shown for DENV2 is considered to be a specific characteristic of the dengue virus, since viremia produced by the closely related JEV continues for 24 h in mice injected intraperitoneally with live JEV [37]. Although DENV2 viremia can last only in a short period immediately after injection of the live virus, monitoring viremia would be an acceptable model also in mice. Viremia levels are generally believed to correlate with disease severity [4,5] and also considered an important factor involved in efficiency of virus transmission to vector mosquitoes.

The present study showed that reduction in viremia level was related to the neutralizing antibody titers in sera. It is considered that the outcome of titrating the plasma samples from mice injected with live virus mainly represents the actual titer existing in circulation, but is partly affected by in vitro neutralization of the virus that may occur following blood collection if the blood contains neutralizing antibodies. The effect of neutralizing antibodies contained in serum/plasma samples on the result of titration is unavoidable when the level of viremia following challenge is used for evaluating candidate vaccines even in monkey models. In the present study, the effect was minimized by using plasma samples (for eliminating incubation at 37 °C to isolate serum) and keeping samples on ice until the use for titration. Pilot experiments using mixture of DENV2 containing 5×10^4 PFU/ml with an equal volume of immune mouse sera showing neutralizing antibody titers of 1:640–1:1280 against DENV2 indicated that incubation for 30 min on ice still maintained the titer at more than 30% of the original without incubation and a mean decrease during the process of titration (incubation at 37 °C for 60 min on Vero monolayer cells) due to the presence of neutralizing antibody was approximately 50% (data not shown). Nevertheless, in vitro neutralization shown in the plasma samples containing neutralizing antibody represents the event occurring in the blood of immune mice, indicating the role of neutralizing antibody in reducing viremia levels.

Co-immunization with a protein vaccine can increase the effectiveness of the dengue tetravalent DNA vaccine, irrespective of whether the protein vaccine is derived from the same or a different serocomplex. Since a higher dose of a protein vaccine (1/5 dose of JEVAX) increased the effectiveness, more effective co-immunization protocols may be established for animals larger than mice. We are planning evaluation of our vaccine strategy using non-human primates. A combination vaccine of different types against different viruses would be an effective strategy to control more than single flavivirus diseases prevailing in one area.

Acknowledgments

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Vol. 22, No. 9, 2006 化学療法の領域
特集 ワクチンの今日的問題点

2. 日本脳炎ワクチン

竹上 勉*

これまでの継続したワクチン接種，衛生環境の改善等によって本邦における日本脳炎患者発生は抑制され，現在の日本では患者は年間10人に満たない数で推移している。しかし，ウイルスがいなくなった訳ではない。野外蚊，豚からのウイルス分離例は毎年報告されている。しかも，最近のウイルスの遺伝子型は変化しているのだ。他方で日本脳炎ワクチンに対する評価が厳しくなっている。副反応として確率は低い（70～200万回に1回）が，急性散在性脳脊髄炎(ADEM)との関連が疑われている。マウス脳由来の原材料が問題となっている。それらの問題点について整理し，検討することが重要であろう。ウイルス感染防御のためのより良い方策を求めて行くのがウイルス研究者や医療現場の立場と考える。

Key Words：日本脳炎／副反応／有効ワクチン／遺伝子型

Ⅰ はじめに

2005年5月に厚労省から提示された「日本脳炎ワクチン接種の積極的勧奨の差し控え」は果たして妥当な決定だろうか。日本脳炎の大きな流行はないのだろうか。今や日本脳炎ウイルス(JEV)は日本の地からほとんどいなくなっているのだろうか。いくつかの疑問が出ている。それらについて考察を加えたい。

Ⅱ 日本脳炎ワクチンの問題点

現行ワクチンはJEV北京株をマウスに接種し，そのマウス脳を破砕し，さらに遠心機を用いて分画を重ね，高度に精製されたものを最終的にホルマリン処理し，その不活化ウイルスをワクチンとして用いている(図1)。こうした不活化ワクチンを接種された個体には複数の種類の抗体が得られる。抗体の中でもウイルスエンベロープ(E)蛋白に対する抗体がウイルス感染を顕著に阻害すると考えられている。ただ，正確なところ，どの抗体がウイルス感染阻止作用を起こすか，詳細は不明であるが，少なくともウイルスに対する感染防御効果があることは明確である。マウスでのワクチン効果をみる限り，ウイルス中和抗体の上昇は質/量共に高く，間違いなくウイルス感染を防いでいる。ヒトに対しても同様であろうと推定されて，長年にわたって接種が続けられてきた。ワクチン接種後の神経合併症等の副反応(副作用)は開発(1954年)初期には多くの報告があったが，その後改良があり，改善が進められ，数は減少している[1,2]。最近の調査においても確認されたが，JEV抗体上昇という面では極めて有効なワクチンといえる[3]。

しかし，マウス脳由来ということで随分以前からアメリカからはクレームがついていた。微量の脳成分の混入がアレルギー症状として出るのではないかというのだ[4,5]。ヒトに対するウイルス感染防御効果については台湾での調査（26万人規

Japanese encephalitis vaccine

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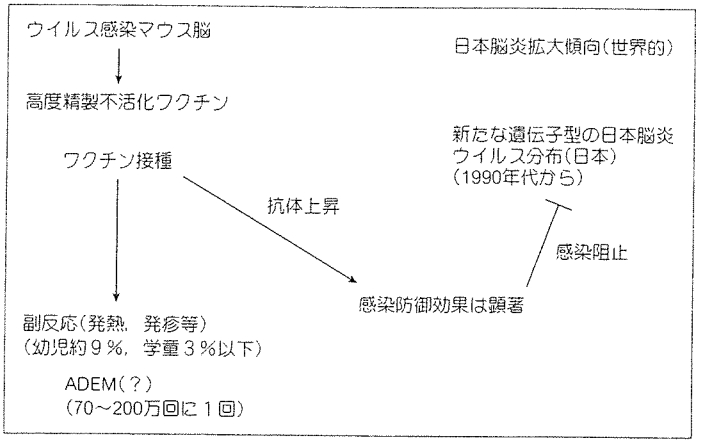

図1　日本脳炎ワクチンに関わる状況：有効性と安全性

現行ワクチンはJEV北京株をマウスに接種し，そのマウス脳を破砕し，さらに遠心機を用いて分画を重ね，高度に精製されたものを最終的にホルマリン処理し，その不活化ウイルスをワクチンとして用いている。

模，1965年）があるが，日本での調査は不十分である。ベトナムでポリオ様急性弛緩性麻痺を起こした可能性が報告されている[6]。歴史的にみれば現行の日本脳炎ワクチンは有効ワクチンとして問題はないと思えるが，科学的調査の不足している状況が結論に至らないもどかしい状況を作っている。後述するように，副反応についての問題が生じている。歩行困難や手足のしびれ，痙攣，てんかんなどの後遺症をもたらす急性散在性脳脊髄炎（ADEM）に罹患した子どもが日本脳炎ワクチン接種の影響で発症した可能性がある，と報告されている（図1）。日本脳炎ワクチンの原材料が感染マウス脳由来であることから，それに関連した副反応の可能性があるとされたのである。こうした状況の中で，2005年5月に厚生労働省による「日本脳炎ワクチン接種の積極的勧奨の差し控え」が出された。ワクチンの有効性と安全性については大変重要な問題である。それを議論するためにも日本脳炎およびウイルスについての基礎的な知識の共有が必要であろう。

Ⅲ　日本脳炎の歴史と現状

日本脳炎は不顕性感染が多いのであるが，感染して発症すれば高熱が続き，頭痛，嘔吐，意識障害が生じる。悪化すれば痙攣，神経麻痺等の脳炎・神経症状が出て死に至る。死亡率は20～40%と高い。日本脳炎発症は歴史的には古く，江戸時代から日本で感染者が出ていたが，患者は明治時代から増加しだした。それは水田耕作の増加や増幅宿主動物となる豚の飼育がさかんになりJEV媒介蚊コガタアカイエカ（*Culex tritaeniorhynchus*）の生息数が増えたことにもよるようだ[7,8]。1924年には6,000人以上が感染発症した。これまでに分離された中で最も古いウイルス株のJEV-中山株は1935年に患者の脳から得られた。その後も流行は続き，4,757人の感染者が出た1948年の大流行以降60年代後半まで年間数千人規模の感染者数が20年間も連続した。その後，衛生環境の改善，媒介蚊の生息数の減少，ワクチンの効果等により急激に感染者数が減少した。1972年の22人の患者数以降は2桁台で推移し，1992年以降現在に至るまで年間10人程度の患者発生に留まっている（図2）。ただし，最近の2，3年間は状況が変わってきている。これまで発生が長年なかった地域，例えば石川県等での発生（2002年）がみられたことは注意しなければならない[9]。

