLE 2. Mean temperatures of each case during brain hypothermia

Case no.	CVBT	TMT	NPT	EPT	RT
1	34.9 ± 0.03	33.2 ± 0.3*	33.5 ± 0.4*	34.2 ± 0.5'	34.6 ± 0.6'
2	34.9 ± 0.03	33.8 ± 0.8*	34.2 ± 0.8'	34.5 ± 0.6'	34.5 ± 0.6'
3	34.9 ± 0.03	34.4 ± 0.6*	34.6 ± 0.5*	34.7 ± 0.5*	34.3 ± 0.5*
4	35.1 ± 0.05	34.1 ± 0.6*	34.9 ± 0.3'	34.9 ± 0.3'	34.7 ± 0.6'
Mean of all cases	35.0 ± 0.7	33.9 ± 0.7*	34.4 ± 0.7*	.4.6 ± 0.6*	34.5 ± 0.6*

Significant differences were observed between CVBT and other temperatures in all cases
CVBT, cephalic vein blood temperature; TMT, tympanic membrane temperature; NPT, nasopharyngeal temperature; EPT, esophageal temperature; RT, rectal temperature
* $P < 0.0001$; ' $P < 0.05$

Discussion

Generally, brain hypothermia therapy for infants is performed with monitoring of body temperatures such as TMT, NPT, EPT, and RT. Although NPT and RT are thought to be the markers of brain temperatures in some reports [4], TMT, NPT, and EPT seem to be influenced by environmental factors including a heating body blanket, an overhead radiant heater, and room temperature [4].

The present study revealed that CVBT was higher than the other temperatures, and the differences between CVBT and the other temperatures were larger than expected. Therefore, if NPT or RT is regarded as a marker of brain temperatures, brain temperatures might be underestimated. Moreover, it is reported that the magnitude of the differences between CVBT and the other temperatures correlates with the prognosis [3]. In these reports, the prognosis is poorer as the differences are smaller, and it would be the poorest if the differences become the negative number. Therefore, it is very important to investigate the differences between CVBT and the other temperatures.

In the present study, we performed general body cooling using ECMO by stably cooling the circulating blood. Extracorporeal membrane oxidation can stabilize cardiopulmonary functions during brain hypothermia and supply a stable blood pressure, which results in supporting the circulation that is sufficient for keeping the cerebral blood flow. The uncontrollable low blood pressure and low cerebral perfusion trigger the occurrence of brain thermo-pooling because of the difficulty in washing out the heat in brain tissues, and lead to the increase of brain temperature. It was recently reported that the induction of mild systemic hypothermia using ECMO is feasible, and is achieved four times as rapidly as selective head cooling [5].

In conclusion, we suggest that it is important to monitor CVBT as a marker of body temperatures during brain hypothermia in infants, since brain temperatures seem to be underestimated if TMT, NPT, EPT, and RT are the markers of body temperatures.

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Changes of Blood Glutamate Levels in Hypoxic Ischemic Encephalopathy Patients Undergoing Brain Hypothermia

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Summary. Recently, brain hypothermia therapy (BHT) has been performed for hypoxic ischemic encephalopathy (HIE) of severely asphyxiated infants, but the effect of BHT for HIE has not been established. Brain hypothermia is thought to affect the release of glutamate in the brain tissue. Therefore, we studied the changes of glutamate release in HIE infants with BHT. Brain hypothermia was performed in seven infants. In all patients a catheter was inserted into the internal jugular vein on the head side in order to monitor intracerebral temperature and take blood samples. Four infants with severe cardiopulmonary insufficiency were referred for extracorporeal membrane oxygenation (ECMO), and participated in whole-body cooling by circulating cooled blood using a heat exchanger in the ECMO circuit. Three infants underwent selective head cooling by wrapping a thermo-exchanger blanket around the head. Brain hypothermia was performed for 72 h to keep the intracerebral temperature around 35°C. Blood samples from the radial artery and cephalic vein were obtained before and during BHT. In each case blood glutamate levels were measured and the differences of those between the cephalic vein and radial artery (Δ blood glutamate level) evaluated, to obtain an indication of glutamate release in the brain tissue. Before BHT the blood glutamate level in the radial artery was 446.753 ± 467.178 nmol/ml and that in the cephalic vein 1094.521 ± 1114.748 nmol/ml. During BHT the blood glutamate levels in the radial artery and cephalic vein dropped to 192.129 ± 185.032 and 387.736 ± 425.490 nmol/ml, repectively. The Δ blood glutamate level before BHT was 647.769 ± 719.637 nmol/ml whereas that after BHT was 195.607 ± 289.357 nmol/ml, significantly low compared with before BHT ($P = 0.0465$). It is suggested that BHT seems to inhibit the release of blood glutamate from brain tissue in HIE cases undergoing BHT.

