2. Midazolamの薬理学的特性[3)21)~24)]

1）注射液の性状

Midazolamは酸性で水溶性となるため、midazolam注射液（ドルミカム®）はpH 2.8~3.8の塩酸水溶液（Midazolam 10 mgを含有、2 ml）としている。したがって、5%糖液や生食水で希釈でき、持続静注投与が可能である。さらにpropylene glycolを含有していないため、静脈への刺激が少ない。しかし、pHが高くなると沈殿や白濁を生じるため、thiopental注射液などのアルカリ性注射液やlidocaine注射液との配合を避ける。また、とくに新生児には有害とされるsodium benzoateやbenzyl alcoholも含まれていない。

2）作用機序[21,23)]

Midazolamはbenzodiazepine受容体に働き、benzodiazepine受容体とGABA受容体との相互作用によりGABA受容体でのGABA親和性が増し、間接的にGABAの作用を増強するため、けいれん抑制作用を発揮するとされているが、midazolamはdiazepamの約2倍のbenzodiazepine受容体への親和性を有するため、diazepamより抗けいれん作用が強力である[25)]。

3）脳血管関門通過性[21,22)]

Midazolam注射液は水溶性であるが、体内の生理的pHでは脂溶性となり、容易に脳血管関門を通過するため、抗けいれん作用は速効性である。

4）Bioavailability[21,22)]

Midazolamは筋注でも90%以上吸収され、効果発現も10分以内と速いため、静脈ラインが確保しにくい時には一時的に筋注で代用できる。さらに、midazolamは注腸、点鼻、頬粘膜投与でも吸収が良く、速効性のため、家庭での一次治療などに応用できる可能性がある。しかし、経口投与では肝臓と腸での初回通過効果を受け、小児での吸収率は27%と不良である。

5）体内分布[21,22)]

成人におけるmidazolamの分布容積（Vd）の報告は1.0~3.1 l/kg、0.95~6.6 l/kgと大きく、女性、老人、肥満などで高値となる傾向がある。小児のVd値の報告はみられない。

6）蛋白結合率[21,22)]

Midazolamの蛋白結合率は約97%で、ほとんどがアルブミンと結合し、遊離型は2.5~4%である。

7）代謝[21,22)]

Midazolamはhepatic microsome oxidationを受ける。6カ月未満の乳児では肝臓機能が未熟なため代謝が遅く、半減期が延長する。MidazolamはCYP3A4の基質であるため、他剤との相互作用を認めることがある。

Midazolamの代謝産物は1-hydroxymethyl midozolamが60~70%であり、他には4-hydroxy-midazolamが5%未満で、dihydroxy-midazolamがごくわずかである。主要代謝産物の1-hydroxymethyl midozolamは半減期が約1時間と短く、生物学的活性がほとんどなく、肝臓でグルクロン酸抱合を受け水溶性となって容易に排泄されるため、長期持続投与時にも蓄積による影響を受けない。

8）排泄[21,22)]

Midazolamは主に腎臓から排泄される。主たる尿中排泄型は1-hydroxymethyl midazolamのグルクロン酸抱合型で66.1~87.8%、他には4-hydroxy-midazolamとdihydroxy-midazolamのグルクロン酸抱合型がごく少量排泄され、intact midazolamの尿中排泄は0.03%未満である。

9）半減期[21,22)]

Midazolam静注時の排泄相半減期（t1/2 β）の報告値は、成人で1.7~2.6時間、1.8~6.4時間（平均3時間）、小児では1歳以上が0.8~1.8時間、6カ月~15歳が1.4~4.0時間であり、小児では成人と同等ないしはやや短い。このようにmidazolamは半減期が短いため、1回量静注後のけいれん抑制効果の持続が短く、長時間効果を維持させるためには持続静注投与が必要となる。新生児では肝臓機能が未熟なため半減期が長く、在胎26~42週の新生児の報告値は6.5~12.0時間である。また、重症全身疾患時には肝機能や腎機能の低下により半減期が延長する。一方、半減期が短いためmidazolam投与中止後の覚醒は約4時間と速い。

10）Clearance[21,22)]

Midazolamのplasma clearanceの報告値は、成

人では4.2〜9.0 ml/min/kg、6.4〜11.0 ml/min/kg、小児では1歳以上が4.7〜19.7 ml/min/kg、6カ月〜15歳が3.2〜15.4 ml/min/kgである。この小児の値はdiazepamの15倍である。肝疾患、腎不全、心不全などではclearanceが低下し、蓄積するため、投与量を減らすなどの調整が必要となる。

小児では100時間をこえる長時間持続静注時にはclearanceが増加し[26]、効果が減弱することがあり、途中で増量を必要とすることがある。

11）血中濃度[21,22]

Midazolamの臨床効果と血中濃度の間には直接的な関係は証明されていないが、血中濃度と鎮静効果の間には相関関係が報告されている。報告[27]では100 ng/ml以上では50%以上の確率で鎮静、200 ng/ml以上では50%以上の確率で入眠、最大鎮静効果は229 ng/mlであった。

けいれん抑制効果とmidazolam血中濃度との関係は十分検討されておらず、けいれん抑制に対する有効血中濃度も確立されていないが、200〜500 ng/mlとの報告がみられる[28]。

12）相互作用[21,22]

MidazolamはCYP3A4の基質であるため、CYP3A4の活性を抑制する薬剤との併用でmidazolamの代謝とclearanceが低下し、midazolamの血中濃度が上昇する。このような薬剤として、erythromycin、clarithromycin、ketoconazole、diltiazem、verapamil、cimetidineが知られている。また、phenytoinはmidazolamとの併用で血中濃度が上昇しにくくなるとの報告もみられる[28]。

13）Withdrawal symptom[21,22]

長期持続静注投与後の急激な中止により、けいれん、興奮、幻覚、異常行動、発熱、嘔吐、頻脈などの症状が生じるので、緩徐に減量、中止を心がけることが必要である。

14）副作用[21,22]

小児におけるmidazolam静注時の副作用として、酸素飽和度低下4.6%、無呼吸2.8%、血圧低下2.7%、逆説反応2.0%、吃逆1.2%、けいれん様動作1.1%、眼振1.1%が報告されているが[21]、このうち呼吸、循環系の副作用は併用中枢神経抑制薬剤の影響によると思われる。

また、midazolam治療に関する多数の報告からも、midazolamは呼吸、循環に対する影響が少なく、midazolam自体による呼吸抑制はほとんどみられないので、midazolam治療に先立って人工呼吸管理を開始する必要はないと考える[9〜17,29,30]。

Midazolam過量投与による過剰鎮静時などにはbenzodiazepine拮抗剤であるflumazenilが有効である。flumazenilは最初0.01 mg/kgを静注し、その後効果がえられるまで0.01 mg/kgずつ漸増するが(平均0.017 mg/kg、最大0.05 mg/kg)、効果は10分以内に出現する[31]。しかし、けいれんを誘発させることがあり、日本ではbenzodiazepine系薬剤を長期投与されているてんかん患者では禁忌とされている。

15）けいれん重積状態治療のmidazolam投与量

けいれん重積状態の治療に用いるmidazolam 1回静注投与量と持続静注投与量に関しては、0.15 mg/kgと0.138 mg/kg/hr[9]、0.02〜0.38 mg/kgと0.06〜0.38 mg/kg/hr[32]、0.15 mg/kgと0.12 mg/kg/hr[13]などの報告がある。著者らは通常1回静注投与量を0.15 mg/kg前後、持続静注投与量は平均0.2 mg/kg/hrで最大0.5 mg/kg/hrとしている[29,30]。なお、1 mg/kg/hr以上の大量投与を行うmidazolam昏睡治療の報告[33]があるが、その有効性についての十分な追試はなされていない。

3. Midazolamと他主要治療薬剤との薬理学的特性に関する比較[34]

けいれん重積状態の治療にはけいれん抑制作用の速効性、強力性、安全性および持続性をすべて兼ね備えた治療が必要である。

けいれん治療に対する保険適応の有無を度外視し、現在日本でけいれん重積状態に対して静注で使用されている主要治療薬剤のdiazepam、phenytoin、lidocaine、barbiturates（pentobarbital、thiopental、thiamylal）について、けいれん抑制作用の速効性、強力性、安全性および持続性の4特性に関してmidazolamとの比較を行った。

1）速効性

Midazolamとdiazepamは脂溶性のため、脳血管関門を速やかに通過し、きわめて速効性である[21,22]。ちなみに、lorazepamはこれらより幾分脂

溶性が悪いため多少速効性に劣る[22]。また、lidocaine も容易に脳血管関門を通過するため速効性である[35]。Barbiturates も速効性であるが[6,36]、phenytoin は静注後効果発現まで 15〜30 分かかり[6]、速効性に劣る。したがって、midazolam、diazepam、lidocaine、barbiturates は速効性に優れ、けいれん重積状態の第一次治療に適している。しかし、phenytoin は群発型けいれん重積状態をはじめ頻発性や群発性けいれんの治療で 15〜30 分の猶予が許されるような場合および diazepam によるけいれん抑制後 12 時間程度の再発予防目的としての使用は有用であるが、とくに連続型けいれん重積状態の治療には不向きである。