図2にみられるように，本邦における日本脳炎患者数は1992年以降は年間10人に満たない数で推移している。日本脳炎ウイルス（JEV）はいなくなったのだろうか？そうではないことは明白である。むしろ，世界的見地でみた場合，注意しなければならない状況にあると考えられる。患者数が

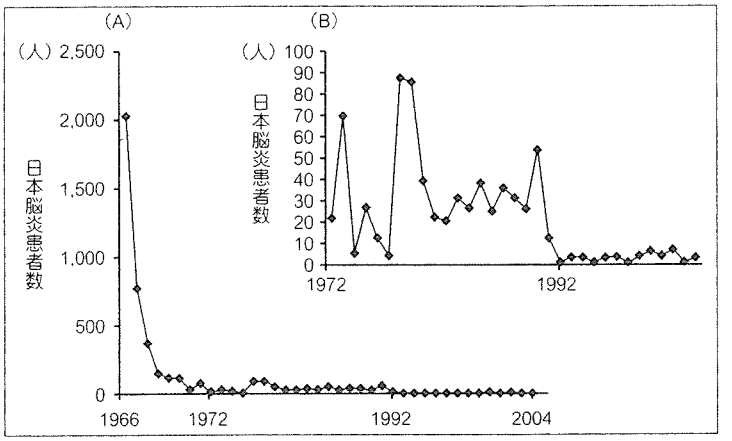

図2　日本脳炎患者数の変遷

（A）は1966年以降，（B）は1972年以降のデータを示している。

年間数万人に上る東南アジア地域，中国南部での流行は依然として継続しているが，それに加え発生地域の拡大がみられる。2005年のインドにおける日本脳炎の大流行がWHOより報告されている。これまでの感染報告がなかったオーストラリアにおける日本脳炎患者の発生（1995年）というのも注意しなければならない[7]。本邦にあっても，先に述べているように過去の歴史を紐解くと，長い間日本脳炎に悩まされていたことが分かる。これまでに大流行の時代があり，1950年には患者数は5,000人を超えていた。1960年代も日本脳炎の流行は続いていたが，1970年代に入ると激減した。その理由は二つ考えられる。一つにはワクチン接種効果がある。二つ目はJEV媒介蚊の生息数減少があり，ウイルス感染の機会が減少したことにもよると考えられている。いずれにしても，本邦における「日本脳炎感患者数が極端に減った」という事実は世界に誇れるものである。

しかし，今日ワクチンについて問題が生じている。日本脳炎ワクチンが不活化ワクチンであり，その材料が感染マウス脳由来である点，それに関連した副反応，さらに2005年5月に出された厚生労働省による「日本脳炎ワクチン接種の積極的勧奨の差し控え」。影響は大きい。他方で以下に述べるウイルス遺伝子型の変動があり，病原性が変化した可能性があると指摘されている[2, 10]。

Ⅳ　日本脳炎ウイルスについて：新たな遺伝子型ウイルスの出現

近年の遺伝子解析の進展によって，新たな視点からウイルスをみることが容易になってきた。JEVおよび近縁ウイルスはフラビウイルス科に総称されている。ワクチンを論議する上で，その対象であるウイルスそのものの性質について理解しておくことは重要である。ここでウイルスの分子生物学について述べる。

JEV遺伝子（ゲノム）は塩基数10,977個（JaGAr01株）のRNAから成り，10個の蛋白質をコードしている（図3）[11]。蛋白質をコードしていない領域（UTR）は5’端に95個，3’端に583個塩基を有している。フラビウイルス特有の遺伝子発現様式を持っている。ウイルス粒子を形成する蛋白質は3種類（C，M，E）のみであり，他の7種類の蛋白質は感染細胞中で主としてウイルスRNAを複製することに用いられている。このウイルスRNA複製時における3’末端UTRの役割は未だ不明であり，機能が注目されている[7, 10, 12]。自らの蛋白質を合成する時はリボソーム，翻訳因子等の宿主の蛋白合成装置を利用している。ただし，最初に大きな蛋白質（ポリ蛋白質）を合成し，それを自ら保有の蛋白分解酵素（非構造蛋白質NS3）で切断（プロセッシング）して各蛋白を生成する点はインフルエンザ等のウイルスとは異なる。最終的にはゲノムRNAにコア（C）蛋

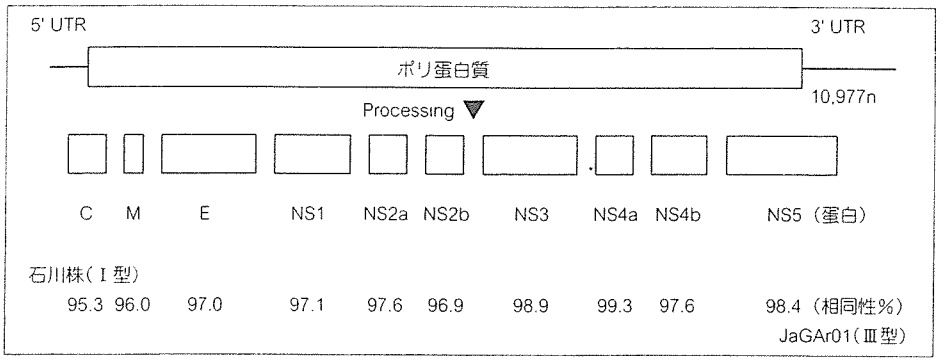

図3　日本脳炎ウイルスのゲノムと蛋白

数字は各ウイルス蛋白における JaGAr01 株（遺伝子型Ⅲ）と石川株（遺伝子型Ⅰ）のアミノ酸レベルでの相同性を示している。

白，M蛋白さらにエンベロープ（E）蛋白が集合し，ウイルス粒子となり細胞外へ放出される。

ウイルス感染時に最も重要な役割を果たすのはE蛋白である。ワクチンを接種して得られる抗体は主にウイルス粒子表面に存在するE蛋白に結合するもの（E抗体）が多い。E抗体は結合その他で作用し，ウイルスを不活性にする。単クローン抗体による解析ではE蛋白に結合することはないが，細胞表面でのウイルス吸着を阻害するという方式で作用する感染阻止抗体の存在が知られるようになった[13]。

ここで近年の日本に新しいウイルスの出現があることに注目しなければならない。1994年に石川県の豚の白血球から分離されたJEV分離株の石川株は遺伝子解析の結果，これまでのウイルス株とは異なっていた。遺伝子型はⅢ型ではなく，Ⅰ型であった[2, 10]。この遺伝子型とは遺伝子解析の結果から提示されているものである。ウイルスゲノム全長解析およびE蛋白領域の遺伝子解析を基にした解析結果をみると，これまでの日本，韓国等での分布ウイルスは遺伝子型Ⅲに属するものであることが分かった。中山株（1935年分離），北京株（1949），JaGAr01株（1959）等の代表株は勿論であるが，日本での分離株全てがⅢ型の遺伝子型であった。新たに分離された石川株ゲノムはJaGAr01株と比較すると相同性は低くなっており，またE蛋白で比べてみても日本での分離株よりもタイでの分離株（ThCMAr）に近縁であることが分かった（図3）[2]。もっとも1998年に石川県での野外蚊より得られた分離株のU1はこれまでの日本での分離ウイルス群と同じく遺伝子型はⅢ型であった[10]。この事実は同一地域において異なる遺伝子型ウイルスが混じって分布していることを示している。最近の分離株の遺伝子解析の結果を合わせると，どうやら1990年代からJEVの分布状況に変動が起こっているようである。なお，新分離株（Ⅰ型）の多くが3' UTR領域において10個以上のヌクレオチドの欠損がみられることは生物活性との関連で注目される[7, 12]。

Ⅴ　新たな問題：ウエストナイルウイルスの侵入に対して

新たな遺伝子型のJEV出現に加え，さらに大きな問題が出現している。ウエストナイルウイルス（WNV）の日本への侵入の問題だ。1999年にニューヨークに突如出現したこのWNVはまたたく間にアメリカ全土に拡大し，2002年には西海岸にまで到達した。いずれ日本に入ってくると恐れられている。水際で食い止めようと空港等での媒介蚊のチェックが行われている。アメリカでは，日本脳炎ワクチンにWNVに対する防御効果が若干あるのではないかと議論されている。