Key words. Brain hypothermia, Hypoxic ischemic encephalopathy, Glutamate, ECMO, Selective head cooling

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Introduction

Despite advances in the antepartum and intrapartum monitoring techniques developed to improve the recognition of the fetus at risk, perinatal asphyxia still occurs in 3–5 per 1000 live births [1]. Perinatal asphyxia continues to be a major cause of death or later neurodevelopmental sequelae. Recent studies have demonstrated that brain hypothermia (BHT) has neuroprotective effects on asphyxiated infants with severe hypoxic ischemic encephalopathy (HIE). As brain-protective measures of BHT, (1) prevention of fever accumulation in the brain, (2) inhibition of brain neurotransmitter release, (3) improvement of brain edema, (4) prevention of tardive nerve cell death, and (5) inhibition of free radicals have been considered. However, the effect of BHT on HIE has not yet been established.

Glutamate surge is regarded as a trigger of ischemic nerve cell death. It is known that the blood glutamate level of cells is controlled inside and outside as an excitability transmission in a normal physiological situation, but it is uncontrollable in the ischemic state, where nerve cell death is caused by persistent surplus excitement of a nerve cell. Brain hypothermia is thought to affect the release of glutamate in the brain tissue.

In the present study, we report on seven severe HIE infants treated with BHT. We measured the blood glutamate levels of different areas in all seven cases and evaluated the effectiveness of BHT in HIE patients.

Patients and Methods

The BHT was performed in infants who were admitted to the neonatal intensive care unit of Kagoshima City Hospital for neonatal asphyxia when the following criteria were present: (1) over 36 weeks' gestation, (2) over 2000 g in weight, (3) pH at birth or on admission <7.00, (4) Apgar score <5 at 10 min, (5) vigorous resuscitation including manual bagging over 10 min after birth, and (6) clinical encephalopathy such as coma, seizure, and abnormal EEG.

We used two methods of hypothermia therapy for these infants. For those with relatively stable respiration and circulation, selective head cooling was performed using a therm-exchanger blanket (Medi-Therm II Hyper/Hypothermia Machine MTA 5900 Series; Gaymar Industries, Orchard Park, NY, USA) wrapped around the infant's head. A cephalic vein catheter, 5.5-F Opticath (Abbott Laboratories, North Chicago, IL, USA), was placed according to the protocol of the selective head cooling, and the blood temperatures from the cephalic vein were monitored by connecting to an Oximetrix SO_2 system (Abbott Laboratories).

If infants with severe cardiopulmonary insufficiency had been referred for extracorporeal membrane oxygenation (ECMO), hypothermia of general body cooling was performed under ECMO. A venovenous (V-V) bypass using a double-lumen catheter (Blood Access UK-Catheter Kit, Argyle) placed through the right internal jugular vein to the right atrium. Another catheter, an 8-F cannula (Fem-FlexII Femoral Venous Cannula, Baxter Healthcare, Irvine, CA, USA), was cannulated through the internal jugular vein to the cephalic vein. Brain hypothermia was performed by regulating temperatures of inflow from the cephalic vein using the heat exchanger. All infants were monitored and cooled before 6 h after birth, if parental consent had been obtained. The cephalic vein temperature was maintained at 35°C for 72 h.

