政ばかりでなく，マクロ経済にも大きな影響を及ぼすため，その抜本的な改革は重要な政策課題となっている。

Ⅲ 医療サービスの機能

医療は公益を目途としたサービス産業であり，受給者（患者）の多くは疾病を獲得した者という，直接受給者が限定されるサービス産業である。医療サービスの供給は受給者に対して新たな価値を創造するのではなく，疾病による受給者の経済活動に関する損失をどれだけ最小化するかが受給者にとっての経済価値となる。つまり，医療の供給を必要とする患者数が増えれば増えるほど，経済損失は増加しているといえる。したがって，医療活動は直接的には経済的損失を前提とした経済活動である。もちろん一方で，社会安全，安心感，健康持続など間接的なマクロ経済への効果も無視はできない。しかし，それでも，誰も病気にならずに寿命まで生きることができる社会があるとして，その社会の効率性(生産性)を超えることはできない。つまり，病気による経済損失をどれだけ軽減できるかで評価されるところに医療サービスの特徴がある。その意味では医療は不採算部門である。そして，その不採算性の軽減が医療政策の重要課題の一つとなる。

Ⅳ 診断群分類の必要性

既に述べたように，不採算部門としての医療費の伸びは，このまま続けば医療保険財政の破綻にととどまらず，マクロ経済にも大きな影響を与えるため，国民医療費の伸びを圧縮することが課題となる。特に国民から見ると患者負担と保険料の増加があり，租税に加えて国民負担は増える一方である（それでも，わが国の社会保障負担率は15%であり，ドイツ〔25.7%〕，フランス〔15.5%〕，スウェーデン〔19.5%〕に比べて負担率は低い)。この様な中で，医療費の中身に対する関心が高まり，特に，医療費に見合った医療の質(治療成績)が確保されているかが重大な関心事となった。そのため，国民に分かりやすい標準化され，施設間比較が可能な医療情報の提供とその透明性が求められてきた。つまり，医療機関は国民が供出した医療費に見合った医療を提供しているかどうかが(value for money) 問われている。

しかし，これまで医療現場から提供されてきた医療情報はそのような期待には応えられていない。むしろ，医療供給の確保とその供給側の既得権益を守るために，そのような比較ができないようなシステムを意識的に構築してきたとも言われる。結果として，診療に関しては膨大な数の傷病名(特にレセプト病名)とそれに対応した治療法があることに加えて，従来の出来高払いでは診療報酬を得るため，多数の傷病名が一患者に与えられ，原疾患の同定すら困難なことが頻繁に見られる。そのため，医療供給機能の評価や施設間比較には困難を極めるため，傷病名（原則的には入院を必要とした傷病名）とその治療法をグループ化して治療成績や経済的評価をする必要性がでてきた。そのような治療成績の公開・提供によって，国民は医療機関の選択を行うことが可能となり，治療成績の悪い医療機関は淘汰され，質のよい医療を提供できる医療機関が生き残ることとなる。標準化された医療情報(経営情報を含む)の提供とその透明化によって，医療情報は国民，患者，医療者，保険者，経営者によって共有化され，効率性を加味した質のよい医療が提供されるシステムとなる。ただし，技術（治療）料・看護料・管理料などの医療コストに関しては治療成績(効果)に応じて国民が決定するものであり，より質の高い医療供給を国民が望む場合は当然のことながらそれに見合って，それらの医療費は増加することとなる。

Ⅴ 診療報酬体系の見直しの原則

包括評価の導入にあたって診療報酬体系を抜本的に見直すことが必要となり，その原則は①医療技術の適正な評価，②医療機関の運営コストの適正な反映，③患者(国民)の視点の導入である。医療技術に関してはドクターフィーとホスピタルフィー的要素に分離し，難易度，時間，技術度，重症化予防，生活指導などの評価が必要となる。医療機関の運営コストに関しては急性期，小児医

療，救急医療，集中治療，精神医療，在宅医療などの機能評価を導入し，治療成績に応じた機能評価を導入している。結果として，治療成績の公開を行うことによって，患者に医療機関の選択の基礎資料を提供し，患者側の選択の拡大が図られることになる。

Ⅵ 包括評価対象医療機関

対象となる医療機関は82の特定機能病院であり，大学病院本院，国立がんセンター本院，国立循環器センターによって構成される。特定機能病院に導入された理由としては診療録管理体制が比較的整備されていること，電子媒体による診療録管理が進んでいること，高度で質の高い，医療が供給されていること，包括評価の改善に関して協力が得られやすいことなどあるが，一方では特定機能病院の「特定機能」の中身が不明確であり，国公立機関は経営努力がされていない，医療情報部や公衆衛生学教室が機能停止に陥っているなど，別の機能改善の意図が含まれていると指摘されることもある。

Ⅶ 包括評価対象患者

包括評価の対象となる患者は一般病棟に入院している患者であり，特定機能病院に入院している患者の9割以上が対象となる。除外されるのは精神病棟，結核病棟に入院している患者，入院後24時間以内に死亡した患者，薬事法での治験対象患者，高度先進医療対象患者，臓器移植の対象患者の一部（皮膚移植術，生体部分肝移植，同種腎移植術，同種骨髄移植，同種末梢血幹細胞移植），回復期リハビリテーション病棟入院料算定患者，緩和ケア病棟入院料算定患者，診断群分類で症例数20未満，変動係数1を超える分類の対象者，その他厚生労働大臣が定める患者である。

Ⅷ 包括評価での診断群分類

包括評価の基礎となる診断群分類は，特定機能病院から収集した診療録及び診療報酬明細書のデータ（平成14年7月～10月の退院患者，26.7万人分）に基づき開発された。診断群分類の対象となるのは575傷病であり，それを2,552に分類した。包括評価の対象となるのは，そのうち1,860分類であり，1,860分類に該当しない患者は，従来通りの出来高算定とされている。

Ⅸ 包括制度の範囲

包括評価での診療報酬は包括部分と出来高部分で構成される。包括評価の範囲はホスピタルフィー的要素部分であり，含まれるものは入院基本料，検査(内視鏡等の技術料を除く)，画像診断，投薬，注射，1,000点未満の処置料，手術・麻酔の部で算定する薬剤・特定保険医療材料以外の薬剤・材料等がある。

出来高評価の範囲はドクターフィー的要素部分であり，手術料，麻酔料，1,000点以上の処置料，心臓カテーテル法による検査，内視鏡検査，診断穿刺・検体採取，指導管理料，リハビリテーション，精神科専門療法等に加えて手術・麻酔の部で算定する薬剤・特定保険医療材料が含まれる。

Ⅹ 包括部分の診療報酬

包括部分の診療報酬は以下の式で決定される。

包括範囲点数＝
診断群分類毎の1日当たり点数×
医療機関別係数×在院日数

診断群分類毎の1日当たり点数は，診断群分類ごとに平均在院日数と包括部分の従来の出来高請求額の平均値をもって算出される。ただし，在院日数に応じた医療資源の投入量を適切に評価する観点から，診断群分類毎の1日当たりの包括点数は，在院日数に応じて逓減される。具体的には診断群分類ごとに以下のように3段階の点数を設定した。

（1）入院日数の25パーセンタイル値までは平均点数に15%加算する。

（2）25パーセンタイル値から平均在院日数までの点数は，平均在院日数まで入院した場合の1日当たり点数の平均点数が，1日当たり平均点を段階を設けずに設定した場合と等しくなるように設定した。

（3）平均在院日数を超えた日から前日の点数の

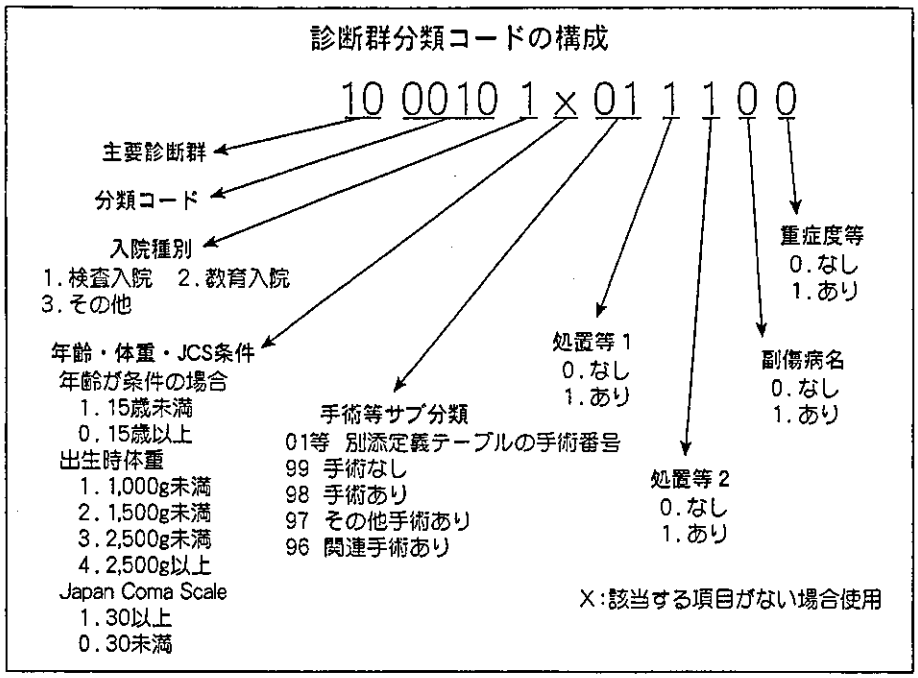

図1 診断群分類コード

診断群分類コードは図のような9種類の数字による構成となっている。これらの数字を合わせたものが診断群分類番号となる。

85%で算定する。

また，包括で決められた入院日数を超えた場合は1日当たり平均の包括点数の85%が1日あたりの入院料として支払われる。入院期間が著しく長い場合（診断群分類毎にみて平均在院日数から標準偏差の2倍以上入院した場合）には，在院日数から標準偏差の2倍を超えて入院した日から全てが出来高により算定される。