Ⅵ　日本脳炎ワクチンの臨床的有効性

では，日本脳炎発症に対する実際のワクチンによる感染防御効果はどうなのか？有効性および有用性について検討してみる。ワクチン接種は3～4歳での初回免疫2回，追加免疫1回，9～12歳

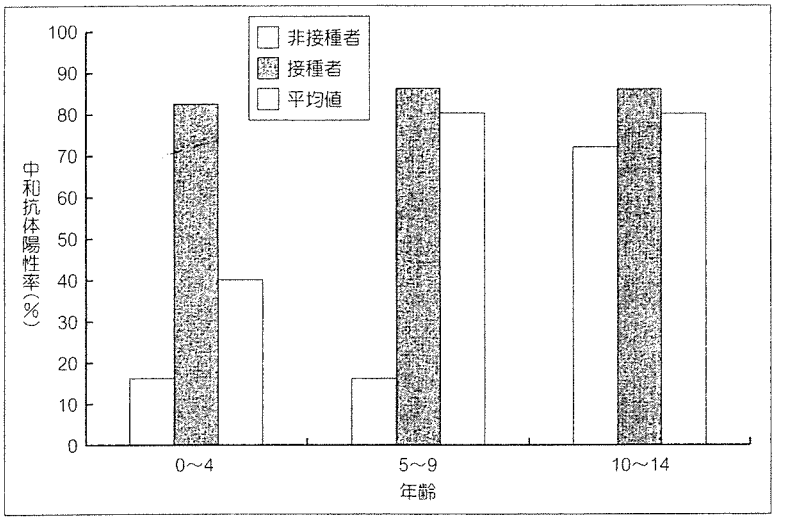

図4　ワクチン接種による抗体陽性率
2000年における調査では，14歳以下でのJEV中和抗体価はワクチン接種者の方が非接種者より顕著に高い。

での追加免疫，さらに14～15歳の追加免疫で行われている。2000年における調査では，14歳以下でのJEV中和抗体価はワクチン接種者の方が非接種者より顕著に高い（図4）[14]。ただ，それがどれほどの感染防御に働いているかは分からないが，歴史的にみれば有効といえる。1965年の台湾での調査（26万人規模）では防御効果が示されている。動物実験では明らかにJEV抗体価が高ければ，ウイルス感染を防ぐという結果が多数得られている。なお，現行ワクチン原材料は1980年以降JEV北京株に変更されており，それ以前に使用されていた中山株より接種量を減らしているがワクチン効果には差はないとされている。しかし，前述したように日本において新たに分布拡大している異なる遺伝子型ウイルスに対する防御効果については検討を加えなければならない，という問題が残る。

Ⅶ　副反応の問題

安全性についてはどうか。日本脳炎ワクチン接種後の反応として軽い発熱，頭痛，発疹，倦怠感等があるが，たいていは2～3日で消失する。神経障害に至る例は稀である。2004年の報告書によればワクチン接種によって軽い発熱が起こる例は幼児で9％，9歳以降の追加免疫では1～3％であった[15]。痙攣症状が接種幼児の0.06％に起こっているという事実には注意しなければならない。日本脳炎ワクチンと脳神経系の病気であるADEMとの関連性が指摘されている。厚労省の報告によれば，1989年から2005年までに日本脳炎ワクチン接種によると考えられるADEMが14例あったとしている（ちなみにこの間の日本脳炎患者は147人で死者数は20人である）。その原因としては微量のマウス脳成分がアレルギー反応を起こしたのではないかと推定されている。確率的には70～200万回接種に1回程度起こる，とされる。しかしながら，実際のところ因果関係は明らかではない。

Ⅷ　今後のワクチン

脳成分の混入という問題点を解消するために，培養細胞Veroを用いてウイルス増殖を行い，上清ウイルス液を集め，それをホルマリン不活化，ワクチン（組織培養ワクチン）作製しようとする試みが進んでいる[16, 17]。今のところ組織培養ワクチンによる中和抗体産生，感染防御効果については現行ワクチンと差異はないようだ。ただ，培養細胞を用いるときはウイルス病原性の変化には注意することが必要であろう。この組織培養ワクチンの実用化には数年がかかりそうであるので，それ

までのつなぎに現行ワクチンの活用は必要ではないか，と考える。他方で，本邦ではヒト以外を対象に生ワクチンの利用も進んでいる。特にJEVの増幅動物である豚についてはこうした感染予防は経済的にも重要な項目となる。現行不活化JEワクチンの問題点の一つに，高価で開発途上国では使えないというものがある。こうした点を解消するために生ワクチン開発が進められ，実際中国ではヒトに対して生ワクチン(SA14-14-2株)使用が行われている。

Ⅸ 新たなワクチン開発

異なる遺伝子型のJEVが存在する状況では，日本脳炎ワクチンについて改めて有効性と問題点について検討することが必要になっている。現行のワクチンについての問題点の一つにウイルス病原性変化への対応不十分が挙げられる。より安価に，かつ効果的ワクチンとしてDNAワクチンが名乗りを上げている[18,19]。黄熱ウイルスとJEVとのキメラワクチン（ChimeriVax-JE）についても検討が進んでいる[5]。DNAワクチンには基本的にJEVの構造蛋白のprM-M-E領域を含んでいるもの（通常は同領域をプラスミドに挿入して発現ベクターを構築）の方が効果的にJEV感染防御する。生体に導入する場合には金粒子にまぶしたりするとより効果が上がるようだ。今のところマウス実験で確認されているに過ぎないが，うまくいけば効果的ワクチンになる可能性がある。

Ⅹ 有効な抗ウイルス剤の開発

日本脳炎には有効な不活化ワクチンがあるが，熱帯地域で猛威を奮うデングウイルス，あるいはWNVなどに対するワクチンはない。従って，フラビウイルスに対する抗ウイルス剤の開発も重要である。最近，小さな2本鎖RNAがフラビウイルス感染増殖阻止に有効と報告され，注目を浴びている[20]。いわゆるRNAi（RNA interference）の活用である。今後の展開が楽しみである。

Ⅺ 今後の展開

先に述べたように，日本脳炎ウイルスJEVは常に身近な場所に存在していることを忘れてはならない。継続してウイルス存在状況を把握し，ウイルスの遺伝子，生物学的性状についての解析を続けていくことが重要である。ウイルスは生きている。病原性も変化している。JEV感染がオーストラリアで起こったように，またウエストナイルウイルス脳炎（ニューヨーク市から広がり，今ではアメリカ全土で流行）の場合のように，環境変化に対応して新たなウイルス侵入もありうる。鳥に対するWNVの毒性が変化したという事実は示唆的である。JEVの場合には神経毒性について注意しなければならない。C型肝炎ウイルスで知られるようなフラビウイルス長期的感染，持続感染の問題も今後生じるかもしれない。こうした状況の中で抗ウイルス剤の開発も必要であるが，ウイルス感染制御の第一義的役割はワクチンにある。副反応の問題はあるが，流行すれば致死率30%の感染症，危険性の高い日本脳炎に対してもっと注意を向ける必要がある。その意味で現段階では「日本脳炎ワクチン接種の積極的勧奨の差し控え」には疑問を感じる。現在，世界的に広がりをみせるJEV感染を防御するためにも安全で，かつ安価で効果的なワクチンが期待される。

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Japanese encephalitis virus RNA synthesis *in vivo* and *in vitro*

Tsutomu Takegami

Abstract: In order to investigate the mechanism of Japanese encephalitis virus (JEV) RNA replication, viral RNA syntheses *in vivo* and *in vitro* were analyzed. First, an appearance of viral specific RNA in JEV-infected cells was examined by northern blot analysis. Minus RNA-probe recognized a negative strand of JEV-specific RNA synthesized in JEV-infected cells as early as 6 hrs post infection (p.i.). Full length genomic 42S positive RNAs were detected in the cells at 12 hrs p.i. Relative amounts of the 42 S positive RNA was much larger in the membrane fraction than the supernatant fraction of JEV-infected cells. To investigate the mechanism of RNA synthesis, it is important to establish the *in vitro* system of RNA synthesis. It was found that JEV specific positive RNAs were efficiently synthesized *in vitro* in the crude membrane and nuclear fractions prepared from the JEV-infected cells. The analyses by SDS-PAGE and immunofluorescence assay indicated that nonstructural proteins NS3 and NS5, considered to be RNA helicase, protease and RNA polymerase, respectively, were membrane associated proteins, even though they were hydrophilic proteins. Maybe other NS proteins, including NS4a and 4b, are responsible to the membrane association, because of the hydrophobicity. The data that monospecific antisera against NS3 and NS5 inhibited *in vitro* RNA synthesis indicate that those proteins strongly contributed for viral RNA synthesis in the JEV-infected cells.