2）強力性

Midazolam、diazepam、phenytoin、lidocaine、barbiturates とも強力な抗けいれん作用を有するが、とくに脳波を complete suppression ないしは burst suppression に至らしめる barbiturates の大量療法はきわめて強力で、脳保護作用も併有する。したがって、Reye 症候群やインフルエンザ脳症など脳浮腫を伴う重篤な急性脳症によるけいれん重積状態では脳圧降下など脳保護作用も併せ持つ barbiturates 昏睡療法が有効と思われる[8]。また、特異な脳炎・脳症後てんかんの一群と theophylline 関連けいれん[37]には他治療が無効で、barbiturates 持続静注治療が導入されることが多い[29,30,37]。Midazolam は強力なけいれん抑制作用を有し、benzodiazepine 受容体への親和性は diazepam の約 2 倍で、薬理学的には diazepam より 3〜4 倍強力といわれている[25]。Midazolam は最初 1 回量を静注後引き続き持続静注を開始し、けいれんが確実に抑制されるまで漸増するが、midazolam は投与量の安全域が広いため増量過程でけいれん重積状態から脱する確率が高い。Barbiturates との効果比較では同等との報告もみられるが[10,38]、臨床的にこれらの有効率を厳密に比較検討した報告は見あたらない。したがって、けいれん重積状態の原因が明らかになるまではまず midazolam のような安全性の高い薬剤による第一次治療を行い、原因が前述の重篤な急性脳症や特異な脳炎・脳症後てんかんの一群あるいは theophylline 関連けいれんと判明した時点で、早めに集中管理のもとに barbiturates 持続静注治療が導入されるべきであると考える。ちなみに、midazolam 大量持続投与による midazolam 昏睡療法の報告[33]があるが、特異な脳炎・脳症後てんかんの一群の 1 例に対して 1 mg/kg/hr の midazolam 大量持続投与を行った筆者らの経験では脳波を burst suppression や complete suppression に至らすことができず、その有効性を確認できなかった。臨床的に diazepam と midazolam の持続静注治療効果を比較した報告ではけいれん抑制効果に有意な差は認めないとするもの[39]もあるが、メタ分析では diazepam は midazolam および pentobarbital、thiopental の barbiturates 静注治療より効果が劣ると報告されており[10]、第一次治療薬としては従来の diazepam よりむしろ midazolam の方がふさわしいと考える。なお、胃腸炎に伴うけいれんでは diazepam が無効で、lidocaine が有効なことが多く[35]、原因疾患や病態によって治療薬の有効性に差があると思われる。

3）安全性

Diazepam は軽度の呼吸抑制や血圧低下をきたすことがあり、とくに急速静注時や barbiturates 先行投与時には注意を要する[2]。また、diazepam 注射液は希釈性が悪く、希釈すると結晶が析出するため、通常原液のまま静注するが、注射液には propylene glycol が添加されており血管刺激性が強く[22]、浸透圧による腎障害の危険も知られている[40,41]。また、diazepam 注射液には sodium benzoate と benzyl alcohol も添加されており、新生児では benzoate がビリルビンと蛋白結合において競合し遊離ビリルビンが増加して核黄疸の危険が生じる[42]。また、新生児に大量の benzyl alcohol が摂取されると gasping syndrome や floppy baby syndrome などがおこることも知られており[13,44]、とくに新生児に対する安全性に問題がある。また、diazepam の反復投与あるいは持続投与時には、生物学的活性を有する主要代謝産物の N-desmethyldiazepam の蓄積による影響も出現する[22]。現在日本で第一次治療に選択されている diazepam には安全性においてこれらの問題点がある。これに対して、midazolam は呼吸、循環に対する影響は非常に少なく、安全性が高い。した

がって、急性脳症などの基礎疾患や重症心身障害児に伴う呼吸障害など患者自身による影響を別にすれば、midazolamの1回量静注および持続静注治療とも人工呼吸管理や昇圧剤は不要であり[17,29,30]、一般病棟での管理が十分可能である。また、midazolamは酸性で水溶性となるため塩酸水溶液である注射液は水溶性で希釈性が良好である[22]。さらに、日本のmidazolam注射液にはpropylene glycol、sodium benzoate、benzyl alcoholとも含まれていないため、血管刺激性がなく、新生児にも安全性が高い。また、midazolamの主要代謝産物である1-hydroxymethyl midazolamには生物学的活性がほとんどなく[21,22]、その半減期も短いため、長期持続投与時でも蓄積による影響を受けない。さらに、半減期が短いため投与中止後の回復が速いなど、安全性にはきわめて優れており、midazolam治療は第一次治療としても非常に適した治療法であると考える。一方、midazolamの長期間持続投与に伴う問題としては、依存性[22]、耐性[22]、clearanceの増加[22]、排泄相半減期の延長[22]などが生じることがあるが、予めこれらの点に対応できるよう配慮することが必要である。また、midazolam使用時には種々の薬剤との相互作用[21,22]にも留意が必要である。Phenytoinは循環系への影響があるため心電図モニターが必要であるが、呼吸抑制は少ない。希釈性が悪くかつ血管刺激性が非常に強いため、生食水で希釈し緩徐に静注後生食水でフラッシュするが、血管痛が非常に強く、誤って動脈に注入した場合や血管から漏れると皮膚や血管の壊死をおこしやすいので十分な注意が必要である[6]。Lidocaineは一般的に呼吸抑制は少ないが、血圧低下や心伝導障害など循環への影響があり、心電図モニターが必要である。また、主として大量投与時ではあるが、けいれんを誘発することが知られている[35]。さらに、Lidocaineの主要代謝産物であるmethylethylglycinexylidideおよびglycinexylidideは生物学的活性を有するため、持続投与時にはこれらが蓄積し、副作用の増強につながる可能性がある[35]。一方、lidocaine注射液の希釈性は良好で、血管刺激性も少ない。また、覚醒度が保たれ、意識状態の評価に支障を来さないことが多い。Barbiturates[6,36]は呼吸、循環に対する影響が強く、1回量静注時でも呼吸抑制や血圧低下などの緊急事態に対応できる体制で行う必要があり、大量持続静注時にはあらかじめ人工呼吸管理下におき、昇圧剤を投与し、厳密に呼吸、循環、および脳波のモニターを行うため、ICU管理が必要となる。また、長期持続静注投与中は腸管麻痺や易感染性なども高率に発生するなど安全性には問題点が多い。さらに、長期間持続投与後は減量時に再発しやすく、中止から覚醒まで長時間を要するなどの不利な点も多い。また、希釈性が悪く、結晶が析出しやすいため、乳糖を含まない溶液で希釈して投与するが、血管刺激性が強く、血管炎をおこしやすいので、中心静脈投与が望ましい。このように、barbiturates治療は安全性には大きな問題があるため、第一次治療には不向きである。

したがって、安全性からはmidazolamが最も優れており、次にlidocaine、diazepamで、phenytoinとbarbituratesは問題が多いと考える。

4）持続性

Midazolamは半減期が短いため、1回量静注ではけいれん抑制効果の持続は3〜4時間と短い[21,22]。しかし、希釈性が良好で持続静注投与が可能なため、1回量静注後引き続きmidazolamの持続静注治療を行うことにより長期間安定したけいれん抑制効果を維持できる。Midozolamと同様、lidocaineとbarbituratesも1回量静注投与では効果の持続性は短いが、持続静注投与により長時間効果を維持できる。とくに、群発型のけいれん重積状態や脳炎・脳症などでは長時間安定したけいれん抑制効果を維持することが必要であり、持続静注治療が最適である。Diazepamは希釈性が悪いため、通常は原液の1回量投与を行うが、けいれん抑制効果の持続は約20分と短い。このため、再発に対して2〜3回反復投与することがあるが、漸次効果が減弱する[3]。したがって、群発型など再発が起こりやすい場合や脳炎・脳症には不向きである。なお、diazepam注射液を大量の溶液で希釈して持続静注投与した報告がみられる[39]が、前述の安全性の面からもdiazepamの持続静注治療は日本では普及していない。Phenytoinは1回量静注後のけいれん抑制効果の持続は8〜12時

間[6]と比較的長いため、短時間以内の再発は予防できる。しかし、希釈性が悪く持続静注投与ができないため、さらに長時間効果を持続させるためには血中濃度を測定しながら追加投与量を決め1回量静注を反復するが、数日間に及ぶ安定した効果を維持することは難しい。したがって、長期間の安定したけいれん抑制効果を維持する必要があるような病態には不向きである。

5）4特性のまとめ

前述したけいれん抑制作用の速効性、強力性、安全性、持続性の4項目に関する各治療薬の評価をまとめて以下に示す。

(1)速効性：Diazepam、midazolam、lidocaine、barbituratesとも速効性に優れるが、phenytoinは劣る。

(2) 強力性：Barbiturates、midazolam、diazepam、lidocaine、phenytoinとも強力であるが、原因疾患によって有効薬剤に差を認める。

(3) 安全性：Midazolamが最も安全性が高い。Diazepam、phenytoin、lidocaineはいくつかの問題点を有し、barbiturates治療は集中管理を要する。

(4) 持続性：Phenytoinは比較的効果持続時間が長いが、diazepamは持続が短い。Midazolam、lidocaine、barbituratesはいずれも半減期が短いため、1回量静注では効果持続は短いが、持続静注投与を行うことにより長期間安定した効果を維持持続できる。