Blood samples from the radial artery and cephalic vein (head side) were obtained before and during BHT. We measured blood glutamate levels in each case and evaluated the differences of those between the cephalic vein and radial artery (Δ blood glutamate levels), which gives an indication of glutamate release from the brain tissue.

TABLE 1. Patient characteristics ($n = 7$)

Gestational age (weeks)	38.8 ± 1.1
Birth weight (g)	3077 ± 765
Apgar Score at 5 min	2.6 ± 1.6
pH at birth	6.87 ± 0.3
BE at birth (nmol/ml)	−21.8 ± 5.6
HIE score	16.6 ± 3.2

BE, brain edema; HIE, hypoxic ischemic encephalopathy

Statistics

Data are expressed as the mean ± standard deviation. Statistical analysis was performed by Student's *t*-test to determine the significance of the difference.

Results

Seven term infants were enrolled in this study, from March 2001 to July 2003, at the neonatal intensive care unit of the Perinatal Medical Center, Kagoshima City Hospital. General body cooling using ECMO was performed in four patients. They were found to have severe cardiopulmonary insufficiency on admission and were treated with ECMO. Selective head cooling was carried out in three patients. The characteristics of the infants are shown in Table 1.

Before BHT, the blood glutamate level in the radial artery was 446.8 ± 467.2 nmol/ml and that in the cephalic vein 1094.5 ± 1114.7 nmol/ml. During BHT blood glutamate level in the radial artery was 192.1 ± 185.0 and that in the cephalic vein 387.7 ± 425.5 nmol/ml. The blood glutamate levels both in the internal cephalic vein and the radial artery during BHT were low compared with before BHT (Fig. 1a,b).

The Δ blood glutamate levels (cephalic vein blood glutamate level minus the radial arterial blood glutamate level) was 647.8 ± 719.6 nmol/ml before BHT and 195.6 ± 289.4 nmol/ml during BHT. The Δ blood glutamate level during BHT was significantly lower than that before BHT ($P = 0.0465$) (Fig. 2).

Discussion

Brain hypothermia has been performed as a therapeutic intervention to reduce secondary neuronal injury after severe perinatal hypoxic ischemia [2]. Animal studies have shown that a reduction in core temperature can reduce both histologic evidence of brain damage and delayed cerebral energy failure, with an improvement in behavioral outcome after hypoxic ischemia [3]. However, the effect of BHT on perinatal hypoxic ischemia has not been established in humans. Furthermore, hypothermia has a significant profile of potential adverse effects, including metabolic, cardiovascular, pulmonary, coagulation, and immunologic complications [4].

Glutamate, an excitatory amino acid, is the major excitatory neurotransmitter in the brain. Glutamate is not degraded, but instead is removed from the synaptic cleft by energy-dependent neuronal and glial uptake transporters. Excitatory amino acid receptors are widespread throughout the neonatal gray matter [5]. Therefore, we have studied changes in glutamate release in HIE infants undergoing BHT.