XI 診断群分類コード

診断群分類は図1のような数字と記号で構成される。

XII 傷病名の記載

包括評価で記載が必要となる傷病名は以下の5傷病名がある。

（1）主傷病名：主治医が最終診断としたもの

（2）入院の契機となった傷病名：主治医が入院を必要と判断し，必要な治療や診療行為を投入すべきと考える傷病名（疑い病名を含む）

（3）医療資源を最も投入した傷病名：医療資源を最も投入したと判断した傷病名

（4）入院時併存症：入院時，又は治療開始時に既に患者が持っていた病態で，主傷病の治療に影響をもたらす疾患，又は全体的な資源投入量に影響をもたらす疾患病態

（5）入院後発症疾患：入院後，又は治療開始後に発症した病態で，主傷病の治療に直接関連した疾患病態

それぞれの傷病名と処置(手術)によって診断群分類が微妙に変化するが，基本的にはもっとも医療資源を投入した傷病名によって診療報酬が支払われる（図2）。

XIII 医療機関別係数

医療機関別係数は医療機関の診療機能に応じた機能評価係数と前年度の診療報酬を保証するための調整係数の2つによって以下のように構成される。

療機関別係数＝機能評価係数＋調整係数

機能評価係数は入院基本料等加算を係数化した機能評価係数であり，以下のような係数がある。

入院時医学管理加算	(0.0103)
紹介外来加算	(0.0257)

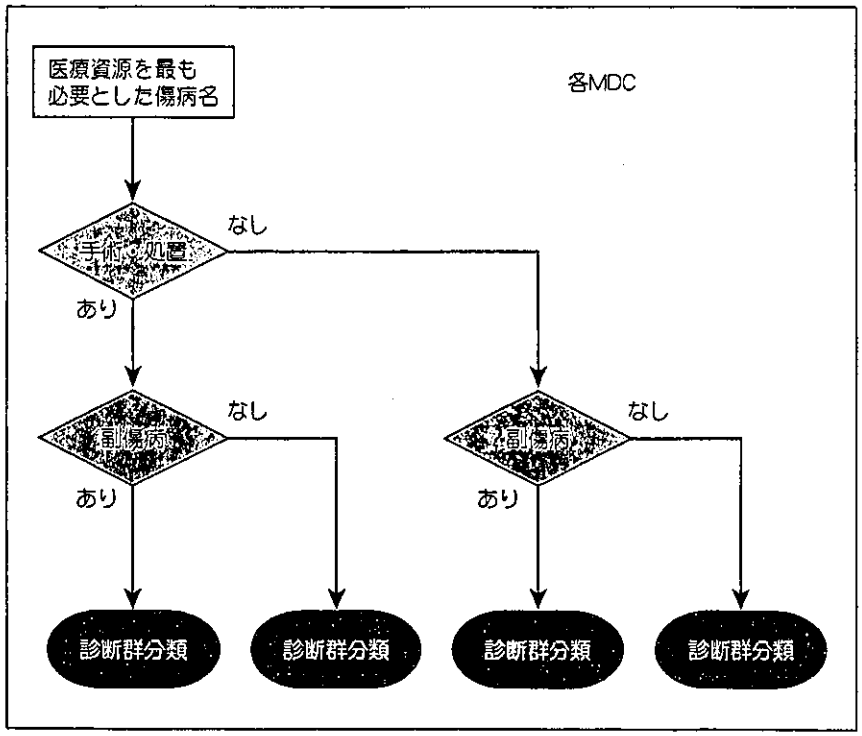

図2 包括評価での診断群分類の基本的考え方
医療資源を最も必要とした傷病名を起点として手術・処置，副傷病名などを検討に加えて，診断群分を行う。

紹介外来特別加算 (0.0086)
急性期入院加算 (0.0030)
診療録管理体制加算 (0.0005)
看護配置・看護補助者配置

調整係数は前年度の診療報酬額を保証するものであり，一時的な係数と考えられ，将来は廃止されると考えられている。調整係数の算定は診断群分類による包括評価に係る医療費が平成14年7月～10月の医療費の実績に等しくなるように医療機関ごとに設定してある。

調整係数＝旧方式による算定÷
新方式による算定－機能評価係数

XIV 特定入院料の取り扱い

急性期の特定入院料の算定対象の患者については，診断群分類による包括評価の対象とし，所定点数の加算を行うこととしている。対象となる特定入院料は以下のごとくである。

救命救急入院料
特定集中治療室管理料
新生児特定集中治療室管理料
総合周産期特定集中治療室管理料
広範囲熱傷特定集中治療室管理料
1類感染症患者入院医療管理料

これらの特定入院料の加算は包括評価に重層して加算されるため，今後は実コストに基づいた特定入院料の算定方式の確立が必要となる。その際は何らかの機能評価係数を加味した1日あたりの算定法になると思われる。つまり，適応がないのに管理量を請求した場合は病院収入は減額され，治療成績が良い場合は病院収入が増加するような評価法が必要となる。

XV 包括評価の今後の課題

包括評価には様々な未解決の問題があるが，診療の透明化と数字による機能評価が可能となった意味で，医療供給システムの評価と改善にとって飛躍的な第一歩を記すことができた。今後さらに検討と改善を必要とするのは以下の点である。

・診断群分類の精緻化

表1 MRSAによる院内感染を獲得した患者での包括支払と出来高の差額(保険点数)

患者名	感染症	転帰	包括支払	出来高	> 2SD	支払い差
A	敗血症	生存	572,470	757,620		△185,150
B	腸炎	生存	376,116	426,574		△50,458
C	胆道感染	生存	465,048	649,567		△184,519
D	創感染	生存	271,081	325,947		△54,866
E	血流感染	死亡	330,657	351,849	+	△21,192
F	腹腔膿瘍	生存	296,078	575,965	+	△279,887
G	創感染	生存	331,243	370,677		△39,434
H	創感染	生存	288,910	293,025	+	△4,115
I	創感染	生存	419,637	817,053		△397,416
J	敗血症	死亡	186,002	508,539	+	△322,537
K	創感染	生存	218,755	216,600		2,155
合計			3,755,997	5,293,416		△1,537,419

- コスト・入院期間に関する「はずれ値」の分析(治療能力と材料外れ値の分離評価)
- 重症度・治療法・合併症・治療能力の評価への組み込み

・在院日数短縮の動機付けの強化
- 傾斜配分とすることを検討する
- 1日当たり入院費から診断群分類当たりに変える

・技術料の包括化
- 熟練度・人数・時間・難易度を加味した技術料の包括化

・コスティングによる評価の達成[4]
- 調整係数の廃止
- 材料差益の消失→技術・サービスの適正評価

・外来の包括化
- 外来診療の機能評価法の確立

・医療の質に関する評価の組み込み
- 患者転帰予測式による重症度(難易度)評価の導入
- 内科診療の機能評価法の確立(Auditの導入)

・特定入院料の算定方式の確立
- ICU, NICUなどの診療機能評価法の確立

・臨床研究, 臨床教育, 患者データ収集などの診療を支える活動に対する加算の検討

この中で調整係数の廃止は早急に実現する必要がある。調整係数がある限り, 前年度の年間病院収入が保証されるため, もともと医療費削減に努力をしてきた医療機関の経営努力が評価されず, 乱診乱療をしてきた医療機関の収益が手厚く保証されることになる。また, 医療の質に関する評価システムを内蔵しない限り, 医療機関に対して必要な検査や与薬を差し控える萎縮医療を招くインセンティブが働く危険性がある。そのため, 診断群分類ごとの治療成績の医療機関別の公開, 予定されない再入院率, 退院患者の救急外来受診率などの臨床指標の算出と開示が必要となる。また, 技術料の包括化や特定入院料の算定には材料コストに加えて人員コストや施設維持コストの算出が必要であり, それに基づいて, 現行の出来高請求額に依拠した包括診療報酬から実コストに依拠した包括診療報酬へと制度設計を変える必要がある。また, 特定入院料の算出には機能評価が必要であり, 何らかの機能評価係数を加えて特定入院料の診療報酬額を決定する必要がある。

加えて, 関連学会や製薬企業が高額な薬剤, 血液製剤, 医療器材の包括からの切り離し(出来高

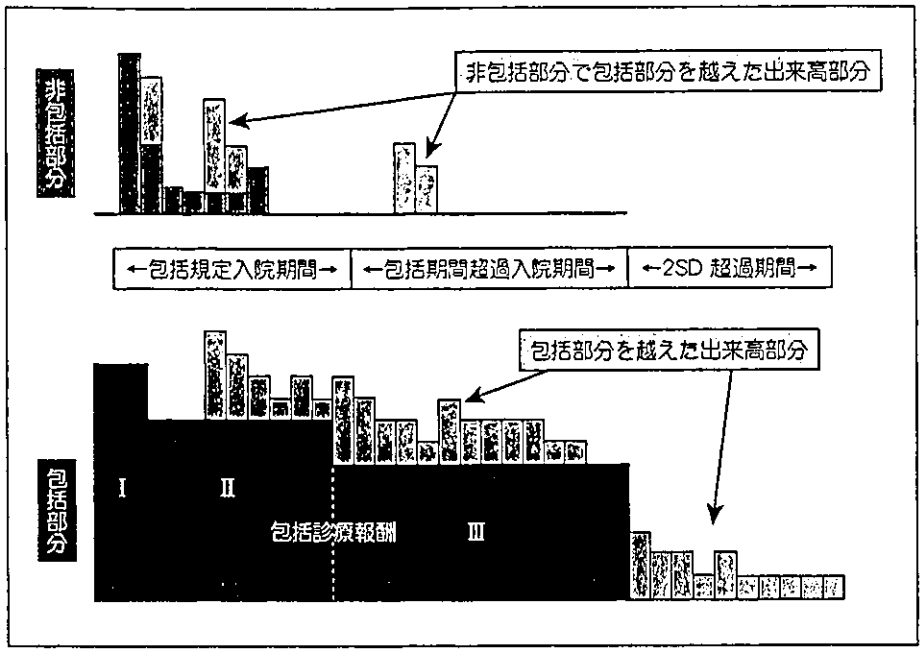

図3 包括評価における院内感染の医療機関の損失と国の損失

包括部分では包括範囲を超えた出来高が院内感染による医療機関の損失となる。事実上はⅠ＋Ⅱの部分が平均的な包括診療報酬であるため，Ⅲの部分が院内感染によって入院期間が延びるとすると，仮にその期間に新規入院患者を得ることができるとすると，新規入院患者の包括診療報酬からⅢの部分を引いた部分が病院の損失となる。

払い）を要求する傾向があるが，有効性が臨床疫学的に証明されているものは包括の中に入れて評価すべきである。有効性が不明な薬剤，血液製剤，医療器材の出来高での支払いを認めると，それらの治療法に対する有効性の検証が遅れ，有効でない治療法に多大な医療費が支払われることとなり，医療供給システム全体の質が劣化することになる。