すなわち、midazolamは持続静注治療法を行うことにより、これら4特性がすべて満たされ、midazolam持続静注治療はけいれん重積状態に対する治療法として非常に有望な選択肢となる可能性が示唆された。

4. 小児のけいれん重積状態治療方式の私案

けいれん重積の第一次治療として、諸外国ではlorazepam静注、日本ではdiazepam静注が行われている。これらの薬剤は軽度の呼吸抑制や血圧低下には注意が必要であるが、速効性と強力性に非常に優れている。Lorazepamは比較的効果が長く持続するが、diazepamは効果の持続が短く、これらの治療に抵抗性の場合には第二次、第三次治療として安定した効果の維持持続が期待できる持続静注治療が選択されることが多い。しかし、従来から行われていたbarbituratesの持続静注治療はきわめて強力ではあるが、人工呼吸管理や昇圧剤投与など集中管理を要するため多くの場合最終手段として選択される。これに対して、midazolamは持続静注治療においても呼吸・循環に対する影響が非常に少ないため人工呼吸管理や昇圧剤の投与が不要であり、一般病棟での管理が十分可能である。さらに、けいれんに対する保険適応はないが、けいれん抑制作用に関しては速効性と強力性にも非常に優れているため、第二次治療、第三次治療としてだけではなく、むしろ第一次治療としてmidazolam持続静注治療を選択する意義は十分あると考える。これによって、多くの場合は第二次治療、第三次治療が不要になることが期待される。実際、著者ら[17]とYoshikawaら[18]はmidazolamを第一次治療に選択した場合でも有効率が高かったことを報告したが、さらにmiidazolam持続静注治療の導入後、lidocaineとbarbituratesの使用頻度が激減したことを経験している。なお、原因疾患や病態によってはlidocaineやphenytoinが第一選択薬となる場合もありうる。第一次治療のmidazolamが無効な場合あるいはlidocaineやphenytoinが無効な場合には、全身管理のもとにbarbiturates持続静注治療を行う。とくに、原因疾患がReye症候群やインフルエンザ脳症などの重篤な急性脳症や特異な脳炎・脳症後てんかんの一群およびtheophylline関連けいれんと診断された場合には早めにbarbiturates持続静注治療を導入することが望ましい。

第一次治療：

(1) Diazepam 1回量静注（Barbiturate先行投与時、急速静注時は呼吸抑制に注意）あるいはMidazolam 1回量静注→Midazolam持続静注

(2) 原因疾患によっては、lidocaine 1回量静注→持続静注（とくに胃腸炎に伴うけいれんに有効）またはphenytoin 1回量静注→（反復静注）

第二次治療：

Barbiturates 1回量静注→持続静注（重篤な

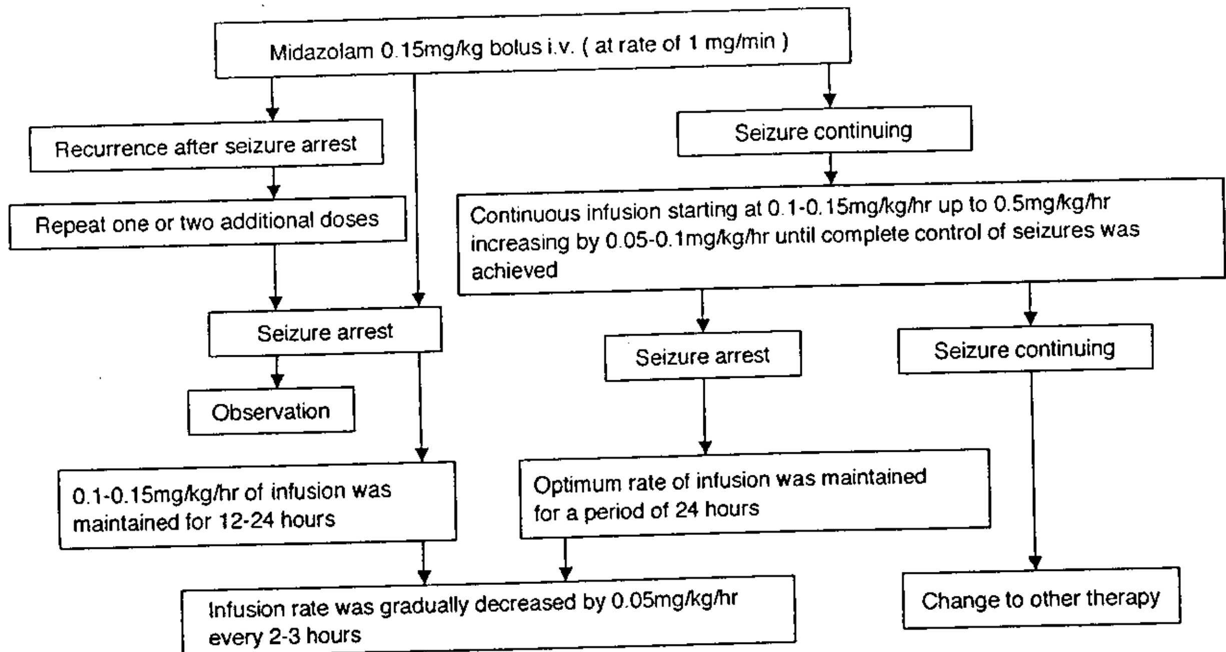

Fig. 1 Protocol of midazolam treatment for status epilepticus

急性脳症、特異な脳炎・脳症後てんかんの一群、theophylline関連けいれんの場合は早めに導入)

5. Midazolam使用方法

1) けいれん重積状態およびけいれん群発時の治療

てんかんおよび種々の基礎疾患（急性脳炎、髄膜炎、急性脳症、脳血管障害、低酸素性虚血性脳症、代謝障害、熱性けいれんなど）に関連する急性症候性けいれんの重積、群発に対するmidazolam治療について、当センターにおける使用方法[29,30]を中心に紹介する。

(1) Midazolam静注治療

けいれん重積状態およびけいれん群発時のmidazolam静注治療方式をFig. 1に示す。

a. 1回量静注治療（第一次治療として施行する場合）：Midazolam注射液（10 mg/2 ml）を生食水か5%糖液で希釈し（1 mg/1 mlが便利）、1回量0.15 mg/kg（0.1～0.3 mg/kg）を基準に1 mg/minの速度で静注する。Midazolam 1回量静注により速やかに重積状態から脱した場合でも、再発予防と脳の安静を保つ目的で、その後12～24時間程度はmidazolamを0.1～0.15 mg. kg/hrで持続静注することが望ましい。ただし、けいれん開始後30分以内ではあるが無治療ではけいれん重積状態になることが予測される場合に初期治療としてmidazolam 1回量の静注を行い、速やかにけいれんが消失、以後再発の危険性がないと判断されるような時にはそのまま経過観察としてもよい。なお、一旦けいれんが消失後に再発をみた場合にはmidazolam 1回量静注をさらに1～2回反復することもあるが、けいれん抑制が不十分な場合には引き続きmidazolam持続静注治療を開始する。また、けいれん群発の場合には引き続き持続静注を行う。

b. 持続静注治療：Midazolam 1回量静注に引き続きmidazolamを0.1～0.15 mg. kg/hrで持続静注を開始する。けいれん重積状態あるいはけいれん群発状態から完全に脱却できるまでmidazolamを0.05～0.1 mg/kg/hrずつ0.3 mg/kg/hrまで増量していくが、治療経過によっては最大0.5 mg/kg/hrまで増量してもよい。増量速度は重積時には急速に、群発時には比較的緩徐とする。ちなみに、midazolam持続静注治療の平均有効投与量は0.2 mg/kg/hr前後である。midazolam持続静注中は呼吸、循環状態をモニターするが、通常投与量の範囲では人工呼吸管理は不要である。ま

た、脳波でもけいれんが抑制されていることを確認するが、脳波の持続的なモニターは必ずしも必要ない。Midazolam持続静注治療によりけいれんが抑制され、その後24時間けいれんの再発がなく全身状態が安定した後には、2～3時間毎に0.05 mg/kg/hrずつ漸減・中止とするが、長期間持続投与した場合には反跳発作を防ぐためさらに減量速度を2～3倍遅らせることが望ましい。一方、midazolam持続静注開始後もけいれん抑制が得られない場合や効果が不十分と判断した場合には原因疾患も考慮してできるだけ速やかに（けいれん重積状態ではmidazolam治療開始後30分を限度とする）barbiturates大量療法など他の治療に切り替える。Midazolamはbarbituratesにみられる脳圧降下など脳保護作用を有さないため脳圧亢進や脳腫脹を伴う重篤な急性脳症では効果が限られる。また、著者らの経験からは特異な脳炎・脳症後てんかんの一群にも有効性は低いと思われる。乳児重症ミオクロニーてんかんのけいれん重積状態や難治性症候性てんかんの強直発作重積状態などでは効果が不十分な場合がありうるが、症例によっては有効な場合もある。なお、theophylline関連けいれんに対するmidazolamの有効性については未だ充分には検討されていない。したがって、このような原因疾患や病態によってmidazolamの有効性に差があることをあらかじめ念頭において治療することが重要である。