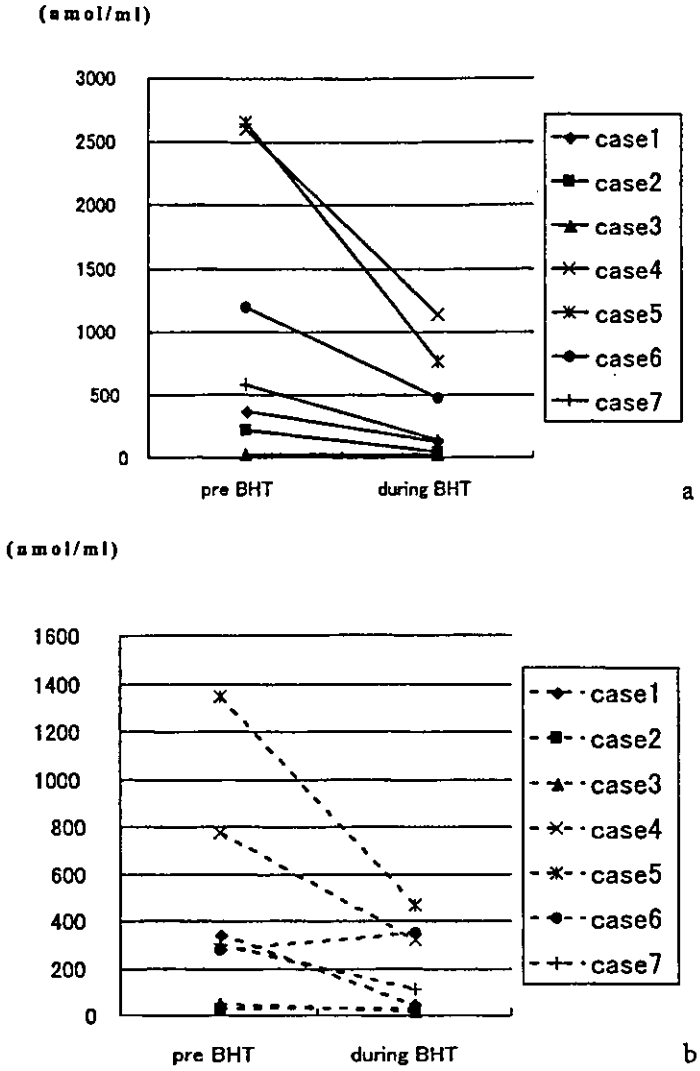

FIG. 1. a Blood glutamic acid level in the cephalic vein (nmol/ml). b Blood glutamic acid level in the radial artery (nmol/ml). *BHT*, brain hypothermia therapy

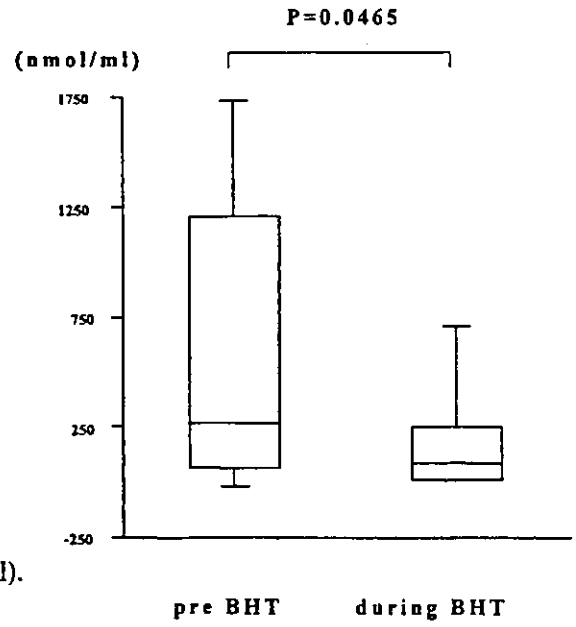

FIG. 2. Changes in glutamate levels (nmol/ml). *Left*, pre-BHT; *right*, during BHT. $P = 0.0465$

In the present study, blood glutamate levels in the cephalic vein and radial artery during BHT were low compared with before BHT. Blood glutamate levels in cephalic vein were more lower compared with radial artery. The change in blood glutamate levels, which might signify the release of glutamate from brain tissues to brain capillary during BHT, was significantly low compared with before BHT. It is suggested that BHT seems to inhibit the release of blood glutamate from the brain tissue in HIE cases undergoing BHT. In conclusion, we suggest that BHT is an effective therapy for neuroprotection in HIE cases.

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Low adjusted serum ionized calcium concentration shortly after birth predicts poor outcome in neonatal hypoxic–ischemic encephalopathy

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Abstract

Aim: Hypoxic–ischemic reperfusion injury causes either necrosis or apoptosis, and the influx of ionized calcium into cells is the major cause of both types of cell death. The aim of this study was to investigate whether or not the serum ionized calcium concentration in neonates with hypoxic–ischemic encephalopathy (HIE) could be used to predict their outcome.

Methods: Serum samples were obtained shortly after birth from 20 HIE neonates who had not urinated or received treatment with calcium. Serum ionized calcium concentrations were adjusted for pH using a correction formula. Twelve neonates without any disease were selected as a control. The results were compared between nine HIE neonates who made a full recovery, 11 who died or had neurologic deficits, and 12 normal neonates.