XVI 包括評価における院内感染対策の重要性

包括評価では診断群分類ごとに決められた入院期間を超過すると医療機関の収益は低下する。これまで，院内感染が発生すると医療機関では入院期間の延長と更なる医療資源の投下が必要となるが，そのほとんどは診療報酬が保証されていたため，大きな損失とはならなかった。むしろ，病床稼働率が上がらない医療機関では収益の補填にすらなっていたと予想される。包括評価の導入によって，医療機関は患者転帰の改善と院内感染の防止が医療機関の経営にとって最重要課題の一つとなる。院内感染だけに限れば，包括評価の導入は医療の質の向上と不必要な医療提供を防止する2つの効用をもっているともいえる。

XVII 包括評価における院内感染の損失

現行では院内感染によって医療機関が損失するのは①包括部分全体で包括支払い枠を超えて使われた薬剤・検査などの余分なコスト，②包括部分で平均的な入院日数を超えて減額される部分（1日当たりの包括額は15%減額），③非包括部分で院内感染のため新たに必要となった再手術や処置となる。従って，システムとしての院内感染防止のインセンティブは不十分である。このインセンティブを強化するには全てを包括化して1日入院日当たりから診断群分類あたりに変更することが必要となる。

平成14年の4カ月間に1,000床規模の病院でMRSAによる院内感染を発症した患者の包括評価制度での診療報酬点数と従来の出来高払いを比

較したのが以下の表1である。包括評価の導入によって，MRSAによる院内感染を発症すると，患者一人当たり約150万円の損失が生じたことになる。

XIII 院内感染に対する実効性のある対策の必要性

従来，院内感染は患者転帰を悪化させて，医療機関の評判を低下させ，集客性を低下させるが，出来高払いのため病院経営には大きな影響を与えることはなかった（集客力の低い病院では損失補填となる）。しかし，包括評価の導入によって院内感染は直接的に医療機関の収益を悪化させることになった（図3）。医療機関の経営者は病院の経営戦略の中に治療成績の向上とコストダウンに加えて新たに医療事故対策と院内感染対策を重要課題として取り上げ，それぞれの実行を図ることが必要となる[4]。これまで，医療機関の経営者の支援を得られずに孤立して院内感染対策を実行してきた医療従事者にとって，初めてその実力を発揮して社会的使命を果たす舞台は整ったといえる。

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Nosocomial Spread of Ceftazidime-Resistant *Klebsiella pneumoniae* Strains Producing a Novel Class A β-Lactamase, GES-3, in a Neonatal Intensive Care Unit in Japan

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Klebsiella pneumoniae **strain KG525, which showed high-level resistance to broad-spectrum cephalosporins, was isolated from the neonatal intensive care unit (NICU) of a Japanese hospital in March 2002. The ceftazidime resistance of strain KG525 was transferable to *Escherichia coli* CSH-2 by conjugation. Cloning and sequence analysis revealed that production of a novel extended-spectrum class A β-lactamase (pI 7.0), designated GES-3, which had two amino acid substitutions of M62T and E104K on the basis of the sequence of GES-1, was responsible for resistance in strain KG525 and its transconjugant. The bla_{GES-3} gene was located as the first gene cassette in a class 1 integron that also contained an *aacA1-orfG* fused gene cassette and one unique cassette that has not been described in other class 1 integrons and ended with a truncated 3′ conserved segment by insertion of IS*26*. Another five ceftazidime-resistant *K. pneumoniae* strains, strains KG914, KG1116, KG545, KG502, and KG827, which were isolated from different neonates during a 1-year period in the same NICU where strain KG525 had been isolated, were also positive for GES-type β-lactamase genes by PCR. Pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus-PCR analyses displayed genetic relatedness among the six *K. pneumoniae* strains. Southern hybridization analysis with a GES-type β-lactamase gene-specific probe showed that the locations of bla_{GES} were multiple and diverse among the six strains. These findings suggest that within the NICU setting genetically related *K. pneumoniae* strains carrying the bla_{GES} gene were ambushed with genetic rearrangements that caused the multiplication and translocation of the bla_{GES} gene.**

Resistance to β-lactam antibiotics mainly depends on the production of β-lactamases. To date, a large variety of β-lactamases which were classified by their amino acid sequences and functional substrate specificity profiles in various gram-negative bacilli such as *Pseudomonas* spp. and members of the family *Enterobacteriaceae* have been documented (6). Since the late 1980s, extended-spectrum β-lactamases (ESBLs) derived from TEM- and SHV-type penicillinases capable of hydrolyzing the oxymino-cephalosporins have been spreading globally, mainly in the *Enterobacteriaceae*, including *Klebsiella pneumoniae* and *Escherichia coli* (5, 23, 29). Moreover, various non-TEM-, non-SHV-type class A β-lactamases exhibiting extended-spectrum activities, including CTX-M-type (13, 31, 38, 39, 41), SFO-type (18), VEB-type (12, 20, 25), and GES-type (10, 11, 19, 24, 28, 37) β-lactamases, have also been reported in various gram-negative bacilli. Among the GES-type β-lactamases, GES-1, which was found to be produced by *K. pneumoniae* ORI-1, identified from a child transferred from French Guiana to France in 1998, was the first report of the GES-type class A β-lactamase (24); and GES-1-producing *K. pneumoniae* strains have caused nosocomial infections in Portugal (9). IBC-1 was identified in an *Enterobacter cloacae* clinical isolate from Greece in 1999 (11), and IBC-1-producing *E. cloacae* has also been reported to cause nosocomial infections in a neonatal intensive care unit (NICU) (17). GES-2, which displayed more extended-spectrum activity against imipenem compared with that of GES-1, was reported in *Pseudomonas aeruginosa* from South Africa (28) in 2000, and GES-2 producers also caused a nosocomial infection (27). All three genes, bla_{GES-1}, bla_{GES-2}, and bla_{IBC-1}, were found to be located as a gene cassette within similar class 1 integrons.

Recently, six clinical isolates of *K. pneumoniae* showing high-level resistance to various broad-spectrum cephalosporins, including ceftazidime, were identified from the NICU of a Japanese hospital, and conventional PCR analyses for TEM-derived ESBLs and CTX-M enzymes failed to specify their genetic determinants. In the present study, therefore, we characterized the molecular mechanism underlying the multiple-cephalosporin resistance among these six strains, as well as the organizations of their genetic environments.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Six *K. pneumoniae* clinical strains had been isolated from neonates over 1 year, from September 2001 to August 2002, and were stored in the clinical microbiology laboratory of the hospital until this study. Biochemical phenotypic identification of these strains was carried out by the analytical profile index procedure (API 20E system; bioMerieux, Marcy l'Etoile, France). A pre-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or Reference
K. pneumoniae KG914, KG1116, KG525, KG545, KG502, KG827	Clinical isolates from neonatal specimens	This study
E. coli		
CSH-2	*metB* F⁻ nalidixic acid^r rifampin^r	T. Sawai, Chiba University
XL1-Blue	*supE44 recA1 endA1 gyrA96 thi hsdR17*(rK⁻ mK⁺) *relA1 lac* [F⁻ *proAB*⁺ *lacI*qZΔM15::Tn*10*(Tet^r)]	Stratagene
BL21(DE3)pLysS	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
Plasmids		
pKGC525	A natural plasmid carrying bla_{GES-3} of *K. pneumoniae* KG525	This study
pKGB525	A recombinant plasmid carrying a 6.7-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	This study
pKGM525	A recombinant plasmid carrying a 11.6-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	This study
pTAGES3	A recombinant plasmid carrying a PCR fragment with the entire bla_{GES-3} sequence and its promoter region cloned into the pCR2.1 vector	This study
pGES3	A recombinant plasmid carrying EcoR1 fragment from pTAGES3	This study
pIBC1	A recombinant plamid carrying bla_{IBC-1} constructed from pGES3	This study
pBCSK⁻	A cloning vector; chloramphenicol^r	Stratagene
pCR2.1	A cloning vector; ampicillin^r kanamycin^r	Invitrogen
pET29a(+)	An expression vector; kanamycin^r	Novagen
pET-GES3	A recombinant plasmid carrying PCR-amplified bla_{GES-3} gene ligated to pET29a(+)	This study

liminary double-disk synergy test was carried out with disks containing ceftazidime and amoxicillin-clavulanate. Bacteria were grown in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, unless specified otherwise.

Antibiotic susceptibility testing. The following antibiotics were obtained from the indicated sources: ampicillin, amoxicillin, and cefminox, Meiji Seika Kaisha, Ltd., Tokyo, Japan; piperacillin, Toyama Chemical Co., Ltd., Toyama, Japan; cephaloridine and moxalactam, Shionogi & Co., Ltd., Osaka, Japan; cefmetazole and chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; cefotaxime and cefpirome, Aventis Pharma, Ltd., Tokyo, Japan; ceftazidime and clavulanic acid, GlaxoSmithKline K. K., Tokyo, Japan; sulbactam, Pfizer Pharmaceutical Inc., Tokyo, Japan; tazobactam, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan; cefepime, Bristol Pharmaceuticals K. K., Tokyo, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo Japan; and rifampin, Daiichi Pharamaceutical Co., Ltd., Tokyo, Japan. The MICs of the β-lactams were determined by the agar dilution method, according to the recommendations of National Committee for Clinical Laboratory Standards document M7-A5 (21). *E. coli* ATCC 25922 and ATCC 35218 were purchased from the American Type Culture Collection (ATCC) and served as control strains in the antimicrobial susceptibility testing.

PCR amplification. To amplify the broad-spectrum β-lactamase genes from the six clinical strains, PCR analyses were performed with sets of primers specific for various β-lactamase genes found in Japan—including the TEM-derived extended-spectrum β-lactamase (39); CMY-2-, MOX-1-, and DHA-1-type β-lactamases (8, 40, 41); and CTX-M-1-, CTX-M-2-, CTX-M-9-, IMP-1-, IMP-2-, and VIM-2-type β-lactamases (13, 26, 30, 31, 33, 39)—under the conditions described elsewhere (33). Detection of the SHV-type β-lactamase gene was not performed because most clinical *K. pneumoniae* strains carry the LEN-1 and/or SHV-1 β-lactamase gene on their chromosomes (1, 7). In order to detect the GES-type β-lactamase gene, an 827-bp internal fragment of the gene was amplified with primers GES-A (5′-CTT CAT TCA CGC ACT ATT AC-3′) and GES-B (5′-TAA CTT GAC CGA CAG AGG-3′) under the conditions described above.