(2) 筋注、注腸、頬粘膜投与、経鼻投与

Midazolamは静注以外の投与経路として、筋注、注腸、経鼻、経頬粘膜投与が利用でき、いずれも速効性かつ強力なけいれん抑制効果をえることができる。したがって、医療機関においても静脈確保に時間がかかるような場合には応急的にはmidazolamの筋注[45,46]、経鼻[47~50]、頬粘膜投与[50~52]が有用である。Midazolam注射液を用いた投与量は筋注0.15～0.3 mg/kg、注腸0.15～0.3 mg/kg、経鼻投与0.2～0.3 mg/kg、頬粘膜投与0.3 mg/kgと報告されているが、実際の臨床使用にあたってはさらに症例の蓄積と詳細な検討を要すると思われる。

2) てんかん発作頻発時の治療[29,30]

Lennox-Gastaut症候群をはじめとする難治性症候性てんかんでは強直発作などのてんかん発作が頻発し、その抑制に難渋することがある。このような場合にmidazolam持続静注治療を長期間継続しながら、急性発作の鎮静化に加え経口抗てんかん薬の調整による発作抑制維持をはかることが可能である。0.1～0.3 mg/kg/hrのmidazolam持続静注では日常生活動作が可能であるため長期間継続しても大きな支障がなく、非常に有用な治療法である。さらにmidazolam持続静注治療中にphenytoin静注など他治療を平行して行うことも可能である。

6. Midazolam治療の今後の問題点

Midazolam治療の今後の問題点として、1回量静注時と持続静注時の至適投与量や最大投与量などの至適投与設計を行うために、有効血中濃度、薬物動態パラメーターの検索が必要である。また、原因疾患や病態によるmidazolamの有効性の差についての検討も必要である。さらに、新生児けいれんの治療にもmidazolamが使われる場合があるが、その適応および使用基準などについては今後の検討課題である。

7. 結　　語

諸外国では小児のけいれん重積状態に対する第一次治療および第二次治療として使用されているlorazepamm静注製剤とfosphenytoin静注製剤が日本には導入されていないため、今後これらの薬剤が日本にも導入されるよう働きかけることが必要である。また、現在日本で使用できる薬剤としてはmidazolam持続静注治療が薬理学的および臨床的にも非常に優れた治療法であり、けいれん重積の第一次治療ならびに第二次治療薬となりうることが示唆され、midazolamがけいれんに対する保険適応となるようさらに臨床研究を進めることも重要であると考える。

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Summary

Effectiveness of Midazolam in the Treatment of Status Epilepticus in Children

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Status epilepticus in children is a medical emergency that requires prompt intervention. Diazepam is most commonly administered initially, whereas phenytoin is the anticonvulsant most used subsequently, in Japan. Intravenous diazepam is often associated with respiratory depression and hypotension and duration of its anticonvulsive effect is very brief. During intravenous infusion of phenytoin, cardiopulmonary depression can occur as well as venous complications and appearance of its anticonvulsive action is slow.

Midazolam has many clinical and pharmacological advantages compared with other antiepileptic agents. It acts more rapidly and it is safer and more effective.

Midazolam should be considered as an initial treatment for status epilepticus in children.

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In Vivo Studies of Phenylalanine Hydroxylase by Phenylalanine Breath Test: Diagnosis of Tetrahydrobiopterin-Responsive Phenylalanine Hydroxylase Deficiency

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ABSTRACT

Tetrahydrobiopterin (BH_4)-responsive phenylalanine hydroxylase (PAH) deficiency is characterized by reduction of blood phenylalanine level after a BH_4-loading test. Most cases of BH_4-responsive PAH deficiency include mild phenylketonuria (PKU) or mild hyperphenylalaninemia (HPA), but not all patients with mild PKU respond to BH_4. We performed the phenylalanine breath test as reliable method to determine the BH_4 responsiveness. Phenylalanine breath test quantitatively measures the conversion of L-[1-^{13}C] phenylalanine to $^{13}CO_2$ and is a noninvasive and rapid test. Twenty Japanese patients with HPA were examined with a dose of 10 mg/kg of ^{13}C-phenylalanine with or without a dose of 10 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} of BH_4 for 3 d. The phenylalanine breath test [cumulative recovery rate (CRR)] could distinguish control subjects (15.4 ± 1.5%); heterozygotes (10.3 ± 1.0%); and mild HPA (2.74%), mild PKU (1.13 ± 0.14%), and classical PKU patients (0.29 ± 0.14%). The genotypes in mild PKU cases were compound heterozygotes with mild (L52S, R241C, R408Q) and severe mutations, whereas a mild HPA case was homozygote of R241C. CRR correlated inversely with pretreatment phenylalanine levels, indicating the gene dosage effects on PKU. BH_4 loading increased CRR from 1.13 ± 0.14 to 2.95 ± 1.14% (2.6-fold) in mild PKU and from 2.74 to 7.22% (2.6-fold) in mild HPA. A CRR of 5 to 6% reflected maintenance of appropriate serum phenylalanine level. The phenylalanine breath test is useful for the diagnosis of BH_4-responsive PAH deficiency and determination of the optimal dosage of BH_4 without increasing blood phenylalanine level. ***(Pediatr Res* 56: 714–719, 2004)**

Abbreviations

BH_4, tetrahydrobiopterin,
CRR, cumulative recovery rate,
HPA, hyperphenylalaninemia,
PAH, phenylalanine hydroxylase,
PKU, phenylketonuria,
PTPS, 6-pyruvoyl-tetrahydropterin synthase

Phenylketonuria (PKU) is an autosomal recessive disorder caused by deficiency of hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1). The disease causes mental retardation unless the affected child is maintained on a strict low-phenylalanine diet (1). Newborn mass screening for PKU is performed worldwide, and patients with a wide spectrum of clinical severity have been identified with almost 100% probability. The incidence of PKU in Japan is 1/120,000 (2) and is much lower than in whites (1/10,000) (3) and Chinese (1/18,000) (4). PKU is a heterogeneous metabolic disorder at both clinical and genetic levels.

The diagnosis of PKU is based on the presence of high concentration of phenylalanine in the serum and lack of deficiency of tetrahydrobiopterin (BH_4), rather than by measuring hepatic PAH activity. The clinical severity of PAH deficiency is also determined mainly by serum phenylalanine level, al-

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though blood phenylalanine concentration is influenced by the dietary protein intake. For an appropriate diet therapy influenced by the social environments and personal situations, it is important to determine directly the clinical severity of PKU and PAH activity. As a representative autosomal recessive inherited disease, it is important to investigate how both alleles of the PKU gene influence PAH activity and how they give rise to clinical symptoms. We reported in 1991 that the clinical phenotype of PKU patients correlated with the genotype, which was the average of *in vitro* PAH activity of both mutations (5). However, subsequent studies reported that the clinical phenotype in several cases of PKU did not correspond with the genotype (6,7). The metabolism of phenylalanine in the human body not only reflects PAH protein but also is influenced by many factors, such as absorption and excretion of phenylalanine and the regulation of transcription and/or translation on PAH gene.

Various methods are available for *in vivo* measurement of PAH activity. ^{2}H-tyrosine in blood is measured after administration of ^{2}H-phenylalanine using gas chromatography mass spectrometry. The results of analysis using this method correlated with hepatic PAH activity and clinical phenotype (8–10). However, this method requires the use of a large amount of phenylalanine as a loading dose (10–200 mg/kg) and frequent blood sampling. Treacy *et al.* (11) described the phenylalanine breath test, a rapid noninvasive test for measurement of the actual phenylalanine tolerance. The test is based on quantitative measurement of the conversion of L-[1-^{13}C] phenylalanine to $^{13}CO_2$ through tyrosine by PAH. The phenylalanine breath test provides information on the whole-body phenylalanine oxidative capacity, as an index of *in vivo* PAH activity.

Recently, BH_4-responsive PAH deficiency was characterized by a decrease of blood phenylalanine after a BH_4 loading test (12) and patients with this deficiency have been treated with long-term BH_4 (13–17). Most patients of BH_4-responsive PAH deficiency have mild PKU and mild hyperphenylalaninemia (HPA), but not all patients with mild PKU respond to BH_4. In fact, even patients with similar mild mutations exhibit different response to BH_4 (12,17). Using the phenylalanine breath test, Muntau *et al.* (17) reported recently that BH_4 increases PAH activity in patients with BH_4-responsive PAH deficiency. The present study was designed to determine whether phenylalanine oxidation capacity is consistent with the clinical phenotype and genotype and whether determination of this parameter is useful for the diagnosis of BH_4-responsive PAH deficiency.

METHODS

Subjects. The subjects of this study were 20 Japanese patients (11 male individuals, aged 1–23 y) who were confirmed to have PAH deficiency on the basis of clinical and biochemical evaluation at the participating institutions. The patients, except for an adult patient with mild and another with classical PKU, were on phenylalanine-free milk and low-protein food ranging from insufficient to sufficient. Serum phenylalanine levels measured before the breath test in the two untreated patients with mild and classical PKU were 0.97 and 1.45 mM, respectively. The mean serum phenylalanine concentration were 0.31 ± 0.14 mM (± SD; range: 0.11–0.46 mM) in si treated patients with mild PKU and HPA and 0.43 ± 0.28 m (range: 0.068–0.95 mM) in 12 treated patients with classica PKU. Two patients with 6-pyruvoyl-tetrahydropterin synthas (PTPS) deficiency were diagnosed by analysis of urinary pteri dine, biopterin loading test, and measurement of PTPS activit and treated with BH_4, 5-hydroxytryptophan, L-dopa, and car bidopa. The criterion for classical PKU is serum phenylalanin concentration of ≥1.2 mM before initiation of a phenylalanine restricted diet or in the absence of dietary restrictions later i life. The serum phenylalanine concentrations in mild PKU an mild HPA were 0.6–1.2 mM and <0.6 mM, respectivel without phenylalanine-restricted diet. Serum phenylalanin was measured using ion-exchange chromatography.