Results: Considered together, the two HIE groups had lower serum ionized calcium concentrations (1.05 ± 0.10 mmol/L) than the control group (1.22 ± 0.07 mmol/L; $P < 0.0001$). Moreover, serum ionized calcium concentrations in the group with the poor outcome (0.99 ± 0.07 mmol/L) were lower than those in the group that made a full recovery (1.13 ± 0.06 mmol/L; $P = 0.0016$).

Conclusions: The serum ionized calcium concentrations shortly after birth were significantly lower in neonates with HIE who had a poor outcome. Low concentrations may reflect multiple organ damage, particularly involving the brain.

Key words: hypoxic–ischemic encephalopathy, multiple organ damage, neonate, outcome prediction, serum ionized calcium.

Introduction

Neonatal hypoxic–ischemic encephalopathy (HIE), the most common neurologic complication in the perinatal period, is a major cause of chronic disability in childhood.[1-3] The reported incidence of HIE per 1000 births at term delivery is 2–9; as for poor outcome as a result of HIE, the incidence of cerebral palsy (CP) is 1–2 and the incidence of death is 0.3 per 1000 births.[1] However, prognostic assessment of HIE is difficult even after

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diagnosis, and the severity of neuronal damage often remains unclear until later in the postnatal course. HIE is associated with simultaneous damage to multiple organs, presumably because of severe systemic hypoxemia of a degree expected to result in cell death from hypoxic–ischemic reperfusion injury.

Hypoxic–ischemic reperfusion injury can cause either necrosis or apoptosis, and a rapid influx of Ca^{2+} into the cell has been reported to be the major cause of both types of cell death.[4,5] Hypoxic–ischemic reperfusion experiments with *in vitro* preparations have shown a simultaneous increase in intracellular Ca^{2+} and a decrease in extracellular Ca^{2+} during both necrosis and apoptosis in organs including the brain,[6–9] kidney,[10,11] heart,[12–14] and liver.[15,16] These findings suggest that a widespread Ca^{2+} influx into cells of multiple damaged organs in neonatal HIE patients might lower the serum ionized Ca^{2+} concentration ($[Ca^{2+}]_s$). If this hypothesis is correct, the $[Ca^{2+}]_s$ should be abnormally low shortly after birth in severe cases of HIE. However, we know of no previous report concerning the severity of neonatal HIE in terms of $[Ca^{2+}]_s$. We therefore evaluated the relation between neonatal HIE outcome and $[Ca^{2+}]$.

Materials and Methods

Patients

Forty-four neonatal HIE patients born after the 37th week of pregnancy were treated in the Neonatal Intensive Care Unit at Kagoshima City Hospital and Toyama Medical and Pharmaceutical University between October 1997 and June 1999. All patients in this study had brain edema demonstrated by ultrasonography, and developed seizures within 72 h of birth. We diagnosed neonatal HIE using the criteria of abnormal muscle tone, feeding difficulties, altered alertness, and at least three of the following: (i) late decelerations on fetal monitoring or meconium staining; (ii) delayed onset of respiration; (iii) arterial cord blood pH <7.1; (iv) Apgar score <7 at 5 min; and (v) multiple organ failure.[17]

Classification of these 44 neonates according to the criteria of Sarnat and Sarnat placed 36 in stage 2 and eight in stage 3.[18] Parental informed consent for treatment was obtained for all patients after sufficient explanation of the likely outcomes. Cerebral hypothermia was not used to treat any of these patients. We excluded six neonates who were affected by asymmetrical intrauterine growth restriction (IUGR). The biophysical profile scores in the remaining 38 cases were 10 points prior to the onset of labor (Fig. 1). Accordingly, the hypoxic–ischemic reperfusion injury in these cases was thought to have occurred mainly during labor or after birth.