Conjugal transfer of β-lactam resistance. Conjugal transfer of the ceftazidime resistance of *K. pneumoniae* KG525 to a recipient *E. coli* strain, strain CSH-2 (F⁻ *metB*, resistant to nalidixic acid and rifampin), was performed by the filter mating method. Transconjugants were selected on LB agar plates containing ceftazidime (2 μg/ml), rifampin (100 μg/ml), and nalidixic acid (50 μg/ml).

Cloning experiment and DNA sequencing. Basic recombinant DNA techniques were performed as described by Sambrook et al. (32). Total DNA of *K. pneumoniae* KG525 was extracted and digested with BamHI. The resultant fragments were ligated into cloning vector pBCSK+ (Stratagene, La Jolla, Calif.) restricted with the same enzyme. Transformants were selected on LB agar plates containing chloramphenicol (30 μg/ml) and ampicillin (50 μg/ml) or ceftazidime (2 μg/ml). The nucleotide sequence of the cloned fragment was determined with BigDye terminator cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.) by using custom sequencing primers.

Site-directed mutagenesis for comparison of GES-3 with IBC-1. PCR-based site-directed mutagenesis of the bla_{GES-3} gene was performed with the LA PCR In Vitro Mutagenesis kit (TAKARA Bio Inc., Ohtsu, Japan). In brief, the entire bla_{GES-3} gene and its promoter region were amplified by PCR and cloned into plasmid pCR2.1 with the TA cloning kit (Invitrogen, NV, Leek, The Netherlands). One plasmid, pTAGES3, was selected after it was confirmed that it contained no amplification error and was then digested with EcoRI. The resultant fragment was recloned into pBCSK+. The resultant plasmid, pGES3, with an insert carrying the bla_{GES-3} gene and its promoter region was used to introduce a single nucleotide mutation (C to T) at nucleotide position 167, which leads to an amino acid substitution (T to M) at position 62 in GES-3, resulting in the conversion of the gene product from GES-3 to IBC-1 expressed under the same promoter.

Pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus (ERIC)-PCR analyses. Total DNA was prepared from six *K. pneumoniae* strains (34) and digested overnight with XbaI (New England Biolabs, Beverly, Mass.). The digested DNA was electrophoresed with a CHEF-DRII Drive Module (Bio-Rad Laboratories, Hercules, Calif.) under the following conditions: pulses ranging from 10 to 40 s at 6 V/cm for 20 h at 16°C. Six *K. pneumoniae* strains were also typed with the primer ERIC-2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′). The PCR was carried out under the conditions described elsewhere (36).

Southern hybridization. Large plasmids were prepared from six *K. pneumoniae* strains by the procedure described by Kado and Liu (16). The chromosomal DNA was extracted from each isolate by the method of Stauffer et al. (35). Both plasmid and chromosomal DNA preparations were separately subjected to Southern hybridization experiments. The 827-bp DNA probes were amplified by a PCR with primers 5′-CTT CAT TCA CGC ACT ATT AC-3′ and 5′-TAA CTT GAC CGA CAG AGG-3′. The PCR amplicons were labeled with digoxigenin (DIG) by a random priming labeling method with the PCR DIG detection system, as recommended by the manufacturer (Roche Diagnostics, Tokyo, Japan). Southern hybridization was performed by the protocol of the manufacturer (Roche Diagnostics).

Purification of GES-3 β-lactamase. To overproduce GES-3 β-lactamase in *E. coli*, the bla_{GES-3} gene was amplified by using two primers, primer GES-F (5′-CAT ATG CGC TTC ATT CAC GCA CTA TTA CTG-3′), which was designed to add an NdeI linker (underlined), and primer GES-R (5′-GTC GAC

TABLE 2. MICs for six *K. pneumoniae* clinical isolates from a NICU

K. pneumoniae strain	Date of isolation (mo/day/yr)	Site of isolation	MIC (μg/ml)[a]													
			AMX + CLA	PIP	PIP + TZB	CAZ	CAZ + CLA	CTX	ATM	CMZ	FEP	IPM	GEN	AMK	LVX	CIP
KG914	9/14/01	Bronchial secretion	>128	>128	128	>1,024	512	64	64	16	32	0.13	1	32	<0.06	<0.06
KG1116	11/16/01	Bronchial secretion	>128	>128	128	>1,024	128	64	64	16	32	0.13	0.5	32	<0.06	<0.06
KG525	3/4/02	Stool	>128	128	128	>1,024	256	64	64	16	16	0.13	2	64	<0.06	<0.06
KG545	3/7/02	Nasal mucosa	>128	>128	>128	>1,024	1,024	128	128	128	64	0.5	2	64	0.25	0.25
KG502	5/2/02	Pus	>128	128	64	1,024	512	16	32	>128	8	8	2	32	0.13	<0.06
KG827	8/27/02	Bronchial secretion	>128	128	32	>1,024	256	16	64	>128	32	0.5	2	32	2	1

[a] Abbreviations: AMX, amoxicillin; CLA, clavulanic acid; PIP, piperacillin; TZB, tazobactam; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; CMZ, cefmetazole; FEP, cefepime; IPM, imipenem; GEN, gentamicin; AMK, amikacin; LVX, levofloxacin; CIP, ciprofloxacin.

CTA TTT GTC CGT GCT CAG GAT GAG-3'), which was designed to add an SalI linker (underlined), and DNA polymerase (Expand High Fidelity PCR System; Roche Diagnostics), according to the instructions of the manufacturers. The resulting products were cloned into plasmid pCR2.1 with the TA cloning kit (Invitrogen, NV) and subjected to confirmatory sequencing. One plasmid with no amplification error was selected and was partially double digested with NdeI and SalI and then subcloned into pET-29a(+) (Novagen, Madison, Wis.), which had been digested with the same enzymes. The expression vector constructed, named pET-GES3, was introduced into *E. coli* BL21(DE3) pLysS (Novagen). *E. coli* BL21(DE3) pLysS carrying plasmid pET-GES3 was cultured in 1 liter of LB broth containing kanamycin (50 μg/ml). Isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) was added when the culture reached an A_{600} of 0.6, and the culture was incubated for an additional 2 h. The cells were harvested by centrifugation and were suspended in 5 ml of 20 mM bis-Tris buffer (pH 6.5). The suspension was passed through a French pressure cell twice and was then centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used for subsequent chromatographic purification. Size-exclusion chromatography was performed on a HiLoad 16/60 Superdex 200 prep-grade column (Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 20 mM bis-Tris buffer (pH 6.5). Fractions containing β-lactamase activity were collected and applied to an anion-exchange Hitrap Q HP column with the same buffer. β-Lactamase activity was recovered in the flowthrough and was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) overnight at 4°C. This partially purified enzyme was loaded onto a Hitrap Q HP column (Pharmacia Biotech) preequilibrated with the same buffer and eluted with a linear gradient of NaCl. Fractions presenting high levels of activity were pooled and dialyzed against 50 mM phosphate buffer (pH 7.0).

Isoelectric focusing (IEF). Fifty milliliters of the bacterial culture was centrifuged, and the cell pellet was suspended in 5 ml of distilled water. A crude periplasmic preparation containing β-lactamase was obtained by freezing-thawing the bacterial suspension three times, followed by ultracentrifugation (40,000 × *g*) for 1 h. The supernatant was condensed to 1/10 volume with an Ultrafree-15 Centrifugal Filter Device (Millipore Corporation, Bedford, Mass.). To determine the isoelectric point (pI), 5 μl of the condensed supernatant containing β-lactamase was loaded onto an Ampholine PAG plate (pH 3.5 to 9.5; Pharmacia Biotech) with a Multiphor II electrophoresis system (Pharmacia Biotech). The pI of the β-lactamase was measured by staining the gel with a 0.05% solution of nitrocefin. Purified GES-3 β-lactamase was also electrophoresed on the Ampholine PAG plate and stained with Coomassie blue.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession number AB113580.

RESULTS

Characteristics of six *K. pneumoniae* clinical isolates. The susceptibilities of the six isolates to β-lactams are presented in Table 2. All isolates were resistant to piperacillin, ceftazidime, and aztreonam. The MICs of cefotaxime, cefmetazole, cefepime, and imipenem for the isolates were variable. Despite the addition of clavulanic acid, these isolates kept their high-level resistance to ceftazidime (MICs, ≥128 μg/ml). This observation was consistent with the negative results of the double-disk synergy test with two disks containing ceftazidime and amoxicillin-clavulanate, respectively. Metallo-β-lactamase production was not detected by using a thiol compound (2). PCR analyses performed preliminarily to detect broad-spectrum β-lactamase genes including TEM derivatives, CTX-M-1, CTX-M-2, CTX-M-9, MOX-1 (CMY-9), CMY-2, DHA-1, IMP-1, IMP-2, and VIM-2, all of which had already been identified in Japan, failed to give positive results.

Transfer and cloning of β-lactamase genes. The ceftazidime resistance determinant of representative strain *K. pneumoniae* KG525 was successfully transferred to a recipient strain, *E. coli* CSH-2; and this finding indicated that the genetic determinant was located on a transferable plasmid. Two ceftazidime-resistant *E. coli* clones, each of which harbored a plasmid containing BamHI fragment inserts of approximately 6.7 and 11.6 kb, respectively, were obtained as a result of the cloning experiment. These two recombinant plasmids contained the same 864-bp open reading frame (ORF) encoding a putative β-lactamase which had conserved structural features of the active site of Ambler class A β-lactamases. The deduced amino acid sequence of the β-lactamase showed an amino acid substitution of M62T (a point mutation of T to C at nucleotide position 167) compared with the amino acid sequences of GES-1 (24), GES-2, and IBC-1, as well as an additional E104K substitution in comparison with the amino acid sequences of GES-1 and GES-2 (Fig. 1). Moreover, an N170G substitution was found in GES-3 compared with the amino acid sequence of GES-2, although the G residue at amino acid position 170 was conserved in IBC-1 and GES-1, as well as in GES-3. Therefore, we named this novel class A β-lactamase GES-3, although GES-1 is based on "Guiana extended spectrum" (24).