Phenylalanine breath test. For phenylalanine breath test 99% enriched L-[1-^{13}C] phenylalanine was administered orall at a dose of 10 mg/kg and a maximum of 200 mg afte overnight fast. Breath samples were collected into aluminu bags at 0, 10, 20, 30, 45, 60, 90, and 120 min. 6R-BH_4 (Sunto Co., Tokyo, Japan) was administered orally at a dose of 1 mg/kg with a maximum of 200 mg, divided into two doses pe day at −2 and −1 d and another dose of 10 mg/kg, with maximum of 200 mg, 3 h before the breath test, as shown i Figure 1. The breath test was repeated twice in seven patient (two classical PKU and five mild PKU), before and after BH_4 The administered ^{13}C-phenylalanine is metabolized by PA and is exhaled as $^{13}CO_2$. $^{12}CO_2$ (m/z 44) and $^{13}CO_2$ (m/z 45 were measured using gas chromatograph/mass spectromete (Breath MAT Plus; Finnigan MAT, Bremen, Germany) (18) Results of the $^{13}CO_2$ breath tests were expressed as ^{13}CO excess permillage (Δ^{13}C, ‰) and cumulative recovery rat (CRR; %) (11). The Δ^{13}C (‰) was expressed as the ratio o $^{13}CO_2/^{12}CO_2$. The CRR was expressed as the ratio of tota amounts of $^{13}CO_2$ (moles) in expiration during 120 min fo administered dose of ^{13}C-phenylalanine. Total CO_2 productio speed is calculated from the body surface area (5 mmol · m^{-} · min^{-1}) (19), and the body surface area (BSA; in m^2) i calculated by the formula [body weight $(kg)^{0.5378}$ × heigh $(cm)^{0.3964}$ × 0.024265] (20). Serum phenylalanine was mea sured before administration of L-[1-^{13}C] phenylalanine and 1 after administration using Hitachi automatic amino acid ana lyzer L8800 (Hitachi Co., Hitachinaka, Japan).

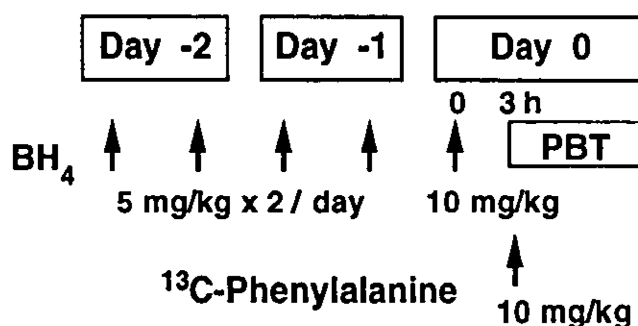

Figure 1. Time schedule of ^{13}C-phenylalanine ingestion and BH_4 dosin PBT, phenylalanine breath test.

DNA analysis. PAH mutations were determined by using denaturing gradient gel electrophoresis and DNA sequencing, as described previously (21,22). Genomic DNA was isolated from lymphocytes or EBV transformed lymphoblasts. Thirteen exons and flanking intronic regions of the PAH gene were amplified by PCR with GC-clamped primers according to Guldberg *et al.* (23). The target exons with mutations were amplified from genomic DNA by PCR with biotinylated primers and were purified to single-strand DNA using magnetic beads coated with streptavidin M280 (Dynal, Oslo, Norway). The purified single-stranded DNA was sequenced by the dye erminator method using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT).

Statistical analysis and ethical issues. All data were expressed as mean ± SD unless otherwise stated. Differences between groups were examined for statistical significance using the *t* test. A $p < 0.05$ denoted the presence of a statistically significant difference. Statistical analyses were performed using the Statview program version 4.5 (Abacus Concepts, Berkeley, CA).

All protocols described in the above studies were approved y the institutional review boards of Osaka City University Graduate School of Medicine, and informed consent for the breath test and genetic analysis was obtained from all patients or their parents.

RESULTS

Identification of genotype. Table 1 shows the results of genetic analysis of 13 patients with classical PKU, six patients with mild PKU, and one patient with mild HPA. A total of 18 mutations were identified in 40 PKU alleles of the 20 patients, except for one allele. Four mutations of A202V (GCT → GTT), R252P (CGG → CCG), Q301H (CAG → CAT), and D415H (GAC → CAC) have not been reported. The genotypes in mild PKU cases were from compound heterozygotes with mild (L52S, R241C, R408Q) and severe mutations, whereas in the mild HPA case, it was homozygote of mild mutation (R241C/R241C). In this study, we could not find cases with discordance between genotype and clinical phenotype.

Serum phenylalanine concentration. We also examined the influence of ^{13}C-phenylalanine loading (10 mg/kg; maximum 200 mg) on serum phenylalanine concentration. No large increase was noted in serum phenylalanine concentration before and after administration of ^{13}C-phenylalanine in both classical PKU and mild PKU/HPA patients (an increase from a predosing value of 0.51 ± 0.39 to 0.62 ± 0.38 mM at 1 h after phenylalanine dosing and from 0.41 ± 0.29 to 0.44 ± 0.26 mM, respectively).

$^{13}CO_2/^{12}CO_2$ ratio in breath test. Figure 2 shows the changes in $^{13}CO_2/^{12}CO_2$ ratio ($\Delta^{13}C$) in expired air. The peak level of $\Delta^{13}C$ in the control occurring between 20 and 30 min after dosing (42.3 ± 10.4; range: 28.7–53.4‰) was significantly higher than that of the heterozygotes at the same time interval (27.2 ± 6.0; 21.1–32.5‰; $p < 0.05$). These results indicated that the administered ^{13}C-phenylalanine dose reached the liver at 20–30 min after dosing, where it was mainly metabolized to tyrosine, *p*-hydroxyphenylpyruvate, and homogentisic acid in the liver with a first-pass effect. Patients with mild HPA showed a small peak of $\Delta^{13}C$ at 45 min, reflecting the residual PAH activity. Low PAH activity was observed in classical PKU and mild PKU, and no peaks were noted for $\Delta^{13}C$. After BH_4 loading, mild PKU showed a peak $\Delta^{13}C$ level of 8.87 ± 8.99‰ (range: 3.83–21.62‰) at 20–30 min. Mild HPA showed a peak $\Delta^{13}C$ (24.45‰) at 20–30 min, a pattern similar to that noted in heterozygotes. BH_4 loading markedly improved phenylalanine oxidation in mild PKU and mild HPA.

Table 1. *Genotypes and* in vitro *PAH activity in 20 patients with PAH deficiency*

Genotype	*n*	%	PAH activity (%)
Severe type			
R413P	12	30	0[29]
IVS4nt-1	3	7.5	0
R111X	2	5	0
Del5&6	2	5	0
T278I	2	5	1[22]
Y77X	1	2.5	0
A202V	1	2.5	ND
E6nt-96a>g	1	2.5	0
R243Q	1	2.5	10[30]
R252P	1	2.5	ND
R261X	1	2.5	0
IVS10nt-14	1	2.5	0
Q301H	1	2.5	ND
D415H	1	2.5	ND
L430P	1	2.5	ND
Mild type			
R241C	6	15	25[25]
L52S	1	2.5	27[19]
R408Q	1	2.5	55[26]
Total	39/40	97.5	

ND, not determined.

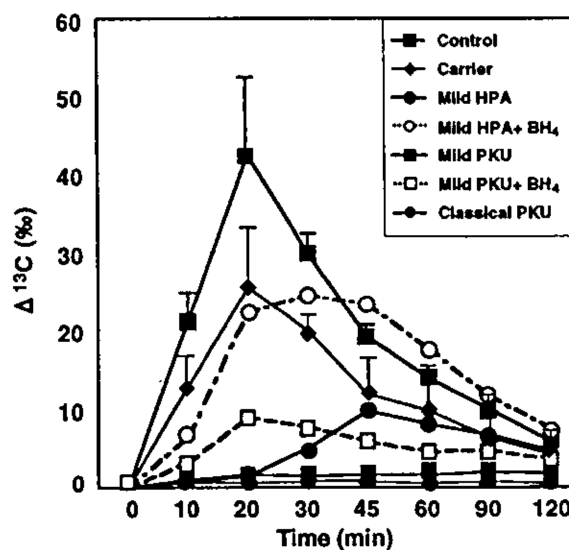

Figure 2. Time course of $^{13}CO_2$ excretion during phenylalanine breath test in control subjects, heterozygotes, and patients with PAH deficiency. $\Delta^{13}C$ (‰) values during 120 min after ingestion of ^{13}C-phenylalanine are expressed as solid lines without BH_4 dosing and as dashed lines with BH_4 dosing. Data of the control and heterozygotes are expressed as mean ± SD, whereas those of patients with PAH deficiency are expressed as mean values.