Key Words : Japanese encephalitis virus, RNA replication, *in vitro* system

Introduction

Japanese encephalitis (JE) is an acute viral infection of the central nervous system in humans, with an estimation of 50,000 cases of encephalitis and 10,000 death annually in the world (1-3). Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis in Asia. Even though recently JE cases in Japan are less than 10 in each year, still JE viruses circulate in wide area including Ishikawa (4). The genome of JEV, a member of the Flaviviridae, is a single-stranded, positive sense RNA ca 11kb in length which has no poly A at the 3'untranslated region (UTR) (5). Several process of JEV-RNA synthesis in the infected cells is unknown, although some studies related to this problem have been reported (6-8). Generally, it is thought that a negative stranded RNA must be synthesized in the early stage of JEV reproduction in cells, similarly to the early events of other positive RNA virus infections. The negative strand copied from positive genomic RNA leads to the formation of double-stranded replicative form (RF), which may have recycling role as a template (6). In order to examine the early events during JEV-RNA replication, it is essential to detect and distinguish the negative and positive strand.

The entire nucleotide sequence of JEV genomic RNA has been determined (9-11). It is suggested that JEV genomic RNA has a specific secondary structure at the 3' end, and this structure plays a significant role in the RNA replication of JEV and other flaviviruses (5-6,11-12). Sequencing data indicate that the genome organization of several flaviviruses is very similar to each other, though not identical (6), and suggest that these viruses have common features in their mechanism of RNA replication. The functions of nonstructural proteins including NS3 and NS5 in the replication complex (RC) is gradually clarified but not enough. Here we report the feature of JEV-RNA synthesis in JEV-infected cells and *in vitro* system using membrane fraction derived from JEV-infected cells.

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Materials and Methods

Cells, Viruses and RNA:

Mosquito cells C6/36 and Vero cells were cultured in the medium MEM containing 5-10% fetal bovine serum. Those cells were infected with JEV, JaGAr01 or Nakayama strains. Culture fluids were taken at proper time post-infection (p.i.) and subjected to the virus titration. Virus titer was estimated by plaque forming using confluent monolayer of BHK cells, as previously described (13). JEV virion were prepared from culture fluids by ultra-centrifugation method using discontinuous density gradient containing 10-40% sucrose. Viral RNA was extracted from virions with SDS-phenol/chroloform and precipitated with ethanol. To prepare RNA from JEV-infected cells, cells were washed with phosphate buffered saline (PBS) and harvested by the addition of 50 mM Tris buffer (pH7.5) containing 0.5% SDS and 1 mM EDTA.

Preparation of cell extracts

To obtain subcellular fractions, infected cells were dissolved in Tris buffer (pH7.5) containing 1.5 mM $MgCl_2$ and 10 mM KCl, and disrupted by 20 strokes in a Dounce homogenizer. Cellular homogenates were subjected to successive centrifugations, i.e. 500 x g for 10 min and then 10,000 x g for 30 min (7). The resulting pellets, nuclear and membrane fractions, were resuspended in 50 mM Tris buffer (pH8.0) containing 1.5 mM $MgCl_2$ and 50 mM KCl, and used for *in vitro* assay reactions.

Procedure of hybridization including Northern blot

Plasmid pJB11 has JEV cDNA sequences corresponding to the 3' end of JEV genomic RNA (9). DNA fragment at nucleotides 9183 to 10883 was prepared from pJB11, and inserted into pSPT18 and pSPT19, and then constructed new plasmids pJT18V and pJT19V (8). Each plasmid was then digested with restriction enzymes to prepare fragments. RNA probes were prepared through the RNA synthesis reaction carried out in the presence of T7 RNA polymerase (Toyobo, Tokyo) and α-^{32}P-UTP. The JT18V and JT19V probes could detect intracellular viral specific negative-sense RNA and positive-sense JEV-RNA including virion RNA, respectively.

For northern blot analysis, sample RNAs were denatured at 50℃ for 60 min in the presence of 1 M glyoxal and 50% DMSO and then were electrophoresed on 1% agarose gel in 10 mM phosphate buffer (pH7.0) which was recirculated continuously. Molecular weight standards were 23S (3.3 kb) and 16S (1.7 kb) *E. coli* rRNAs. RNAs were transferred to a nylon membrane (Pall, USA) with 20 x SSC (1xSSC:0.15M NaCl and 0.015M sodium citrate) and baked. The baked nylon membrane was treated with prehybridization buffer and then hybridized with ^{32}P labeled RNA probe at 42℃ for 16 hr. After hybridization, the nylon membrane was washed repeatedly with 2 x SSC containing 0.1% SDS. Slot hybridization was performed as similar protocol as described above. The dried filters were exposed to Fuji RX films.

In vitro assay of RNA synthesis.

In vitro RNA synthesis was carried out in 30 μl reaction mixture containing 50 mM HEPES (pH8.0), 3.5 mM MgCl2, 50 mM KCl, 5 mM DTT, 10 μg/ml actinomycin-D (Act-D), 0.7% NP40, 0.5 mM each of ATP, CTP and GTP, 10 μCi α-^{32}P-UTP and subcellular fractions (ca 5 μg protein). After an incubation at 30℃ for 60 min, 120 μl of 0.5% SDS solution was added, followed quickly by 150 μl of phenol. The RNAs in aqueous phase were precipitated with ethanol. To detect *in vitro* RNA product, 1% native agarose gels were used. Electrophoresis was carried out in TBE buffer (90 mM Tris-borate (pH 8.0) and 2 mM EDTA). To identify ds- and ss-RNA, RNAs were treated with 2M LiCl and fractionated by a centrifugation after the overnight-incubation. In ultra-centrifugation using 15-30% sucrose density gradient, ultra-centrifuge (Beckman) was used. The dried gels were exposed to Fuji RX films.

Labeling of protein with ^{35}S methionine

In a long labeling experiment, cells were pretreated with 1 μg/ml Act-D. After 4-hr pretreatment, cells were infected with JEV and added with 5 μCi/ml of ^{35}S-methionine (Met), and then cultured for further 24 hr and harvested. Subcellular fractions were prepared by centrifugation and subjected to SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE) (7). The gels were exposed to X ray films.

Indirect immunofluorescence antibody assay (IFA)

For the IFA, JEV-infected Vero cells were fixed with acetone and incubated with specific antisera, as previously described (14). The specific antisera including anti-E, anti-NS3 and anti-NS5 were prepared from rabbits which were immunized with JEV proteins purified by the method using SDS-PAGE (14-15). After incubation with FITC conjugated goat anti-rabbit IgG, the cells were observed by a fluorescence microscope.

Results

Viral RNA in JEV-infected cells

JEV specific RNA in the infected cells was detected by hybridization using RNA probes. In order to examine the size of JEV-specific in the infected cells, RNA samples were subjected to glyoxal gel electrophoresis. The JT19V probe could detect positive-sense JEV-RNA including virion RNA, while the proves JT18V recognized only intracellular viral

specific negative-sense RNA. Since 2 kinds of genome sized RNA (42S) were detected in the hybridization with ^{32}P-plus (JT19V) and ^{32}P-minus probes (JT18V), we used both probes to examine viral RNAs in the infected cells (Fig.1). By using the minus probes, it was elucidated that the negative stranded viral RNA (42S) was synthesized in the early stage of infection. As shown in Fig. 1B, the amounts of

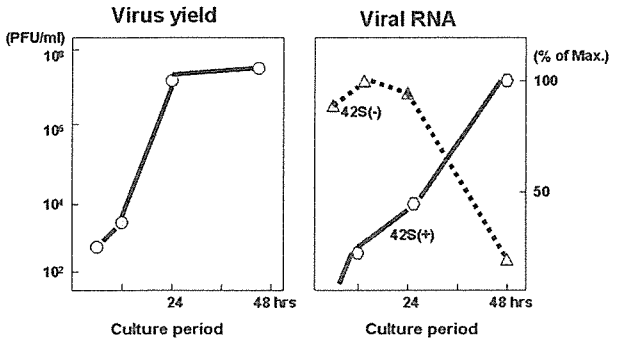

Fig. 1. Virus reproduction and appearance of JEV-specific RNAs in the infected cells.
(Left) JEV growth in C6/36 cells. Cells were infected with 10 m.o.i. of JEV and virus titers were assayed by plaque forming method.
(Right) RNAs were prepared from JEV-infected C6/36 cells at different times after virus infection. After glyoxal gel electrophoresis, RNA samples were transferred to nylon membrane and hybridized first with plus, ^{32}P-JT19V and second with minus probes, ^{32}P-JT18V. The amount of 42S vRNA were estimated and relative amounts are shown.