Antibiotic susceptibilities. The MICs of the β-lactams for parent strain *K. pneumoniae* KG525, transconjugant *E. coli* CSH-2(pKGC525), and transformant *E. coli* XL1-Blue(pKGB525) are listed in Table 3. Parental strain *K. pneumoniae* KG525 was resistant to most β-lactams except the cephamycins and carbapenems. The tranconjugant and transformant were resistant to ceftazidime, and the MICs of the other β-lactams were lower for the tranconjugant and the transformant than for the parent strain. The changes in the MICs of cefotaxime and ceftazidime for parent strain KG525 were apparently observed by the addition of β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, while decreases in the MICs of amoxicillin, ampicillin, and piperacillin, as well as cefotaxime and ceftazidime, were observed for the *E. coli* tranconjugant and transformant in the presence of the inhibitors.

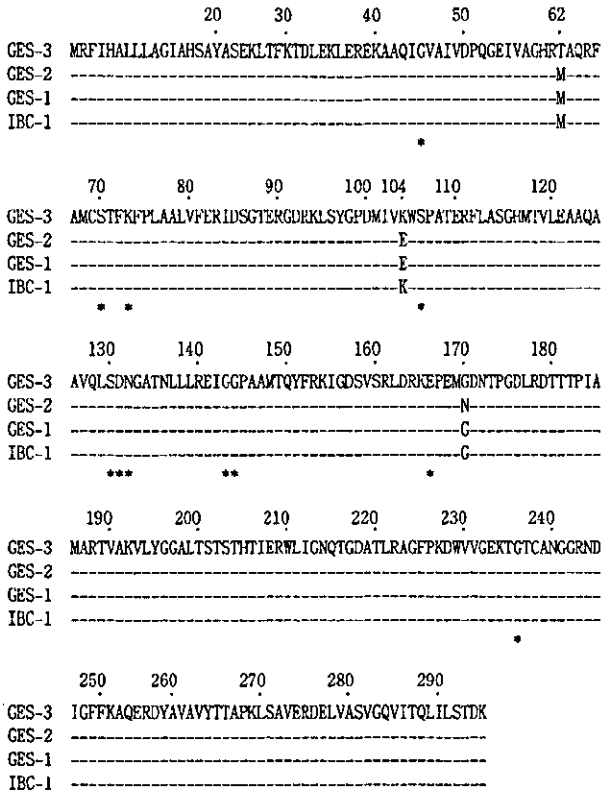

FIG. 1. Comparison of the amino acid sequence of GES-3 with those of the GES-1, GES-2, and IBC-1 β-lactamases. Only the substituted amino acid residues are indicated by the single-letter amino acid code. Dashes represent positions where no amino acid substitution was observed among the four enzymes. The amino acid residues conserved among class A β-lactamases are indicated with asterisks.

Genetic environment of bla_{GES-3}. Two distinct BamHI fragments carrying the bla_{GES-3} gene were cloned; their sizes were approximately 6.7 and 11.6 kb, respectively, and pKGB525 had the 6.7-kb fragment. Sequencing analysis of the entire insert on pKGB525 revealed that the bla_{GES-3} gene was located as a gene cassette within a class 1 integron structure, as was observed in the other GES-type β-lactamase genes, bla_{GES-1}, bla_{GES-2}, and bla_{IBC-1} (Fig. 2). The 59-base element downstream of the bla_{GES-3} gene was made up of 110 bp and was different from that of the truncated 59-base element of bla_{GES-1} on pTK1 (GenBank accession number AF156486), but shared it 99% nucleotide identity with those of bla_{GES-2} on pLAP-1 (GenBank accession number AF326355) and bla_{IBC-1} on pHT9-2 (GenBank accession number AF208529).

The second gene cassette adjacent to the bla_{GES-3} gene was a fused *aacA1-orfG* gene cassette. The results of the disk diffusion test indicated that the presence of an *aacA1* component, which encodes aminoglycoside-6′-*N*-acetyltransferase, conferred kanamycin resistance to the transformant *E. coli* XL1-Blue(pKGB525) (data not shown). The nucleotide sequence of this fused gene cassette shared 100% identity with that in a class 1 integron on plasmid pCMXR1 (GenBank accession number AB061794). The function of the product encoded by *orfG* has not been characterized in detail. The third gene cassette is 327 bp and was named *orfA*. The *orfA* gene was suggested to be a gene cassette by recognition of the features typical of these elements: (i) the presence at the cassette boundaries of 7-bp core site sequences that completely fit the consensus sequence and (ii) the presence of a 59-base element of 78 bp downstream of the *orfA* gene. However, no remarkable similarity between the hypothetical protein encoded by *orfA* and any other known protein sequences was detected in a search performed with the BLAST program. The 3′ conserved segment of this integron showed a characteristic organization. The *qacE*Δ*1* gene was truncated at nucleotide position 114 by the insertion sequence IS*26*. In the region downstream of IS*26*, an IS*6100* element and two ORFs of unknown function, i.e., *orf5* and *orf6*, were found. The nucleotide sequence of the 1.4-kb region containing IS*6100*, *orf5*, and *orf6* was identical to that seen downstream of the *sulI* gene of the class 1 integron in the chromosomal multidrug resistance locus of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (GenBank accession number AF261825). Sequencing analysis of pKGM525 carrying the second bla_{GES-3} gene on an 11.6-kb fragment was also done, and the bla_{GES-3} gene was also found in a class 1 integron structure with the same gene cassette configuration as in pKGB525. The nucleotide sequence of the region from *intI1* to IS*26* was the same as that found in pKGB525 carrying a 6.7-kb BamHI fragment.

Construction of IBC-1 by site-directed mutagenesis. Only one amino acid substitution, M62T, was found between the sequences of GES-3 and IBC-1 (Fig. 1). Therefore, to examine whether this amino acid substitution affects the MICs of β-lactams for the *E. coli* clones producing each enzyme, we constructed plasmid pIBC1, which encodes the IBC-1 enzyme under the same promoter as that for GES-3, by site-directed mutagenesis of the bla_{GES-3} gene within the parental plasmid, pGES3. However, this single substitution did not markedly influence the MICs for the *E. coli* clones (data not shown).

PCR detection and genotypic comparison. The remaining five nonrepetitive ceftazidime-resistant *K. pneumoniae* strains, strains KG914, KG1116, KG545, KG502, and KG827, were all found to be bla_{GES} positive by PCR. The results of PFGE analysis of all six isolates are shown in Fig. 3. Their fingerprinting patterns were very similar but in some cases were distinct. We examined the fingerprinting patterns from 48.5 to 194 kb in detail under other conditions (data not shown). Overall, there were from three to seven band differences among the six strains examined. The ERIC-PCR patterns amplified with the ERIC-2 primer were indistinguishable from one another (data not shown). Taken together with the fact that these isolates were collected over a 1-year period, we speculate that they were genetically related and had probably spread via nosocomial transmission of an endemic clone.

Plasmid profiles and Southern hybridization. The plasmid profiles of the six bla_{GES}-positive strains showed the presence of a large plasmid of approximately similar size in five of the six strains (Fig. 4A), while some of them possessed additional plasmids which were smaller and more diverse in size. Hybridization analyses with the probe specific for the GES-type β-lactamase genes, including bla_{IBC-1}, revealed that the location of this gene varied among the strains tested. Hybridization signals for large plasmids were detected for strains KG914, KG1116, and KG502 (Fig. 4B). Hybridization signals for both plasmids and chromosomal positions were observed for

TABLE 3. MICs of antimicrobial agents for the parental strain, transconjugant, and transformant

β-Lactam[a]	MIC (μg/ml)				
	K. pneumoniae KG525	*E. coli* CSH-2(pKGC525)[b]	*E. coli* CSH-2	*E. coli* XL1-Blue(pKGB525)[c]	*E. coli* XL1-Blue(pBCSK+)
Ampicillin	>128	>128	4	>128	4
Ampicillin + sulbactam	>128	2	2	2	2
Amoxicillin	>128	>128	8	>128	4
Amoxicillin + clavulanate	>128	32	4	32	4
Piperacillin	128	16	1	16	1
Piperacillin + tazobactam	128	0.5	1	0.5	0.5
Cefotaxime	64	2	0.13	2	0.13
Cefotaxime + clavulanate	8	0.06	0.06	0.06	0.06
Cefotaxime + sulbactam	32	0.06	0.06	0.06	0.06
Cefotaxime + tazobactam	64	0.06	0.06	0.06	0.06
Ceftazidime	>1,024	128	0.13	128	0.13
Ceftazidime + clavulanate	256	4	0.13	4	0.06
Ceftazidime + sulbactam	>128	0.25	0.13	0.5	0.13
Ceftazidime + tazobactam	>128	0.5	0.13	0.5	0.13
Cephaloridine	>128	16	2	16	2
Cefminox	8	0.5	0.5	1	0.5
Moxalactam	4	0.25	0.13	0.5	0.13
Cefpirome	>128	1	0.06	2	0.06
Cefepime	16	0.13	0.06	0.25	0.06
Aztreonam	64	4	0.06	4	0.06
Imipenem	0.25	0.5	0.25	0.13	0.13
Gentamicin	2	0.13	0.13	<0.06	<0.06
Amikacin	64	2	0.25	4	0.25
Levofloxacin	<0.06	0.13	0.13	<0.06	<0.06
Ciprofloxacin	<0.06	<0.06	<0.06	<0.06	<0.06

[a] Clavulanate, tazobactam, and sulbactam were used at a fixed concentration of 4 μg/ml each.
[b] pKGC525 is a resident plasmid found in *K. pneumoniae* strain KG525, and it carries the bla_{GES-3} gene.
[c] pKGB525 is a recombinant plasmid that carries a 6.7-kb BamHI insert that mediates the bla_{GES-3} gene.

KG525. One of the hybridized plasmids from each of KG914, KG1116, and KG525 were similar in size. A single hybridization signal corresponding to the chromosomal position was detected for each of the strains KG545 and KG827 (Fig. 4C).

pIs of β-lactamases. The pI value of the purified GES-3 enzyme was determined to be 7.0 (Fig. 5A). IEF of crude extracts from six GES-type β-lactamase-producing clinical strains revealed two bands with pIs of 7.6 and 7.0 (Fig. 5B). The band with pI 7.0 was also detected in a GES-3-producing *E. coli* transformant which harbored the bla_{GES-3} gene of *K. pneumoniae* strain KG525. The band with a pI of 7.6 corresponds to the chromosomally encoded LEN-1 (1) or SHV-type penicillinase of *K. pneumoniae*.