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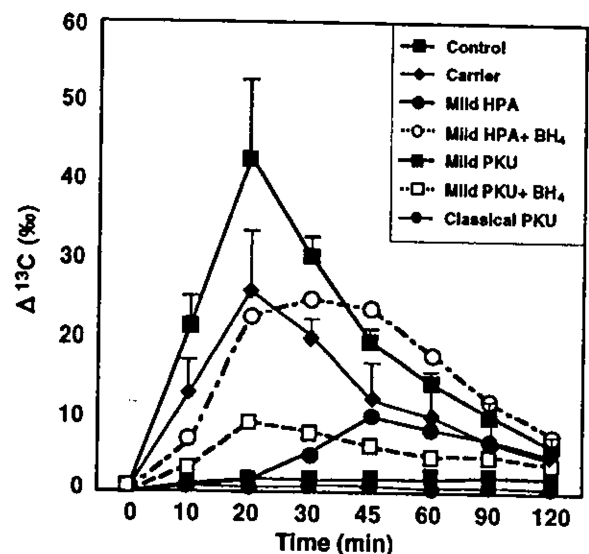

Figure 2. Time course of $^{13}CO_2$ excretion during phenylalanine breath test in control subjects, heterozygotes, and patients with PAH deficiency. $\Delta^{13}C$ (‰) values during 120 min after ingestion of ^{13}C-phenylalanine are expressed as solid lines without BH_4 dosing and as dashed lines with BH_4 dosing. Data of the control and heterozygotes are expressed as mean ± SD, whereas those of patients with PAH deficiency are expressed as mean values.

CRR in breath test. As shown in Figure 3, the phenylalanine breath test exhibited continuous levels of CRR in PAH deficiency from 0 (classical PKU) to 2.74% (mild HPA). These results are in agreement with various clinical manifestations of PKU. The CRR (*in vivo* PAH activity) could distinguish control subjects (15.4 ± 1.5%; range: 13.79–17.44%); the heterozygotes (10.3 ± 1.0%; 9.00–11.42%); and mild HPA (2.74%), mild PKU (1.13 ± 0.14%; 1.01–1.40%), and classical PKU (0.29 ± 0.14%; 0–0.93%) patients (Fig. 3). We found consistency between the CRR and clinical phenotype in the Japanese patients who were tested in this study. Patients with compound heterozygotes of mild (L52S, R241C, R408Q) and severe mutations had CRR of 1.0–1.4% and mild PKU phenotype, whereas patients with severe mutations for both alleles had CRR of <0.93% and classical PKU phenotype. In this study, we did not find inconsistency among clinical phenotype, CRR, and genotype.

BH_4 loading increased CRR from 1.13 ± 0.14 to 2.95 ± 1.14% (2.6-fold) in all four patients with mild PKU and also increased it from 2.74 to 7.22% (2.6-fold) in mild HPA patients. All patients with mild PKU and mild HPA in this study responded to BH_4. Two patients with classical PKU showed no increase in the CRR after BH_4 loading. BH_4-induced activation was proportional to residual PAH activity. In PTPS patients 1 and 2, serum phenylalanine was effectively controlled to ≤0.12 mM after administration of BH_4 at 3.4 and 6 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively, with regular food. The CRR values in these two patients were 5.88 and 19.0%, respectively.

Correlation between CRR and phenylalanine levels without dietary treatment. Correlation between CRR and serum phenylalanine levels without dietary treatment was examined in 26 patients. Serum phenylalanine levels of four control subjects, four heterozygotes, and two patients with PTPS deficiency were measured before administration of phenylalanine in the breath test. Plasma phenylalanine levels of 16 patients with PAH deficiency were examined before phenylalanine-restriction therapy. As shown in Figure 4, CRR correlated inversely with phenylalanine concentration ($1/y = 0.69 + 1.02\,x$; $p < 0.0001$). This result indicates that phenylalanine levels can decrease steeply with a slight increase of CRR (from 0% or near 0% to 1–2%), and the clinical phenotype changes from classical PKU to mild HPA.

DISCUSSION

In the phenylalanine breath test, the administered ^{13}C-phenylalanine is absorbed in the intestine and transported to the liver cells through the portal vein. In the liver, ^{13}C-phenylalanine is converted to ^{13}C-tyrosine by PAH, then to homogentisic acid by tyrosine aminotransferase and dioxygenase, and is finally exhaled as $^{13}CO_2$. Thus, this test not only simply measures PAH activity but also evaluates the overall state of phenylalanine metabolism in humans, *i.e.* phenylalanine oxidation capacity. This test is expected to reflect the clinical phenotype of PKU. The phenylalanine breath test used in the present study does not require blood sampling or special

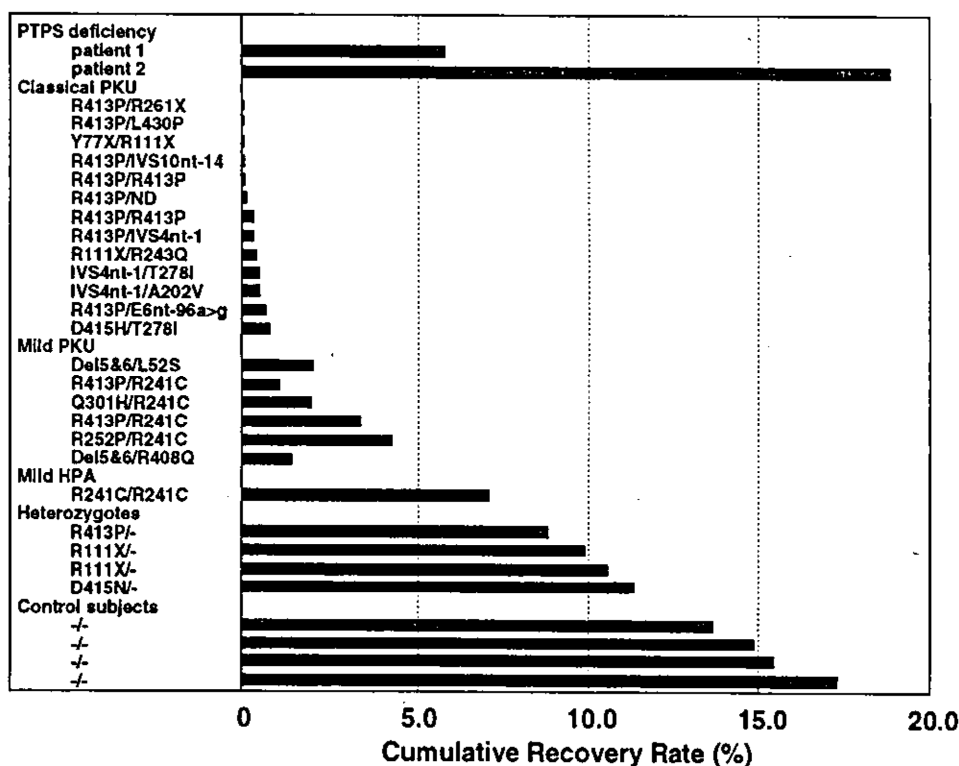

Figure 3. Phenylalanine oxidation capacity in control subjects, heterozygotes, patients with PAH deficiency, and patients with PTPS deficiency. ▤, CRR (%) values determined during 120 min after the ingestion of ^{13}C-phenylalanine without BH_4 dosing; ■, CRR (%) values determined during 120 min after the ingestion of ^{13}C-phenylalanine with BH_4 dosing. The detected mutations in PAH-deficient patients and heterozygotes are indicated in the left panel. -, no mutation; ND, not determined.

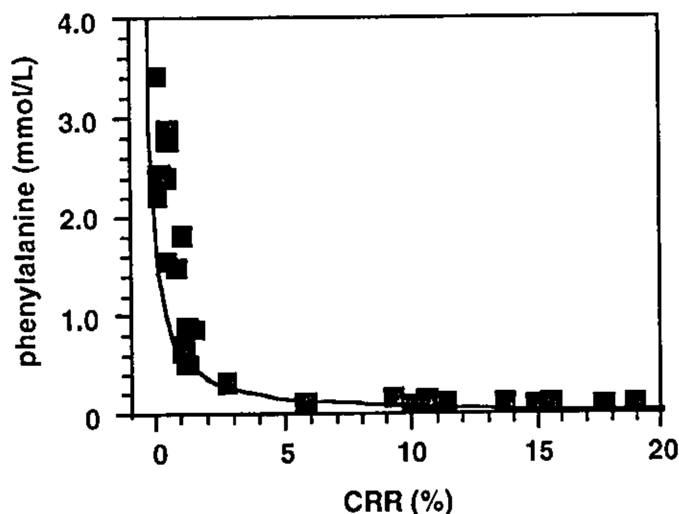

Figure 4. Correlation between pretreatment phenylalanine levels and CRR in control subjects, heterozygotes, patients with PAH deficiency, and patients with PTPS deficiency ($n = 26$).

conditions such as high serum phenylalanine levels. The results are deduced from analysis of the expired gas collected over a short period of time. Thus, the phenylalanine breath test is simple and noninvasive and can be repeated several times.