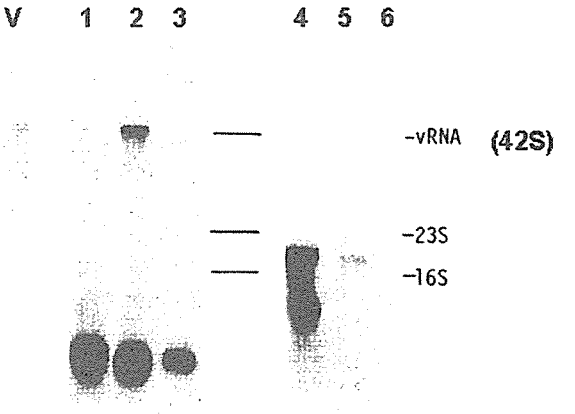

Fig. 2. Northern blot analysis of JEV-specific RNAs from the infected cells.
RNAs were extracted from virions or from the subcellular fractions of JEV-infected cells (48 hr p.i.) and electrophoresed using glyoxal gel, as described in the text. Lane V, JEV-vRNA. Lanes show RNAs from supernatant (lanes 1,4), crude membrane (lanes 2,5) and nuclear fractions (lanes 3, 6), respectively. After the transfer, hybridization was carried out by using RNA probes as described in the legend to Fig.1. Lanes V, 1-3 hybridized with JT19V. Lanes 4-6, JT18V.

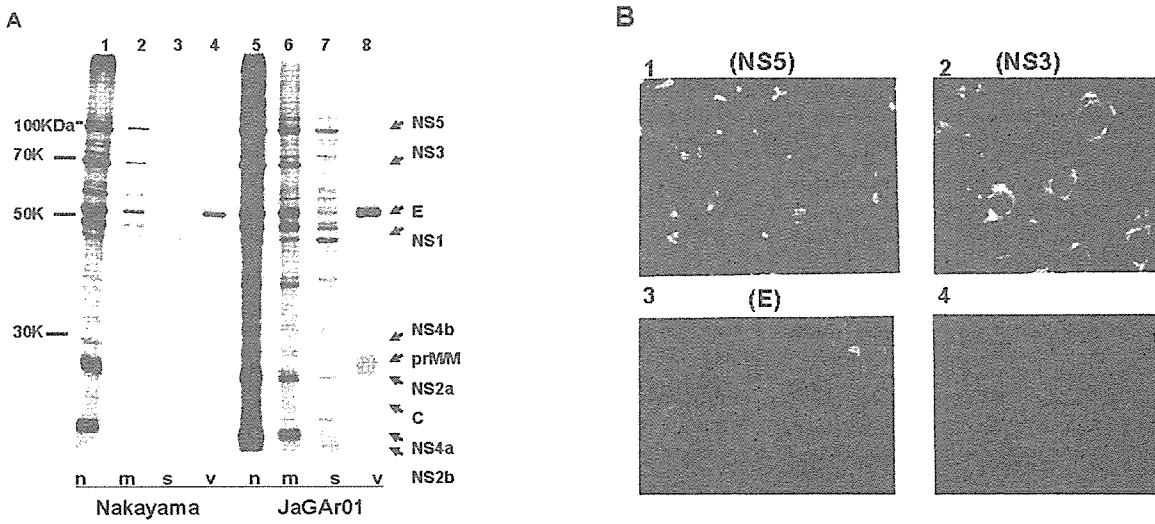

Fig. 3. Localization of viral proteins in JEV-infected cells.
A: JEV-infected cells labeled with ^{35}S-Met for 2hr were disrupted and fractionated into subcellular fractions including nuclear (lanes 1, 5), crude membrane (lanes 2, 6) and supernatant fractions (lanes 3, 7) by the centrifugation. Here we used 2 kinds of JEV strains for analysis. Lanes 4 and 8 indicate the partially purified JEV virion.
B: Indirect immunofluorescence assay (IFA) of JEV-infected cells.
Feature of JEV-proteins NS5, NS3 and E were shown in panels 1, 2 and 3. Panel 4 indicates IFA using preimmune rabbit sera.

negative stranded viral RNA reached a maximum level around 12 hr p.i. and then decreased. Appearance of the positive RNA (42S), however, was delayed, and it was obviously detected as 12 hr p.i. and then increased in amounts gradually. The accumulation of 42 S (+) RNA was similar to that of the virus growth curve assayed by plaque forming methods, as shown in Fig1.

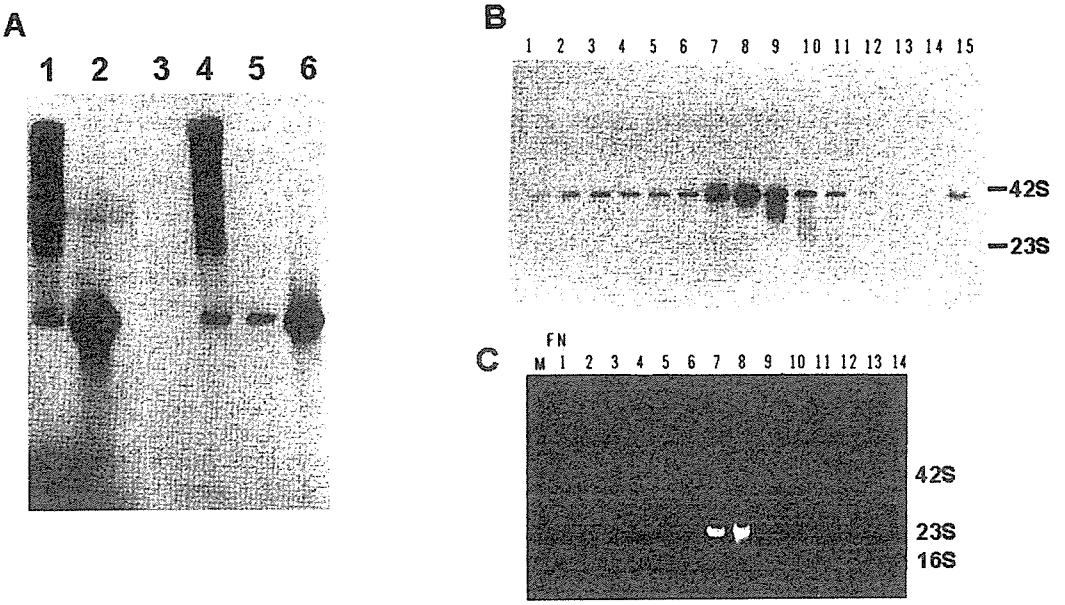

Fig. 4. Electrophoretic analysis of RNA products synthesized *in vitro*.
A: RNA synthesis *in vitro* using the crude membrane fraction were carried out in the absence (lane 1) or presence (lane 2) of NP40, and then ^{32}P RNA products were analyzed on the solubility in 2M LiCl. Lanes 3 and 5 were insoluble, and lanes 4 and 6 were soluble fractions.
B: ^{32}P RNA products were fractionated by the centrifugation using 15-30% sucrose density gradient.
C: *E.coli* ribosomal RNA was used as size marker in the fractionation by the ultra-centrifugation. 42S genomic RNAs were fractionated in the same fraction as *E. coli* rRNA by the ultra-centrifugation, since they formed duplex.

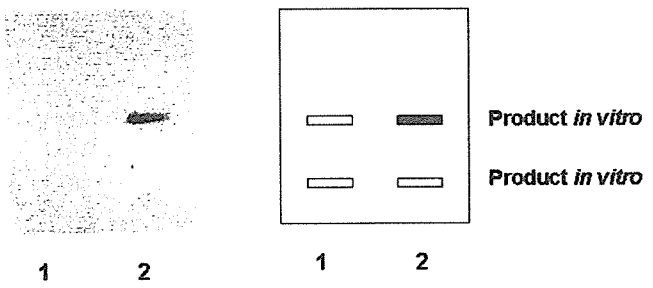

Fig. 5. Northern blot analysis of ^{32}P RNA *in vitro* products.
To determine polarity of ^{32}P RNA *in vitro* products, slot hybridization method was carried out. In the hybridization, both RNA fragments, JT18V (lane 1) and JT19V (lane 2) unlabeled were reacted with ^{32}P-RNA *in vitro* products used as probe. RNA fragment in upper side of figure contains 2 times higher than that of bottom.