DISCUSSION

Considerable differences in the levels of resistance to various cephalosporins were observed among the *E. coli* clones pro-

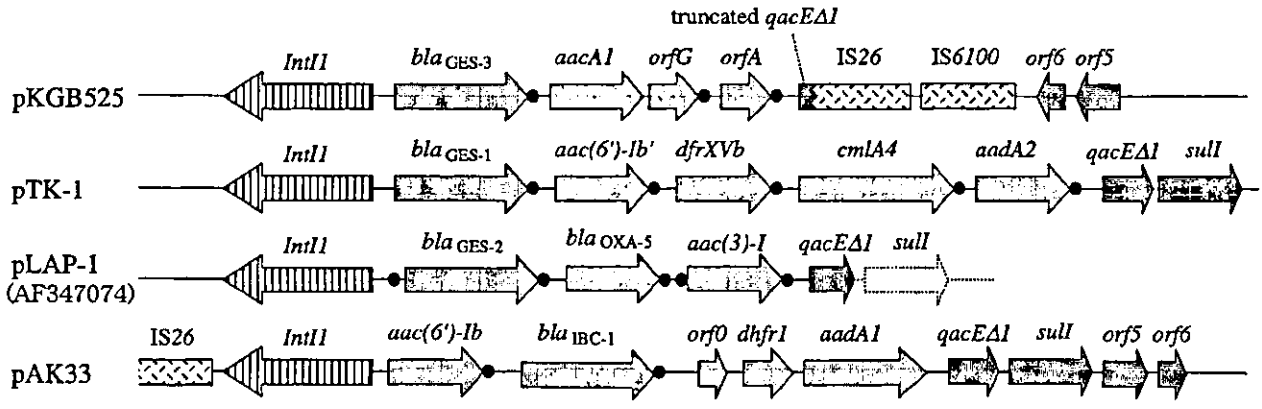

FIG. 2. Schematic comparison of the class 1 integron on pKGB525 with those on pTK1 (GenBank accession number AF156486), pLAP-1 (GenBank accession number AF326355), and pAK33 (34). Filled circles indicate the positions of GTTRRRY (core site) or the 59-base elements around the gene cassettes. pKGM525, which carries the 11.6-kb BamHI insert, was also sequenced; and the nucleotide sequence from *intI1* to IS*26* was the same as that found in pKGB525.

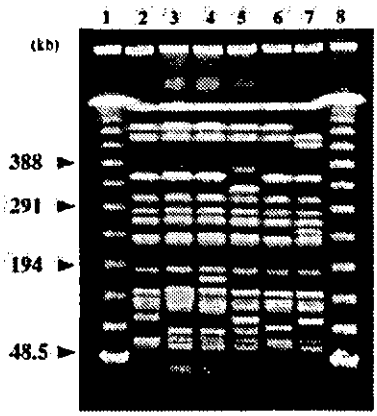

FIG. 3. PFGE analysis of *K. pneumoniae* isolates. Lanes: 1 and 8, PFGE marker; 2, *K. pneumoniae* KG914; 3, *K. pneumoniae* KG1116; 4, *K. pneumoniae* KG525; 5, *K. pneumoniae* KG545; 6, *K. pneumoniae* KG502; 7, *K. pneumoniae* KG827.

ducing GES-1, GES-2, and IBC-1, although the level of production of each enzyme may differ in individual clones. For instance, the MIC of ceftazidime for an *E. coli* clone producing GES-1 was 128 μg/ml (24), while that for an *E. coli* clone producing GES-2 was 8 μg/ml (28). The single amino acid substitution in the Ω loop observed between GES-1 and GES-2, G170N, may well contribute to the difference in the substrate specificities of these enzymes. On the other hand, the MIC of ceftazidime for an *E. coli* clone producing IBC-1 was >256 μg/ml (11). The only amino acid substitution observed between GES-1 and IBC-1, E104K, might well also be attributed to a higher level of resistance of IBC-1 than that of GES-1 to ceftazidime, cefotaxime, and aztreonam (11, 19, 24). The MIC of ceftazidime for an *E. coli* clone producing GES-3 was 128 μg/ml, and GES-3 has a single M62T substitution compared with the sequence of IBC-1, which also confers high-level resistance to ceftazidime in an *E. coli* clone (MIC, >256 μg/ml). We investigated whether this one amino acid substitution observed between GES-3 and IBC-1 affects the MICs for *E. coli* clones expressing each enzyme. However, this substitution did not result in significant changes in the MICs for the *E. coli* clones. This finding suggests that the amino acid substitution at position 62 may not play a crucial role in the extended substrate specificity of GES-3 against ceftazidime and that those at positions 104 and 170 would be crucial for extended-spectrum enzyme activity.

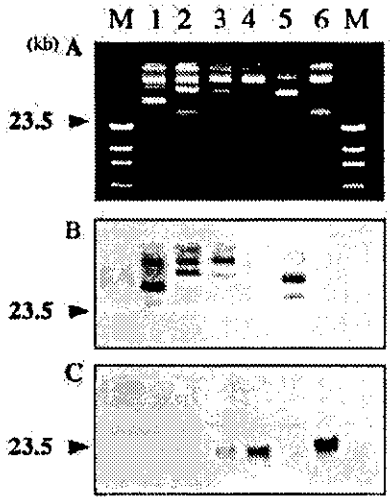

FIG. 4. Plasmid profiles and Southern hybridization analysis. (A) Plasmid profiles of each strain prepared by the method of Kado and Liu (16); (B) hybridization to large plasmids harbored by each strain; (C) hybridization to the chromosomal position of each strain. The photograph of the results of gel electrophoresis of chromosomal DNAs prepared by the method of Stauffer et al. (35) was omitted. The large plasmids and chromosomal DNA were separately extracted by using freshly prepared reagents to avoid cross contamination of nicked or physicochemically amputated DNA fragments. For strains KG545 and KG827, the bla_{GES} gene was suggested to be encoded by the chromosome. In strain KG525, the bla_{GES} gene was suggested to be encoded by both the plasmid and the chromosome. Lanes: M, HindIII-digested DNA marker; 1, *K. pneumoniae* KG914; 2, *K. pneumoniae* KG1116; 3, *K. pneumoniae* KG525; 4, *K. pneumoniae* KG545; 5, *K. pneumoniae* KG502; 6, *K. pneumoniae* KG827.

In the present study, we also isolated a novel GES-type class A enzyme, GES-3, from *K. pneumoniae* strains which caused neonatal nosocomial infections in 2002 in Japan. Sequence analysis of the genetic environments of the bla_{GES-3} genes on pKGB525 carrying a 6.7-kb insert and pKGM525 carrying a 11.6-kb insert revealed that the bla_{GES-3} genes were located as gene cassettes in class 1 integrons, as observed in other GES-type β-lactamase genes, including bla_{IBC-1} (Fig. 2). Integrons are very sophisticated site-specific recombination systems that capture various gene cassettes, including antibiotic resistance genes, between their 5′ and 3′ conserved segments (14, 15, 22). The gene cassettes for the GES-type enzymes with a very close phylogenetic relationship might have originated as a single clone and then disseminated worldwide with the help of class 1 integrons possessing very similar genetic organizations. These integrons are mediated by self-transmissible plasmids with a wide host range. Since very similar GES enzymes have so far been found in French Guiana, Greece, South Africa, Portugal, and Japan, these GES-type β-lactamase-producing strains might have been scattered globally by the recent extensive international travel or dissemination of humans, foods, and animals.

In the present study, we analyzed genetic relatedness using PFGE and ERIC-PCR of all six GES-type β-lactamase-producing *K. pneumoniae* strains isolated in a NICU over a 1-year period. Since the fingerprinting patterns obtained by PFGE and ERIC-PCR were very similar, these isolates were suggested to belong to the same genetic lineage that caused the nosocomial spread. The minor differences in the fingerprinting patterns obtained by PFGE might be due to the occurrence of genetic rearrangements over the course of the nosocomial spread. Interestingly, the results of Southern hybridization suggested that the locations of the bla_{GES} genes were multiple and diverse among the six strains studied. By consideration of the results of PFGE, ERIC-PCR, and Southern hybridization, it can be speculated that an endemic strain containing the bla_{GES} genes might have spread within the NICU setting over the 1-year period and might have undergone genetic rearrangements, including translocation and multiplication of the bla_{GES} gene.

The presence of multiple bla_{GES-3} genes in strain KG525 is

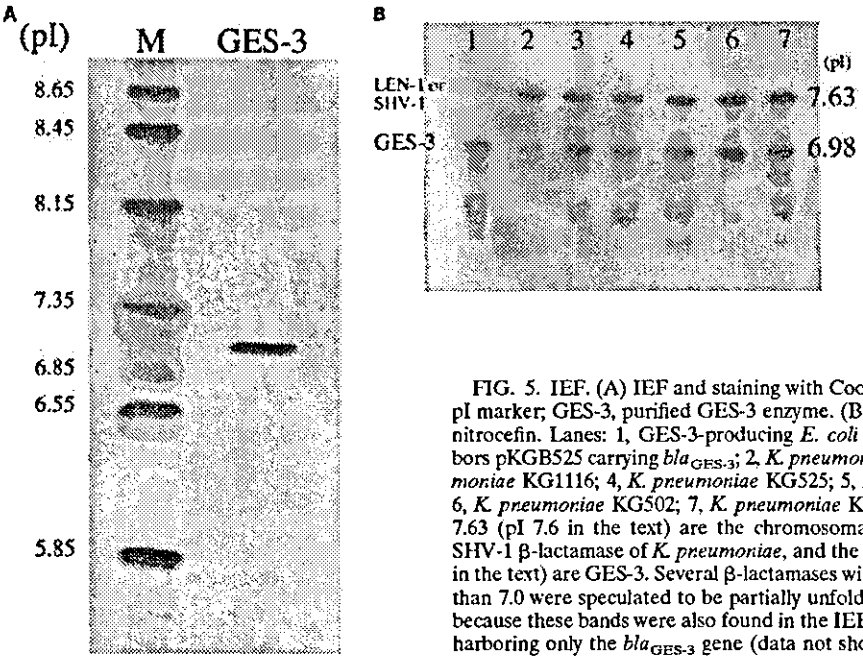

FIG. 5. IEF. (A) IEF and staining with Coomassie blue. Lanes: M, pI marker; GES-3, purified GES-3 enzyme. (B) IEF and staining with nitrocefin. Lanes: 1, GES-3-producing *E. coli* transformant that harbors pKGB525 carrying bla_{GES-3}; 2, *K. pneumoniae* KG914; 3, *K. pneumoniae* KG1116; 4, *K. pneumoniae* KG525; 5, *K. pneumoniae* KG545; 6, *K. pneumoniae* KG502; 7, *K. pneumoniae* KG827. The bands of pI 7.63 (pI 7.6 in the text) are the chromosomally encoded LEN-1 or SHV-1 β-lactamase of *K. pneumoniae*, and the bands at pI 6.98 (pI 7.0 in the text) are GES-3. Several β-lactamases with activities at pIs lower than 7.0 were speculated to be partially unfolded GES-3 β-lactamase, because these bands were also found in the IEF gels of an *E. coli* clone harboring only the bla_{GES-3} gene (data not shown).