Ionized calcium

The serum ionized calcium concentration, considered the best indicator of physiologic blood calcium activity, averages 1.25 mmol/L, with a 95% confidence limit of 1.1–1.4 mmol/L.[19] Physiologically active $[Ca^{2+}]_s$ is difficult to assess in cases with severe acidosis or alkalosis, but within a blood pH range of between 7.2 and 7.6 an adjusted $[Ca^{2+}]_s$ at pH 7.4, designated $[Ca^{2+}]_{s(7.4)}$, can be calculated using the following formula:[20,21]

$$\text{adjusted } [Ca^{2+}]_{s(7.4)} = \text{actual } [Ca^{2+}]_s \times [1 - 0.53 (7.4 - \text{actual blood pH})].$$

Neonatal HIE patients show a rapid decrease in $[Ca^{2+}]_s$ shortly after birth. This is likely to result from calcium excretion into the urine, reflecting the severe renal dysfunction caused by hypoxic–ischemic reperfusion injury.[22,23] In the present study, the total quantity of calcium excretion into the urine on the first day was significantly greater in the HIE group (0.171 ± 0.085 mEq/day) than in the control group (0.038 ± 0.024 mEq/day; $P < 0.0001$). For these reasons, we determined $[Ca^{2+}]_{s(7.4)}$ shortly after birth from serum samples obtained before the first urination. To minimize the effect of calcium excretion in the bladder, balloon catheters were put on all patients at admission. The urinary volume of these cases was almost zero, so the loss of calcium through the bladder could not affect the serum calcium concentration. Thus, two conditions, blood pH > 7.2[21] and no excretion of calcium from the body prior to serum sampling, are necessary for an informative comparison of $[Ca^{2+}]_{s(7.4)}$. From the 38 neonates not excluded because of asymmetric IUGR, 14 failed to meet one of the above criteria and were excluded. We also excluded four patients who had received either calcium-containing agents for emergent resuscitation on admission or sodium bicarbonate (for reducing severe metabolic acidosis with base excess ≤–20), as these treatments could influence $[Ca^{2+}]_s$ (Fig. 1).

Twenty HIE patients remained who satisfied all conditions for the study. Full neurologic recovery was made in nine (group 1), while death or CP occurred in 11 (group 2). With informed parental consent, 12 neonates without HIE or other significant illness were selected randomly as a control group. We compared $[Ca^{2+}]_{s(7.4)}$ shortly after birth between these three groups.