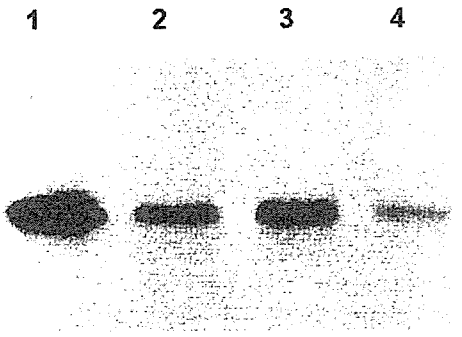

Fig. 6. Effect of antisera, anti- NS3 and -NS5 on *in vitro* RNA synthesis.
Preincubation of the crude membrane fraction and antisera was carried out prior to the addition of α-^{32}P-UTP. The procedure for *in vitro* reaction was the same as described in the text. Lane 1 (control); Products *in vitro* by treatment with preimmune sera at dilution 1:5. Lanes 2-3; treatment with anti-NS5 at dilution of 1:5 and 1:10, respectively. Lane 4; treatment with anti-NS3 at dilution of 1:5.

Localization of JEV-specific RNA in the infected cells.

To examine the localization of JEV-specific RNA in the cells, RNAs prepared from each of the subcellular fractions were subjected to Northern blot analysis. Fig. 2 shows the data of the hybridization using JT18V (right) and 19V (left) probes, respectively. In this experiment, the same sample filter was used and hybridized with both plus and minus probes. The 42S (+) RNA was abundantly in the crude membrane fraction (Fig. 2, lane 2). On the other hand, the amounts of the 42 S (-) RNA in the membrane fraction was almost same as those in the supernatant fraction (Fig.2, lanes 4 and 5). A large amount of small RNAs was observed in plus and minus hybridization shown in Fig. 2. Positive small 10S RNA was now clarified to contain the fragment at 3' end of genomic RNA (16) (data not shown).

Distribution of JEV-specific proteins in infected cells

To clarify the function of viral proteins, it is important to know the distribution of proteins in the cells. Fig.3A indicates the localization of viral proteins of 2 kinds of JEV strains (Nakayama and JaGAr01) labeled with ^{35}S-Met. Most of viral proteins were detected in nuclear and membrane fractions (Fig.3A, lanes 1, 2, 5 and 6). This nuclear fraction means not only nucleus but also nuclear membrane associated fraction. RNA dependent RNA polymerase (replicase) (NS5, 100KDa) and RNA helicase (NS3, 70KDa) were detected in the nuclear and membrane fractions. IFA indicated that both NS5 and NS3 were localized perinuclear fraction (Fig.3). NS5 appears to form some kinds of aggregates at the perinuclear site of the infected cells, as shown in Fig.3B, panel 1. NS3 also indicated the similar aggregates (Fig.3B, panel 2), which was different from the case of the distribution of E protein (Fig.3B, panel 3). E proteins were dispersed in the cytoplasm.

Viral RNA synthesis in vitro

In order to investigate viral RNA synthesis, it is useful to develop *in vitro* reaction system. By using crude membrane fractions from JEV-infected cells, we developed viral RNA synthesis system *in vitro*. As shown in Fig. 4, RNA products were clearly detected in cell-free fraction from JEV-infected cells at 48 hr p.i. In the presence of 0.7% NP-40 (Fig.4A, lane 2), larger quantities of RNA were synthesized. Next, RNA products were analyzed on solubility in LiCl, to identify ds- or ss-RNA. In the presence of 2M LiCl, most of RNA products were in the LiCl-soluble fraction (lanes 4 and 6), meaning dsRNA. By the ultra-centrifugation using sucrose density gradient 15-30%, products were fractionated at the position about 20S (Fig.4B and 4C). These indicate that *in vitro* RNA products are mainly of ds-RNA, may be replication form (RF). Next, in order to identify whether RNA product is positive RNA or negative, hybridization method using same probes JT18V (Fig.5, lane 1) and 19V (lane 2) was performed. Only JT19V probe could hybridized with ^{32}P-products *in vitro*, meaning that the products are positive strand (Fig.5).

Viral proteins influencing in vitro reaction of RNA synthesis

Viral proteins NS3 and NS5 are thought as helicase and RNA-dependent RNA polymerase, respectively and the important members of replication complex. In order to know the functions of NS3 and NS5 in viral RNA synthesis, we examined the effect of antisera, anti-NS3 and anti-NS5 on RNA synthesis *in vitro*. In the presence of antisera, anti-NS3 and anti-NS5, RNA synthesis level *in vitro* was decreased (Fig.6). The inhibitory effect of anti-NS3 on RNA syntheis was much more remarkable (Fig.6, lane 4). This was confirmed by repeated experiments.

Discussion

As described in the result, JEV-RNAs in the infected cells were clearly detected by the hybridization using the specific probes. The data indicated that the negative stranded JEV-RNA was synthesized before 6hr p.i. It is reasonable that the negative stranded RNA appears earlier than the positive stranded RNA during JEV infection. The facts that the positive JEV-RNAs (42S) were accumulated in the membrane fraction in the infected cells and it differed from the distribution of the negative RNAs suggest that the positive RNAs are continuously synthesized at the membrane, maybe endoplasmic reticulum (ER) and outer nuclear membrane during viral replication, as reported else about flavivirus replication (6). This fact seems to be related with the data of JEV-RNA synthesis *in vitro* that the membrane fractions have a high activity of RNA (mainly positive-stranded) synthesis (Fig.4).

Although several reports have been published describing flavivirus RNA synthesis, the mechanism of RNA synthesis is not yet fully understood (6-7,8,14). Here we described the *in vitro* RNA synthesis using the crude membrane fraction. The data indicated that RNAs produced *in vitro* were JEV specific, ds-RNA including positive strand labeled with ^{32}P. This *in vitro* RNA synthesis system, however, includes endogenous RNA template, so that most of the RNA polymerase reaction is probably a chain elongation. It is not obvious that the initiation occurs during the *in vitro* reaction (17).

The amounts of *in vitro* RNA products in the presence of non-ionic detergents such as NP40 and

Triton X 100 increased, and clear bands were detected in the agarose gel. It is thought that RNA synthesized *in vitro* formed duplex structure like replication intermediate (RI) or replication form (RF) under the native condition (6-7,18-19). Actually *in vitro* synthesized poliovirus RF (19) migrated slower than did genomic ssRNA in the native agarose gel. Most of products synthesized here, in the presence of NP40 seem to be RF, which migrates to the similar site with ssRNA (42S) in 1% native gel. The data that *in vitro* synthesized RNAs were in the LiCl-soluble fraction and fractionated at the similar site to rRNA of *E. coli* in the centrifugation also indicate that most of products are of dsRNA. The result that the clear band was observed in the agarose gel (Fig.4) is explainable as follows; It is likely that during the incubation in the presence of NP40, RI was degraded by some endogenous RNase and only RF was accumulated, since in the absence of NP40 some larger bands including RI were detected (Fig.4A).

It should be noted that the *in vitro* JEV-RNA synthetic activity was high in the crude membrane fraction including outer nuclear membrane. The study on West Nile virus RNA synthesis also showed that outer nuclear membrane contained RNA dependent RNA polymerase (20). Here, ^{35}S-labeling and immunofluorescence experiments indicated so much NS3 and NS5 proteins were accumulated in the crude nuclear and membrane fraction (Fig.3). It is obvious that NS3 is strongly associated with the nuclear and membrane fractions, although sequence data suggest that NS3 is hydrophilic and not a transmembrane protein (21). This phenomenon seems to be due to the function of hydrophobic and transmembrane protein NS4a/b (22). NS4 may work as an anchor for NS3. Actually we could see the NS4 band in the crude nuclear and membrane fractions as shown in the result (Fig.3A). Now it is well known that NS3 has protease activity at amino terminal region, and ATPase and RNA helicase in other region (21,23). Recently it has been reported that flavivirus (HCV) NS3 influences IFN pathway (24). Putative viral replication proteins NS3 and also NS5 actually work together in RNA replication (25), because anti-NS3 and anti-NS5 could inhibit RNA synthesis *in vitro* (Fig.6).