probably the result of these genetic rearrangements. Translocation and multiplication of the bla_{GES} gene might be facilitated by mediation of a site-specific recombination system of an integron or a transposon. A similar example of multiple locations of the same antibiotic resistance gene has been reported by Yagi et al. (38). In their study, a single clinical *E. coli* isolate was found to carry three distinguishable Toho-1-like β-lactamase genes, which were later identified as $bla_{CTX-M-2}$, by their restriction digestion patterns on the chromosome. These multiple locations of the same β-lactamase gene would be beneficial to bacteria, since they increase the chance of amino acid substitutions necessary for extension of the substrate profiles of β-lactamases as well as the multicopy effect of gene expression. Bradford et al. (3) reported that point mutations leading to ESBLs (ESBLs TEM-1 to TEM-10 and TEM-12) occurred on the plasmids of a single *K. pneumoniae* clinical isolate. A notable finding presented in that report was the distinct hydrolyzing activity between TEM-10 and TEM-12. TEM-10 had hydrolyzing activity against ceftazidime, while TEM-12 also hydrolyzed cefotaxime and aztreonam, in addition to ceftazidime. A variety of susceptibility profiles for cephamycins were also observed among the six *K. pneumoniae* strains in the present study. For instance, strains KG914, KG1116, and KG525 were susceptible to cefmetazole (MICs, 16 μg/ml), whereas strains KG545, KG502, and KG827 were resistant to this agent (MICs, ≥128 μg/ml). In particular, strain KG502 showed high-level resistance to other cephamycins, such as cefoxitin (MIC, >128 μg/ml), cefminox (MIC, >128 μg/ml), and moxalactam (MIC, 128 μg/ml). An evolutionary event similar to that observed in the TEM enzymes (3) might have occurred in these *K. pneumoniae* strains to give them further resistance to a broad range of antibiotics. The MIC of imipenem for strain KG502 was 8 μg/ml, and this might be due to the hyperproduction of some β-lactamase with an extended substrate specificity as well as the loss of some outer membrane protein, as reported by Bradford et al. (4). Further molecular characterization of the cephamycin resistance observed in strain KG502 will be undertaken in the next study.

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Ⅴ．　参考資料、その他

Evaluation of Systemic Inflammatory Responses in Neonates with Herpes Simplex Virus Infection

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Neonatal herpes simplex virus (HSV) infection is a severe disease with high mortality and morbidity. To investigate the pathogenesis of neonatal HSV infection, we examined inflammatory responses and markers of apoptosis in patients with neonatal HSV infection. Concentrations of inflammatory cytokines and markers of apoptosis were significantly higher in patients with disseminated HSV infection and were correlated with HSV load. It appears that the immunopathological damage that results from host responses to viral infection leads to organ dysfunction in patients with neonatal HSV infection.

Neonatal herpes simplex virus (HSV) infection is a severe disease with high mortality and morbidity [1]. Neonates develop 3 types of infection, which are classified according to the clinical extent of disease: localized skin, eyes, or mouth (SEM) infection; central nervous system (CNS) infection; and disseminated infection, which involves several organs, such as the lungs, liver, and adrenal glands, with or without CNS involvement. The prognosis for neonatal HSV infection has improved with the advent of effective antiviral drugs, such as vidarabine and acyclovir [2]. However, the mortality for patients with disseminated infection remains unacceptably high. Patients with disseminated infection have sepsislike symptoms, with fever, hepatic failure, and disseminated intravascular coagulopathy (DIC), which often lead to death [1].

It is known that viral and bacterial infections cause systemic inflammatory response syndrome (SIRS). A frequent complication of SIRS is the development of multiple organ dysfunction syndrome (MODS) [3]. Investigations into the pathophysiology of SIRS have indicated that proinflammatory cytokines, such as interleukin (IL)–6 and tumor necrosis factor (TNF)–α, play a central role [4]. Many studies have shown that the serum concentrations of proinflammatory and anti-inflammatory cytokines are elevated in patients with SIRS. Concentrations of these cytokines are reported to be elevated in neonatal patients with severe bacterial infection [5] and have been correlated with poor clinical outcome. The occurrence of organ damage is thought to be related to immunopathological damage as the result of overexuberant host responses. Furthermore, some proinflammatory cytokines have the potential to enhance apoptosis in organ tissues and endothelial cells. Recently, it has been reported that apoptosis plays a role in the development of MODS in patients with SIRS [6].

Although the criteria for SIRS in neonates have not been established, we hypothesized that neonatal disseminated HSV infection corresponds to SIRS, because the clinical manifestations of these patients were similar and because they often developed MODS. To evaluate the inflammatory responses, we measured the serum concentrations of IL-6 and soluble TNF receptor 1 (sTNF-R1). We also measured the concentrations of mitochondrial aspartate aminotransferase (mAST) and cytochrome *c*, which are considered to be markers of apoptosis [7].

Methods. Informed consent was obtained from the parents of all children in this study. Nineteen patients with neonatal HSV infection were enrolled in this study, all of whom had been referred to the Nagoya University Graduate School of Medicine for the diagnosis of HSV infection. Their ages ranged from 33 to 41 weeks (mean, 38.6 weeks), and their birth weights ranged from 1208 to 4034 g (mean, 2929 g). The day of onset of infection ranged from 3 to 23 days after birth (mean, 7.2 days). Diagnosis was based on the isolation of HSV or the detection of HSV DNA by use of polymerase chain reaction (PCR) [8]. The HSV type was determined with the type-specific monoclonal antibody or by use of restriction fragment–length polymorphism analysis of amplified products [8]. Nine cases were caused by HSV-1, and 10 were caused by HSV-2. The clinical types of neonatal HSV infection were classified according to the National Institutes of Allergy and Infectious Diseases Collaborative Antiviral Study Group [1].

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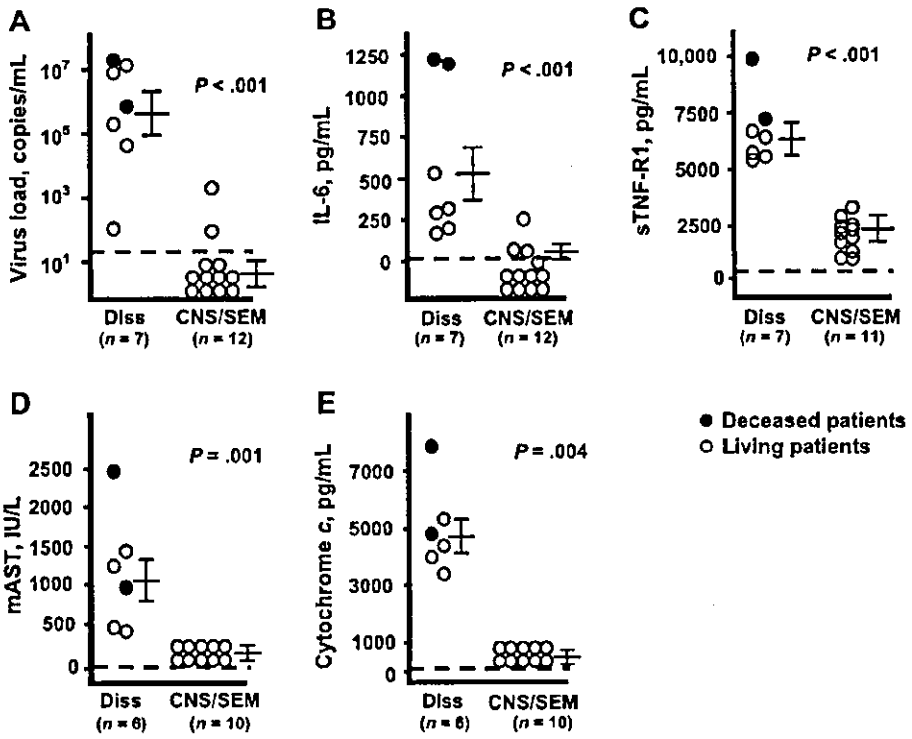

Figure 1. Comparison of herpes simplex virus (HSV) load *(A)* and concentrations of interleukin (IL)–6 *(B)*, soluble tumor necrosis factor receptor 1 (sTNF-R1; *C*), mitochondrial aspartate aminotransferase (mAST; *D*), and cytochrome *c* *(E)* in patients with disseminated (Diss) HSV infection and central nervous system (CNS)/skin, eyes, and mouth (SEM) HSV infection. All of the samples were obtained at diagnosis or within 3 days after the initiation of therapy with acyclovir. *Bars*, Means and SEs for each group. *Dotted lines*, Detection limits for each sample. The statistical method used was the Mann-Whitney *U* test.

Serum HSV load was quantified by use of real-time PCR assay using the TaqMan PCR kit (Applied Biosystems), as described elsewhere [9]. Serum concentrations of IL-6 and sTNF-R1 were determined with 100 μL of serum, using sandwich-type ELISA kits (R&D Systems), as described elsewhere [10]. This assay takes ~5 h. The normal values for IL-6 and sTNF-R1 are <12.5 pg/mL and 500–1500 pg/mL, respectively.

Serum concentrations of mAST were assayed by use of the inhibition method with proteinase K, at the SRL Laboratory (Tokyo). Serum concentrations of cytochrome *c* were determined by use of ELISA, at the Eisai Laboratory (Ibaraki, Japan) [11]. The normal values for mAST and cytochrome *c* are <7 IU/L and <1000 pg/mL, respectively.

Statistical analysis was performed by use of StatView software (version 5.0; SAS Institute). Statistical comparisons of virus loads, serum concentrations of cytokines, and serum concentrations of markers of apoptosis were evaluated by use of the Mann-Whitney *U* test. Regression analyses were used to compare virus loads, serum concentrations of cytokines, and serum concentrations of markers of apoptosis. $P < .05$ was considered to be statistically significant.