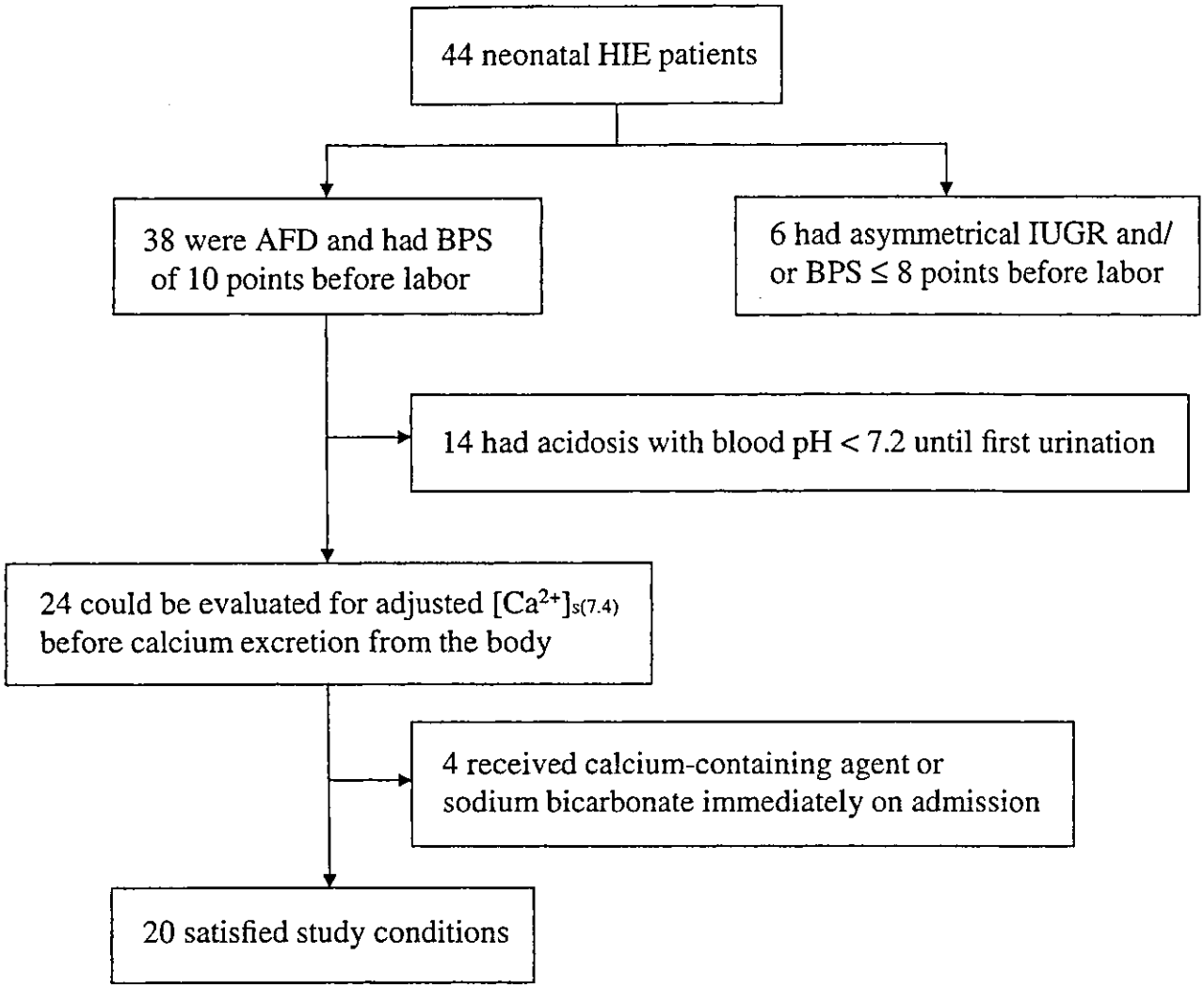

Figure 1 Profile of subject inclusion and exclusion in the study. AFD, appropriate-for-dates infant; BPS, biophysical profile scoring; HIE, hypoxic–ischemic encephalopathy; IUGR, intrauterine growth restriction.

When HIE patients were admitted to our center, an adequate artificial ventilation mode, such as synchronized intermittent mandatory ventilation or high-frequency oscillation, was selected according to the patient's particular respiratory dysfunction. For all, urinary balloon catheters were inserted to detect and monitor urination. Convulsions caused by HIE were treated immediately on occurrence with diazepam (10 mg/kg). Blood tests, including complete blood count, biochemistry, blood gases, and electrolytes, were carried out for all patients at admission. Blood gases and electrolytes, including serum ionized calcium, were measured at 2–4-h intervals until blood pH stabilized.

After placement of a radial or posterior tibial arterial catheter, arterial blood was drawn and the samples were collected in glass capillary tubes (Capillary Tube Kit 82590–10; Instrumentation Laboratory, Lexington, USA) containing heparin (70 IU/mL). Blood pH and $[Ca^{2+}]_s$ were measured simultaneously in all samples (ABL725; Radiometer Medical, Copenhagen, Denmark).

Follow-up neurologic examinations and electroencephalograms (EEG) were carried out at 3, 6, 9, 12, and

24 months of age, or more frequently in a few cases. A normal outcome at a given age was based on the following criteria: the absence of hemiparesis or other focal neurologic deficits, the presence of normal muscle tone and reflexes, and a normal EEG.

Data are presented as mean ± SD. Statistical analysis was carried out using the Mann–Whitney *U*-test between values for any two groups. Significant differences were defined at the 5% level ($P < 0.05$).

Results

The clinical background of the 20 neonatal HIE patients is summarized in Table 1, while short- and long-term clinical outcome is shown in Table 2. Nine patients had a normal outcome (full neurologic recovery; group 1), while 11 had an abnormal outcome (death or development of CP; group 2).