As previously described, even though recently JE cases in Japan are less than 10 in each year, still JE viruses circulate in wide area of Japan (4). In addition, it was found that recently isolated viruses including Ishikawa strains belonged to genotype I which differs from previous JEV strains (genotype III) (4,26). Now we need to be careful about the change in the pathogenesity of new genotype JEV. It is also necessary to develop new drug against JEV infection (27). To develop new drugs, it is important to elucidate the mechanism of virus reproduction including RNA replication.

Taken together the results here indicate that the membrane fractions have high activity for viral RNA synthesis, and contain a large amount of NS3, NS5 and other NS proteins which make the replication complex in the infected cells. In addition, we reported the sensitive probes for the detection of JEV specific RNAs. To know the mechanism of JEV-RNA synthesis, further study will be essential.

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Short communication

Activation of Rac1 by Rho-guanine nucleotide dissociation inhibitor-β with defective isoprenyl-binding pocket

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Abstract

Rho-guanine nucleotide dissociation inhibitor-β (RhoGDIβ), a regulator for Rho GTPases, is implicated in cancer cell progression. We reported that C-terminal truncated RhoGDIβ (ΔC(166–201)-RhoGDIβ) promoted metastasis through activating Rac1 signaling pathway in ras-transformed fibroblast cells. To better understand the mechanism of Rac1 activation by ΔC(166–201)-RhoGDIβ during metastasis, the amount of GTP-bound Rac1 was measured as the activation level of Rac1 in cells expressing various mutant RhoGDIβ with sequential C-terminal deletions. Three C-terminal hydrophobic amino acid residues (Trp191, Leu193, and Ile195) supposed to interact with isoprenyl groups of Rac1, was indispensable for a proper regulation of Rac1 activation/inhibition. Deletion of this region led RhoGDIβ to continuously associate with GTP-bound Rac1, provoking constitutive activation of Rac1. Thus, impaired interaction of RhoGDIβ with Rac1 isoprenyl groups possibly makes RhoGDIβ function as a positive regulator for Rac1 during metastasis.

Keywords: RhoGDI; Rac1; Prenylation; Metastasis

1. Introduction

Rho GTPases play crucial roles in various cellular events, such as cytoskeletal organization, cell polarity, gene transcription, cell cycle progression, microtubule dynamics, and vesicular transport (Etienne-Manneville and Hall, 2002). We had identified Rho-guanine nucleotide dissociation inhibitor-β (RhoGDIβ) lacking the C-terminal 36 amino acids (ΔC(166–201)-RhoGDIβ) as a metastasis-inducing gene (Tatsuka et al., 1997; Ota et al., 2004). In several reports, RhoGDIs are also implicated in the progression of cancer cells, however their roles are still controversial. RhoGDIα was upregulated in ovarian cancer (Jones et al., 2002), and breast cancer (Fritz et al., 2002). RhoGDIβ was also upregulated in ovarian cancer (Tapper et al., 2001) and in metastatic fibrosarcoma cells generated by overexpression of autocrine motility factor (Yanagawa et al., 2004). In contrast, downregulation of RhoGDIα was reported in breast cancer (Jiang et al., 2003) and expression of RhoGDIβ was inversely correlated with the invasive capacity in human bladder cancer cell lines (Seraj et al., 2000). Furthermore, RhoGDIβ was reported as an invasion and metastasis suppressor gene (Gildea et al., 2002). Although these observations collectively indicate that RhoGDIs regulate the metastatic processes, their roles are elusive. We reported earlier that the promotion of metastasis by ΔC(166–201)-RhoGDIβ was mediated by the activation of Rac1 signaling pathway (Ota et al., 2004). In this study, to better understand how RhoGDIβ functions in the regulation of metastasis, we examined which region of the 36 amino acid sequence was important for affecting the Rac1 signaling

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pathway. We found that the deletion of the C-terminal region containing Trp191, Leu193, and Ile195, hydrophobic residues supposed to participate in the interaction with isoprenyl group of Rac1 (Gosser et al., 1997; Hoffman et al., 2000; Grizot et al., 2001), was critical for the promotion of Rac1 activation and the association of ΔC-RhoGDIβ with GTP-bound Rac1. Our results indicate that the destruction of the interaction of RhoGDIβ with isoprenyl group on Rac1 makes RhoGDIβ function as a positive regulator for Rac1 during metastasis.

2. Materials and methods

2.1. Cells and culture

The 1-1ras1000 cell line, derived from BALB/c 3T3 A31-1-1 cells transfected with an activated c-Ha-ras oncogene (Tatsuka et al., 1996), was used for assay. In this cell line, the expression of ΔC(166–201)-RhoGDIβ induces metastatic phenotype by activating Rac1 signaling pathways (Ota et al., 2004). Cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Plasmids and transfection

To construct expression vectors for wild type and C-terminal truncated RhoGDIβ, the entire and truncated sequences were amplified by PCR using pcDNA3.1-LyGDI, which is an expression vector for wild type RhoGDIβ (Ota et al., 2004). The products were then subcloned into Xpress-tagged pcDNA3.1 expression vector. Cells were transfected with plasmids using LipofectAMINE™ plus (Invitrogen, Carlsbad, CA).

2.3. Rac activation assay

Pull-down assays for measuring the Rac activation were performed using Rac activation assay kits (Cytoskeleton Inc., Denver, CO) according to the manufacturer's instructions. The cells were washed with PBS and lysed on the dish in cell lysis buffer. Rac1-GTP was pulled down using the GST tagged PBD (p21 binding domain) of Pak1 (p21/Cdc42/Rac1-activated kinase 1) protein beads.

2.4. Immunoblotting

Samples were lysed with Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were then probed with a primary antibody, followed by a peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using ECL Plus reagents (Amersham Biosciences, Little Chalfont, UK). For densitometric analysis of immunoblots the films were scanned and the intensity of the bands was measured using the ImageJ program (NIH, Bethesda, MD, USA), and the Rac1 and Xpress-RhoGDIβ values in PBD-bound fraction were normalized with respect to those in total cell lysate.

2.5. Antibodies

Anti-Xpress, anti-α-tubulin (clone B-5-1-2), and anti-Rac1 (clone 102) antibodies were purchased from Invitrogen (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO), and BD Biosciences (San Jose, CA), respectively. Peroxidase-conjugated anti-mouse IgG was purchased from DakoCytomation (Glostrup, Denmark).

3. Results and discussion

We had reported that C-terminal truncated RhoGDIβ (ΔC(166–201)-RhoGDIβ) promoted the metastasis by activating Rac1 signaling pathways in *ras*-transformed fibroblast cells (1-1ras1000 cells) (Ota et al., 2004). In this report we attempted to define which region within the 36 residues deleted in ΔC(166–201)-RhoGDIβ was important for Rac signaling. Fig. 1A shows the amino acid sequences of

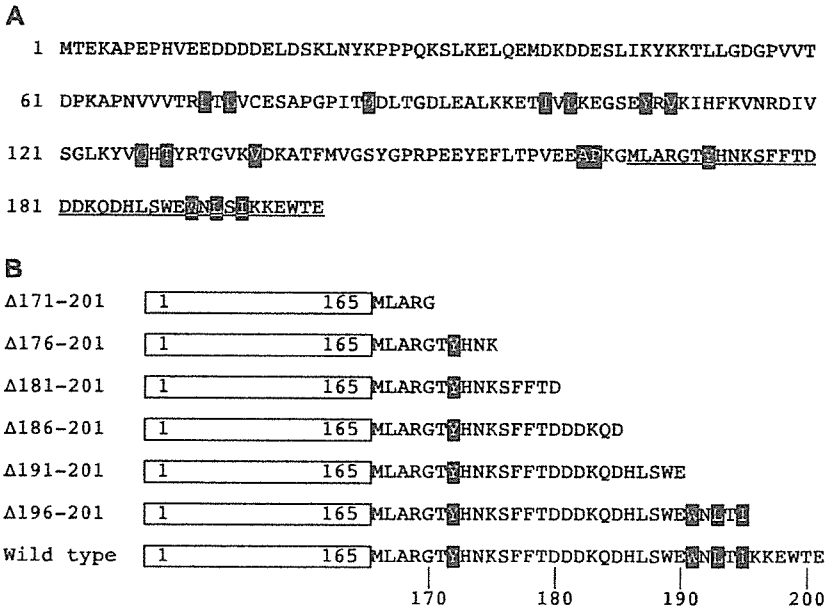

Fig. 1. Amino acid sequences of human RhoGDIβ. (A) Hydrophobic residues of RhoGDIβ corresponding to the residues of RhoGDIα that participate in the interaction with isoprenyl groups of Rho GTPases (Gosser et al., 1997; Hoffman et al., 2000; Grizot et al., 2001) are indicated as black boxes. Residues 166–201, which are deleted in metastasis-inducing ΔC-RhoGDIβ (Ota et al., 2004), are underlined. (B) A schematic representation of sequential C-terminal deleted RhoGDIβ. Four hydrophobic residues in 166–201 of RhoGDIβ proposed to participate in the formation of isoprenyl-binding pocket are indicated as black boxes.