The CRR in phenylalanine breath test showed a continuum from classical PKU to mild HPA (Fig. 3). This finding is in agreement with the clinical notion that PKU is a highly heterogeneous disease for the clinical phenotype, which is caused by the strong heterogeneity of PKU mutations. In fact, 18 different mutations were detected in 20 patients in the present study. Patients with classical PKU, mild PKU, and mild HPA had significantly different CRR values of <1%, 1–1.4%, 2.4%, respectively. Therefore, we can predict the clinical phenotype from the CRR. Furthermore, CRR can be used to determine adherence to diet therapy. Concerning the effects of BH_4 on PAH, all five patients with mild PKU and mild HPA responded to BH_4 loading and showed an increase in CRR, which was proportional to the residual PAH activity after BH_4 dosing. In patients with classical PKU, BH_4 loading did not increase CRR. When the CRR is 1% or higher before BH_4 dosing, the response to BH_4 may be expected (Fig. 3). A CRR value of 5.88% in patients with PTPS deficiency indicated <0.12 mM of serum phenylalanine levels on long-term BH_4 therapy. These findings suggest that the cumulative recovery of 5 to 6% is also sufficient for maintaining serum phenylalanine level (0.12 mM) in patients with PAH deficiency and that our test may be potentially useful for determining the optimal dosage of BH_4 for long-term medication of BH_4-responsive HPA patients.

The frequency and types of PKU mutations differ greatly between whites and East Asians (21,24). Mutations associated with mild phenotype and BH_4-responsive PAH deficiency in East Asians also differ from those in whites. In the present study, all patients with mild PKU phenotype were compound heterozygotes with severe and mild mutations, which included R241C (*in vitro* PAH activity; 25%) (25), L52S (27%) (22), and R408Q (55%) (26), as shown in Table 1. Mild HPA was R241C homozygote. The patient with R408Q (*in vitro* PAH activity, 55%) and Del 5&6 had higher CRR than four patients with R241C (*in vitro* PAH activity, 25%) and severe mutations (R413P, Q301H, and R252P). The phenylalanine oxidation capacity, *i.e.* CRR, determined by our test stands between the clinical phenotype and the genotype and links them together. In the present study of Japanese patients, none of the patients showed any disagreement among clinical phenotype, phenylalanine oxidation capacity, and genotype. It follows that the genotype determined by both alleles mainly specifies PAH activity, which in turn specifies the clinical manifestations in an individual.

However, in East Asians, discordance between BH_4 respon siveness and genotype has been reported (12). Two patient with P407S mutation were described, one as a nonresponde (P407S/R111X) and the other as a responder (P407S/R252W to BH_4. This different responsiveness is thought to be due t another mutation in each patient (R111X: stop codon mutation R252W: missense mutation) on PAH protein synthesis. I whites, inconsistencies associated with BH_4 responsiveness ar reported concerning Y414C, L48S, I65V, and R261Q muta tions, and the BH_4 responders and nonresponders are present i individuals with the same mild mutation (17).

The cause for discordance among clinical phenotype includ ing BH_4-responsive PAH deficiency and genotype is not ye clear. In addition, the mechanism responsible for the recove of defective PAH activity after BH_4 loading remains elusive Two broad factors determine the effect of BH_4 on PAH: *1*) BH site: absorption, distribution, and metabolism of orally admin istered BH_4, and 2) PAH site: interaction between BH_4 an PAH gene and protein. Concerning BH_4 site, absorption o BH_4 is minimal and unstable and differs greatly from on individual to another (Suntory Co., personal communication The optimal dose and the duration of BH_4 administration fo the diagnosis of BH_4-responsive PAH deficiency remain un known. Bernegger *et al.* (27) pointed out that a single dose o 20 mg/kg of 6R-BH_4, the active form, was 5–20 times mo effective than smaller doses of 6R- BH_4 or 6R, S-BH_4 an induced a response in 70% of patients with mild PAH de ciency. With regard to the optimum BH_4 dose for long-ter control of patients with BH_4-responsive PAH deficiency, f vorable blood phenylalanine levels were obtained at a B dose of 5–10 mg/kg. It may be necessary to repeat BH_4 dos over several days for unstable absorption of BH_4. In our stud using BH_4 at 10 $mg \cdot kg^{-1} \cdot d^{-1}$ for 3 d, a rise of PAH activit was noted in all patients with mild PKU and mild HPA.

Concerning PAH site factors, various mutations associate with BH_4 responsiveness have been identified, and some m tations were outside the catalytic domain or the locu associated Km variant for BH_4 of the PAH enzyme. Dire effects of BH_4 are suspected. In other words, BH_4 may u regulate the expression of the PAH gene, stabilize PA mRNA, and facilitate and stabilize the formation of function PAH tetramers (16). Figure 4 provided in this study seems confirm the proposal put forward by Scriver (28): "gene dosa effect in PAH deficiency." Figure 4 may clarify the causes a the mechanisms of the BH_4 responsiveness in mild PA deficiency and the discordance between genotype and clinic

phenotype. Mutations that were identified in cases with discordance between genotype and clinical phenotype were basically related to the mild genotype. Patients with the mild clinical phenotype exist at the turning point of the correlation curve shown in Figure 4. This mild phenotype is produced by a small residual PAH activity, which is specified to the genotype. The formula suggests that a slight increase of the CRR at the turning point by certain effectors should greatly reduce blood phenylalanine level and cause transformation to a mild phenotype. In contrast, a slight decrease of the CRR leads to a rise in blood phenylalanine level and subsequently leads to transformation to a severe phenotype. Patients with mild mutation could become milder or more severe by certain effectors of the PAH enzyme. BH_4 is advocated as a strong effector to influence mutations at the turning point.

The phenylalanine breath test is useful for the diagnosis of BH_4-responsive PAH deficiency and determination of the optimal dosage of BH_4 without increasing blood phenylalanine level. To clarify the discordance between clinical phenotype including BH_4 responsiveness and genotype, it is important to investigate both genotype and phenylalanine oxidation capacity and to further accumulate such data.

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Infection of Different Cell Lines of Neural Origin with Subacute Sclerosing Panencephalitis (SSPE) Virus

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Abstract: **Measles virus is the causative agent of subacute sclerosing panencephalitis (SSPE). The viruses isolated from brain cells of patients with SSPE (called SSPE viruses) are defective in cell-free virus production *in vitro*. To investigate the cell tropism of three strains of SSPE virus (Osaka-1, Osaka-2, Osaka-3), SSPE virus-infected cell cultures were treated with cytochalasin D to prepare virus-like particles (CD-VLPs). All CD-VLPs formed syncytia after infection in CHO cells expressing CD150 but not in those expressing CD46. In addition, an antibody to CD46 did not block the infection of Vero cells by SSPE CD-VLPs. The results were consistent with our previous suggestion that one or more unidentified receptors might be involved in the entry process. Infection with the CD-VLPs from three SSPE strains was further examined in different human cell lines, including those of neural origin, and was found to induce syncytia in epithelial cells (HeLa and 293T) as well as neuroblastoma cells (IMR-32 and SK-N-SH) with varying efficiency. SSPE CD-VLPs also infected glioblastoma cells (A172) and astrocytoma cells (U-251) but syncytial formation was rarely induced. These epithelial and neural cell lines were not permissive for the replication of wild-type MV. Together with our previous observations, these results suggest that the cell entry receptor is the major factor determining the cell tropism of SSPE viruses. Further studies are necessary to identify other viral and/or cellular factors that might be involved in the replication of SSPE virus in specific neural cells and in the brain.**

Key words: **Measles virus, Syncytium formation, Receptor, Cytochalasin D**

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative disease caused by persistent infection of the central nervous system (CNS) with measles virus (MV). SSPE viruses, variant MVs isolated from the brains of patients with SSPE, differ biologically and genetically from MVs isolated from patients with acute measles. When the nucleotide sequences of the genome of SSPE virus strains were compared to the consensus sequences of MV, many mutations were found, particularly in the entire M gene and in part of the F gene (2, 4, 6, 9–13, 33, 47, 59). We have characterized three strains of SSPE virus (designated Osaka-1, Osaka-2, and Osaka-3) (44), compared them with their possible progenitor MV strains, and confirmed some essential SSPE-associated alterations (3, 5, 42, 50). We have also demonstrated the occurrence of SSPE virus-induced acute encephalopathy in hamsters and carefully monitored nucleotide sequence alterations during the course of experiments (29, unpublished observation).

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Abbreviations: CD-VLPs, virus-like particles prepared by treating infected cells with cytochalasin D; CHO, Chinese hamster ovary; CNS, central nervous system; CPEs, cytopathic effects; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; IFN, interferon; MV, measles virus; PFU, plaque-forming units; SSPE, subacute sclerosing panencephalitis; VSV, vesicular stomatitis virus.

SSPE viruses cause neurological disorders in experimental animals (such as hamsters and mice) when inoculated into the brain. Wild-type MVs as well as vaccine or laboratory strains do not usually cause neurological symptoms in genetically unmodified adult hamsters and mice. The molecular mechanisms underlying neurovirulence are not well understood. The involvement of host cell surface receptors for MV in neurovirulence is one of the important factors (48). Two MV receptors have been identified: CD46 (membrane cofactor protein for complement) (17, 40) and CD150 (signaling lymphocyte activation molecule, SLAM) (20, 27, 57).