Table 3 compares the clinical features between the control and the two HIE groups. No significant difference was observed in gestational age or birth weight. The level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) was significantly higher in the HIE groups (74 ± 53 IU/L, 14.5 ± 8.5 IU/L, and 955 ± 450 IU/L, respectively, in group 1; 277 ± 294 IU/L, 63.3 ± 66.3 IU/L, and 2491 ± 1230 IU/L, respectively, in group 2) than in the control group (38 ± 12 IU/L, 5.3 ± 1.5 IU/L, and 405 ± 59 IU/L, respectively). Moreover, the levels in group 2 were significantly higher than those in group 1. The level of creatine kinase (CK) in the HIE groups (1279 ± 1203 IU/L in group 1; 1996 ± 1622 IU/L in group 2) was significantly higher than that in the control group (405 ± 59 IU/L). The concentration of blood urea nitrogen (BUN) and creatinine (14.2 ± 4.7 mg/dL and 0.97 ± 0.28 mg/dL, respectively) was significantly higher in group 2 than in the control group (11.3 ± 2.6 mg/dL and 0.67 ± 0.11 mg/dL, respectively) or group 1 (10.4 ± 1.7 mg/dL and 0.72 ± 0.18 mg/dL, respectively). However, the Apgar scores at 1 min and 5 min, umbilical artery pH, and lactate did not differ significantly between groups 1 and 2.

Figure 2 shows the time-course of changes of unadjusted $[Ca^{2+}]_s$ during the 24 h after birth. While $[Ca^{2+}]_s$ decreased only slightly in the control group, it decreased more markedly and rapidly in groups 1 and 2.

The adjusted $[Ca^{2+}]_{s(7.4)}$ shortly after birth in the HIE groups considered together was 1.05 ± 0.10 mmol/L, significantly lower than in the control group

Table 1 Clinical background of neonatal hypoxic–ischemic encephalopathy patients

Case no.	Sex	Gestational age (weeks)	Birth weight (g)	Abnormality in fetal cardiotocogram	Cause	Apgar score (1 min)	Apgar score (5 min)	Umbilical artery pH
1	M	40	3420	Prolonged bradycardia	Not clear	5	6	6.96
2	M	39	2766	Prolonged bradycardia	Not clear	4	5	ND
3	M	38	2800	Prolonged bradycardia	Abruption of the placenta	2	5	6.98
4	M	41	3240	Prolonged bradycardia	Abruption of the placenta	1	4	ND
5	F	38	2680	Prolonged bradycardia	Breech position	2	4	6.95
6	F	41	2930	Persistent L/D	Abruption of the placenta	2	3	6.90
7	F	37	2631	Prolonged bradycardia	Not clear	2	5	6.88
8	F	41	2930	Prolonged bradycardia	Abruption of the placenta	4	4	6.89
9	F	40	3268	Prolonged bradycardia	Not clear	5	5	6.87
10	M	40	3480	Prolonged bradycardia	Not clear	2	3	ND
11	F	38	3202	Prolonged bradycardia	Abruption of the placenta	2	6	ND
12	F	38	2850	Persistent L/D	Not clear	1	2	6.88
13	F	37	2715	Prolonged bradycardia	Abruption of the placenta	1	2	6.92
14	F	40	3210	Prolonged bradycardia	Prolapse of the umbilical cord	2	4	ND
15	M	40	3567	Prolonged bradycardia	Not clear	3	4	6.98
16	M	39	2860	Prolonged bradycardia	Abruption of the placenta	4	5	6.89
17	M	39	3520	Prolonged bradycardia	Not clear	4	5	6.91
18	F	37	2652	Prolonged bradycardia	Abruption of the placenta	1	3	6.71
19	M	40	3044	Prolonged bradycardia	Abruption of the placenta	1	2	6.90
20	M	40	2950	Prolonged bradycardia	Abruption of the placenta	2	5	6.80

F, female; L/D, late deceleration; M, male; ND, not determined.