Results. Of 19 patients with neonatal HSV infection, 7 were classified as having disseminated infection, 10 as having CNS infection, and 2 as having SEM infection. The mean ± SD time of onset was significantly earlier in the patients with disseminated infection, compared with those with CNS or SEM infections (3.9 ± 0.8 vs. 9.1 ± 5.4 days), as reported elsewhere [1]. Other characteristics (birth weight, gestational age, sex, and HSV type) did not differ among the clinical types.

Most of the patients with disseminated infection had high fever, tachycardia, and tachypnea. At admission, 4 of the 7 patients already had DIC. All but 1 patient received therapy with acyclovir. Two patients died of MODS. The remaining patients recovered without apparent sequelae after 1 year of observation.

The serum HSV loads of the patients are shown in figure 1*A*. The mean ± SE HSV load was significantly higher in patients with disseminated infection ($10^{5.5} \pm 10^{0.7}$ copies/mL) than in patients with CNS and SEM infections ($10^{0.6} \pm 10^{0.4}$ copies/mL).

Serum concentrations of IL-6 and sTNF-R1 were also examined (figure 1*B* and 1*C*). The mean ± SE concentration of IL-6 was significantly higher in patients with disseminated infection (550 ± 180 pg/mL) than in patients with CNS and SEM infections (27 ± 22 pg/mL). The mean ± SE concentration of sTNF-R1 was also higher in patients with disseminated infec-

tion (6600 ± 610 vs. 2400 ± 240 pg/mL). Two patients who had extremely high concentrations of IL-6 (>1000 pg/mL) and sTNF-R1 (>7000 pg/mL) subsequently died.

Serum concentrations of mAST and cytochrome *c* are shown in figure 1*D* and 1*E*. Concentrations (mean ± SE) of both of these markers of apoptosis were significantly higher in patients with disseminated infection than in patients with CNS and SEM infections (1100 ± 320 vs. 4.9 ± 0.7 IU/L for mAST; 5000 ± 650 vs. 130 ± 29 pg/mL for cytochrome *c*).

We then analyzed the correlations between the different parameters. There were positive correlations between HSV load and cytokine concentrations (HSV load vs. sTNF-R1, $r = 0.85$ and $P < .0001$; HSV load vs. IL-6, $r = 0.68$ and $P = .003$). There were also positive correlations between the concentrations of cytokines and markers of apoptosis. Cytochrome *c* correlated with sTNF-R1 ($r = 0.86$ and $P < .0001$) and IL-6 ($r = 0.78$ and $P < .001$). Furthermore, mAST correlated with IL-6 ($r = 0.77$ and $P < .001$) and sTNF-R1 ($r = 0.78$ and $P < .001$). Representative results are shown in figure 2*A* (HSV load vs. sTNF-R1) and figure 2*B* (sTNF-R1 vs. cytochrome *c*).

The concentrations of cytokines and markers of apoptosis were estimated sequentially and plotted against the day after onset of disseminated HSV infection. The concentrations of sTNF-R1 and cytochrome *c* decreased with time (figure 2*C* and 2*D*). The concentration of sTNF-R1 decreased gradually but remained detectable until 15–20 days after onset. On the other hand, the concentration of cytochrome *c* decreased more rapidly and was negligible 10 days after onset. As with cytochrome *c*, the concentrations of IL-6 and mAST decreased rapidly with time (data not shown).

Discussion. IL-6 is an important mediator of the early systemic host response to infection; it reaches peak concentrations rapidly after onset, with a half-life of several hours [12]. The concentration of IL-6 has been shown to be an effective prognostic indicator of clinical severity in SIRS and correlates with mortality states. In addition to IL-6, we examined sTNF-R1, which is one of the extramembraneous fragments of the TNF receptor on cells. TNF-α is one of the major proinflammatory cytokines in the cytokine cascade that is manifested in SIRS. However, the detection of TNF-α in patients with SIRS has not been consistent, and correlations with severity and outcome of disease have been poor because the peak concen-

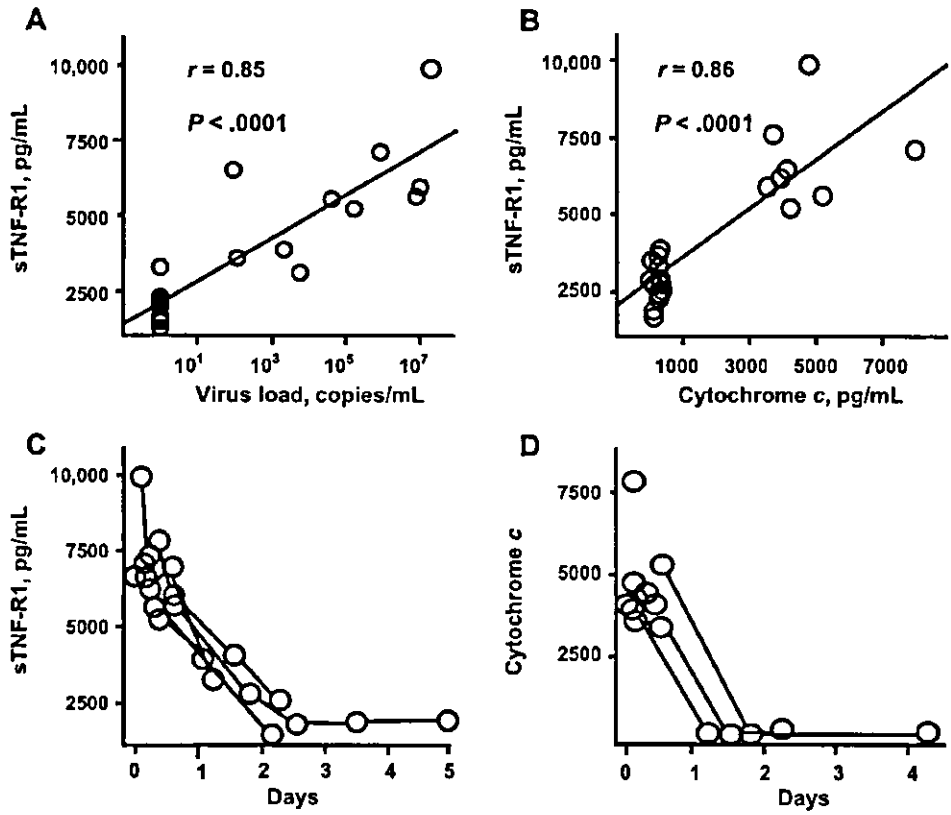

Figure 2. Correlation of soluble tumor necrosis factor receptor 1 (sTNF-R1) concentrations with herpes simplex virus (HSV) load *(A)* and concentrations of cytochrome *c (B)*. Regression analysis was used for the comparison. Dynamics of sTNF-R1 *(C)* and cytochrome *c (D)* of patients with disseminated HSV infection. Lines connect individual data points for each patient.

trations of TNF-α occur rather rapidly after the onset of disease. TNF-α binds to sTNF-Rs that are shed into the circulation [12]. Thus, instead of measuring the concentration of TNF-α, we measured the concentration of sTNF-R1, which is the natural homeostatic regulator of the action of TNF-α. In the present study, most of the patients with disseminated HSV infection had high concentrations of IL-6 and sTNF-R1. The 2 patients who subsequently died had higher concentrations of these cytokines. Furthermore, the concentrations of IL-6 and sTNF-R1 correlated with HSV load. These data suggest that the immunopathological damage caused by the host responses leads to organ dysfunction or death in patients with neonatal disseminated HSV infection and that these immune responses are dependent on the magnitude of HSV infection.

It is known that some of the proinflammatory cytokines have the potential to enhance apoptosis in organ tissues and endothelial cells and that apoptosis plays a role in the development of MODS in patients with SIRS [6]. In particular, TNF-α is the major cytokine implicated in the development of apoptosis, either by signaling through TNF-R1 or via other mechanisms. TNF-α has been shown to mediate apoptosis in endothelial cells, hepatocytes, and other cell types that are dysfunctional in SIRS [6]. To evaluate apoptosis in the patients, we measured the concentrations of cytochrome *c* and mAST. Both cytochrome *c* and mAST are mitochondrial proteins. It has been shown that TNF-α causes rapid release of cytochrome *c* from mitochondria, and convergent evidence suggests that the release of cytochrome *c* from mitochondria is a critical step in the apoptotic process. Early during the apoptotic process, mitochondrial depolarization occurs, and cytochrome *c* and mAST are released from the mitochondria into cytoplasm [7]. In the present study, the concentrations of cytochrome *c* and mAST were significantly higher in patients with disseminated infection than in patients with CNS and SEM infections and correlated with HSV load and concentrations of cytokines. This result suggests that, as the result of host immune responses, apoptosis is associated with the severity of neonatal HSV infection. In fact, apoptosis of liver cells subsequent to HSV infection is shown in mice [13]. In addition, TNF-α is an important factor in the development of endothelial injury and DIC in patients with SIRS. It has been reported that 34% of patients with disseminated infection show DIC [1]. In the present study, 4 of the 7 patients with disseminated infection had DIC at admission. The frequent observation of DIC in patients with disseminated infection might be associated with systemic cytokine responses.

It is not clear why neonates with disseminated HSV infection show high inflammatory responses. Previous studies have shown that deficiencies in the neonatal immune response permit disseminated HSV infection. NK cells and antibody-dependent cellular cytotoxicity are critical components of the early immune response to viral infection [14]. It has been shown that concentrations of transplacentally acquired neutralizing antibodies and antibody-dependent cellular cytotoxicity are lower in patients with disseminated HSV infection. On the other hand, Schultz et al. reported that the cytokine-producing capacity of macrophages was higher in neonates than in adults [15]. It might be speculated that, during disseminated infection, macrophage or other immune cells are activated in an abnormal fashion to eradicate HSV. These uncontrolled immune responses may result in damage to the organs of the patients.

Our findings are important for the development of a strategy for treating neonatal HSV infection. Although an intact inflammatory response is essential for the maintenance of normal host defense mechanisms, the modulation of inflammatory responses or apoptosis could be an appropriate target for therapeutic intervention. Although further studies are necessary, the combination of antiviral and anticytokine or antiapoptotic therapies may improve the prognosis of neonatal disseminated HSV infection.

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