When compared to MVs isolated from patients with acute measles, SSPE viruses are little known in many respects. Kobune et al. (32) reported that susceptibility of B95a cells (a marmoset B lymphoid cell line transformed by Epstein-Barr virus) to MV in clinical specimens is 10,000 times that of Vero cells. On the other hand, SSPE viruses have generally been isolated from cells of non-lymphoid origin, such as Vero (16, 25, 35, 37), BSC-1 (7), and primary human embryonic lung cells (58). We have tried to isolate our three SSPE viruses by cocultivation of brain cells from patients with SSPE with three different cell lines, Vero, B95a, and primary human embryonic lung cells (44). Although two of the three SSPE strains could be isolated by cocultivation with B95a cells, all strains were most successfully isolated by cocultivation with Vero cells. Some reports demonstrate that adaptation to Vero cells is accompanied by specific amino acid replacements within the H protein (Asn to Tyr at position 481 or Ser to Gly at position 546) (28, 46, 52, 60). However, such amino acid replacements were not found in the H proteins from our SSPE viruses isolated and passaged repeatedly in Vero cells (22). Our recent study of the receptor usage of SSPE viruses, using the vesicular stomatitis virus (VSV) pseudotype system, revealed that SSPE viruses can use SLAM, but not CD46, for cell entry (53). Furthermore, the pseudotype viruses with SSPE envelope glycoproteins could enter SLAM-negative cells including Vero and some neural cells, pointing to the existence of yet another entry receptor for SSPE viruses on certain cell types (53). Although it is poorly understood, cell tropism of MV is not determined solely at the cell entry level. Many internal cellular factors as well as viral mutations can affect the replication of MV (48, 49). Takeuchi and colleagues reported that mutations in the P or M gene of a Vero cell-isolate of MV (having genes for envelope glycoproteins identical to a B95a cell-isolate of MV) allow MV replication in Vero cells (55). Because the VSV pseudotype system cannot be used to study the replication of MV after cell entry, we characterized SSPE virus infection of different cell lines including those of neural origin by another approach.

Cells infected with SSPE viruses are usually defective in production of a cell-free progeny virus. Nevertheless, cell-free virus-like particles are needed to study their cell tropism for different cell lines. The freeze-thawing method (43) was unacceptable because our strains could not be reproducibly prepared and the titers were unsatisfactory. Preparation by treating infected cells with cytochalasin D (CD-VLPs) (2) enabled us to infect cell lines and thereby compare the infectivity differences between SSPE virus and MV.

Materials and Methods

Cells and viruses. Vero (African green monkey kidney) cells, Vero/SLAM (Vero cells expressing human SLAM) cells, and HeLa (human uterus cancer) cells were cultured at 35 C in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% fetal bovine serum (FBS) and 4% newborn calf serum. B95a (Epstein-Barr virus-transformed marmoset B lymphocytes) cells were cultured in RPMI 1640 medium supplemented with 5% FBS. Chinese hamster ovary (CHO) and CHO cells stably expressing human CD46 (CHO/CD46) (30) or human SLAM (CHO/SLAM) (57) were grown in RPMI 1640 medium supplemented with 10% FBS. Culture media for CHO/CD46 cells or CHO/SLAM cells were supplemented with 0.7 mg/ml of hygromycin B (Wako Pure Chemicals, Osaka, Japan) and 0.5 mg/ml of G418 (Calbiochem-Novabiochem, La Jolla, Calif., U.S.A.), respectively. In addition, 293T (human embryonic kidney), IMR-32 and SK-N-SH (human neuroblastoma), A172 (human glioblastoma), and U-251 (human astrocytoma) cells were cultured in DMEM supplemented with 10% FBS. For cultivation of IMR-32 and SK-N-SH cells, non-essential amino acid solution (Gibco BRL, Gaithersburg, Md., U.S.A.) was added to a final concentration of 1%.

A Vero cell-adapted laboratory MV strain (Nagahata, genotype C1) and a field MV strain (OCU98-1P, genotype D3) isolated from peripheral blood lymphocytes of a patient with acute measles by cocultivation with B95a cells, and SSPE virus strains isolated from the brains of patients with SSPE have been described previously (5, 36, 44). In this study, we used three SSPE viruses (Osaka-1 Fr/V, Osaka-2 Fr/V, and Osaka-3 Oc/V) isolated from the brains of three patients. These viruses are sibling viruses that were isolated with different cell lines (such as Vero or B95a cells) and from different regions of the brain (such as the frontal or occipital lobe). For isolation and designation of sibling viruses of each strain and detailed patient information, refer to (22, 44).

SSPE strains were usually maintained by repeated

(i.e., 20–30) subcultures of the infected cells without adding fresh cells after isolation. Biochemical and biological characteristics of SSPE viruses used in this study, such as nucleotide sequence of the viral genome, viral protein synthesis in infected cultures, extent of infectious cell-free virus production and neurovirulence in hamsters, were examined at this passage level (29, 42, 50, unpublished observations).

Preparation of infectious cell-free virus-like particles. To test cell tropism of SSPE strains of MV in different cell lines, it was necessary to obtain infectious cell-free virus particles. However, SSPE strains are highly cell-associated and rarely produce a cell-free virus. Cytochalasin D is known to affect the membrane of some cell types and to cause projections to form at the cell surface by a process called "zeiosis" (23). The knobs with a slender stalk are formed and contain cell components such as ribosomes normally resident in endoplasm. When cells infected with SSPE strains are treated with cytochalasin D, the resulting knobs are expected to contain viral nucleocapsids and display F and H glycoproteins on their surface, similar to virus budding at the cell membrane. It was expected that the knobs would be mechanically chopped off the surface by vigorous pipetting. In practice, SSPE virus was cultured in a 25-cm^2 flask until large syncytia were formed (usually two or three days after passage) and the culture medium was replaced by 2 ml of maintenance medium containing cytochalasin D (5 µg/ml; Sigma, St. Louis, Mo., U.S.A.). Cells were incubated at 35 C for 30 min and then pipetted vigorously. The culture medium was collected and clarified by centrifugation at $1,600 \times g$ for 15 min. The resulting supernatant fluid was stored at −85 C until use. We have successfully prepared CD-VLPs from cells infected with any of the three SSPE strains of MV. The titer of the CD-VLPs was around 10^3–10^4 plaque-forming units (PFU)/ml on confluent monolayer cultures of cells used for virus isolation. One of the CD-VLP stocks was filtrated through a 0.45-µm membrane filter and the titer was determined. The approximate size of CD-VLPs was estimated from the reduction in titer after filtration and was compared with that of the MV (Nagahata strain) particles produced naturally in the culture fluid.

Electron microscopy. Morphological examination of CD-VLPs was carried out by electron microscopy as previously described (31). A drop of the CD-VLP's stock was placed for 1 min on a 400-mesh collodion-coated copper grid covered with a carbon film. The resulting samples were negatively stained with 2% phosphotungstate (pH 6.4) for 1 min and were examined in a JEOL transmission electron microscope (JEM-1200EXII) at 80 kV.

Virus infection, syncytium formation, and antibody treatment. Monolayer cultures of various cell lines were prepared in 24-well tissue culture plates. One milliliter of culture media containing the MV strains or SSPE CD-VLPs (50 to 300 PFU/well) was added and then incubated at 35 C for 3 hr. The cells were washed with a culture medium to remove cytochalasin D in the stock solution of CD-VLPs, and the wash solution was then replaced by a fresh culture medium. Syncytial foci were counted at 48 hr post-infection for MV and at 72 hr post-infection for SSPE CD-VLPs using an inverted microscope.

For the antibody-blocking experiment, 300 µl/well of culture medium with or without mouse monoclonal antibodies was added to monolayer cell cultures in 24-well plates and pre-incubated for 30 min at room temperature. Antibodies (M177 and M160 [51] against human CD46 [10 µg] or IPO-3 [Kamiya Biomedical, Seattle, Wash., U.S.A.] against human SLAM [10 or 20 µg]) were used. Then, 700 µl of culture media containing SSPE CD-VLPs (50 to 300 PFU/well) was added without aspirating the media. Ten micrograms of M177 antibody but not M166 antibody was found to completely block syncytium formation by the Nagahata strain of MV.

Indirect immunofluorescence tests. Cells cultured on coverslips were infected, cultured for three or four days, rinsed once with phosphate-buffered saline (pH 7.4), and fixed with acetone. Cells were incubated with a monoclonal antibody against MV N protein at room temperature for 30 min and then stained with anti-mouse IgG rabbit antibody conjugated with FITC for 30 min at room temperature.

Flow cytometric analysis. Expression of CD46 and CD150 of various human cell lines was determined by flow cytometry (FACSCalibur, Becton Dickinson, Tokyo) after staining with a mouse monoclonal antibody against CD46 or CD150, followed by rabbit anti-mouse IgG conjugated with FITC.

Results

Syncytium Formation by SSPE CD-VLPs in Vero Cells and CHO Cells Expressing CD150

We previously characterized the cell entry receptors for SSPE by using VSV pseudotype virus expressing SSPE envelope glycoproteins (53). To further confirm the receptor usage by SSPE viruses under conditions similar to natural virus infection, we prepared virus-like particles by treating SSPE virus-infected cultures with cytochalasin D and mechanical pipetting. Electron microscopic examination of the CD-VLPs prepared from both infected and uninfected cells showed numerous spherical