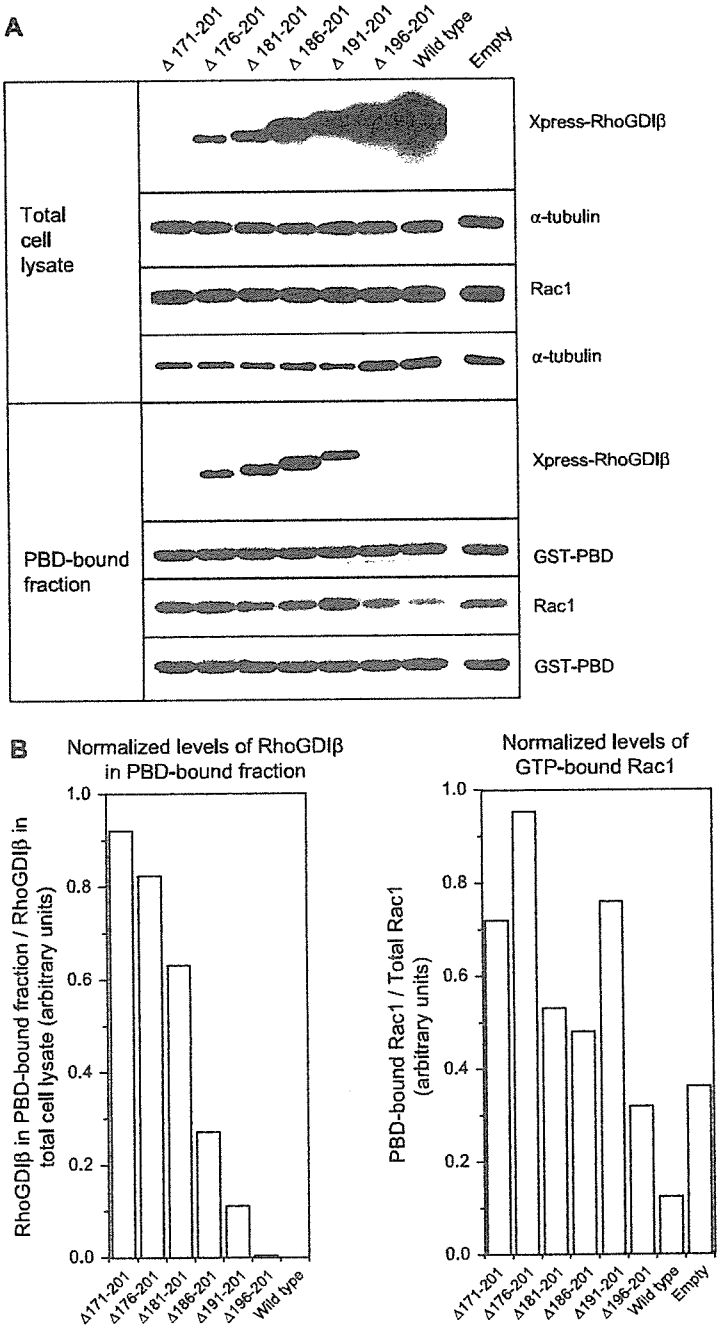

Fig. 2. Increase of GTP-bound Rac1 and association of ΔC-RhoGDIβ with GTP-bound Rac1 in cells expressing ΔC-RhoGDIβ. (A) Cells were transfected with Xpress-tagged pcDNA3.1 expression vectors of various ΔC-RhoGDIβ. After 48 h cells were lysed and Rac1-GTP was pulled down using the GST tagged PBD protein beads. Total cell lysate and PBD-bound fraction were immunoblotted with anti-Xpress antibody or anti-Rac1 antibody. As a loading control, α-tubulin was stained in total cell lysate and GST-PBD was stained with Coomassie brilliant blue in PBD-bound fraction. When Xpress-RhoGDIβ was overexpressed, an unidentified band smaller than expected size was observed in total cell lysate. The amount of this smaller band was less than 5% of major band. (B) The blots in Fig. 2A were analyzed by NIH ImageJ analysis software, and the values of Xpress-RhoGDIβ and Rac1 in PBD-bound fractions were normalized to those values in total cell lysate.

RhoGDIβ. In the deleted region of ΔC(166–201)-RhoGDIβ, four hydrophobic residues (Tyr172, Trp191, Leu193, and Ile195) are supposed to participate in the formation of an isoprenyl-binding pocket involved in interaction with isoprenyl groups of Rac1 (Gosser et al., 1997; Hoffman et al., 2000; Grizot et al., 2001). We constructed Xpress-tagged pcDNA3.1 expression vectors of various length for ΔC-RhoGDIβ with residues sequentially deleted from C-terminus (Fig. 1B). These expression vectors were transfected into the 1-1ras1000 cells and the amount of GTP-bound Rac1 were determined by pull-down assay using GST tagged PBD protein beads. Although the expression levels of introduced

genes varied among transfectants (Fig. 2A), cells expressing ΔC-RhoGDIβ lacking the three C-terminal hydrophobic amino acids Trp191, Leu193, and Ile195 (Δ171–201, Δ176–201, Δ181–201, Δ186–201, and Δ191–201) showed higher levels of GTP-bound Rac1 than empty control cells (Fig. 2A). Furthermore, ΔC-RhoGDIβ was detected in PBD-bound fraction, whereas wild type RhoGDIβ was not detected in PBD bound fraction despite its highest expression level (Fig. 2A). When the amounts of ΔC-RhoGDIβ in PBD-bound fraction were normalized to those in total cell lysate, ΔC(196–201)-RhoGDIβ, which retains C-terminal three hydrophobic amino acid residues, was detectible only at trace level (Fig. 2B). These results further support our previous observation that C-terminal truncated RhoGDIβ (ΔC(166–201)-RhoGDIβ) induces metastasis by constitutive activation of Rac1 and associates with GTP-bound Rac1 (Ota et al., 2004).

Wild type RhoGDIβ and ΔC(196–201)-RhoGDIβ, which retain the three hydrophobic amino acid residues at the C-terminal, could not increase the amount of GTP-bound Rac1 and yielded hardly detectible levels in the PBD-bound fraction (Fig. 2). These results clearly indicate that the absence of the C-terminal hydrophobic amino acids Trp191, Leu193, and Ile195, which are supposed to interact with isoprenyl groups of Rho GTPases, is crucial for ΔC-RhoGDIβ to express its phenotype. The isoprenyl group of Rho GTPase is not essential for the binding of Rho GTPases to RhoGDI (Lian et al., 2000; Faure and Dagher, 2001; Thapar et al., 2002). Indeed, we found that ΔC-RhoGDIβ lacking an intact isoprenyl binding-pocket could associate with GTP-bound Rac1 (Fig. 2). However, the interaction of the isoprenyl groups of Rho GTPases with RhoGDIs are important for the proper intracellular localization and functions of Rho GTPase (Hori et al., 1991; Regazzi et al., 1992; Michaelson et al., 2001; Sun and Barbieri, 2004; Wennerberg and Der, 2004). Therefore, RhoGDIβ defective in the isoprenyl binding pocket probably binds Rac1, but is expected to differ from wild type RhoGDIβ in the intracellular localization and function. Previously, we had shown that ΔC(166–201)-RhoGDIβ anchored Rac1 at the membrane to activate its effector molecules (Ota et al., 2004). In another report, a mutant RhoGDIα, defective in extracting the isoprenyl moiety of Cdc42 off the membrane through an isoprenyl-binding pocket, forms a complex with Cdc42 and retains it on the membrane (Sun and Barbieri, 2004). To explain the ability of RhoGDIα to release Cdc42 from membrane a two-step model has been proposed (Nomanbhoy et al., 1999; Hoffman et al., 2000). In this model, the N-terminal regulatory domain of RhoGDIα binds to Cdc42 on the membrane and subsequently the C-terminal isoprenyl-binding pocket of RhoGDIα extracts the membrane-buried isoprenyl moiety of Cdc42 from the membrane. Furthermore, it has been reported that RhoGDIα more efficiently interacted with GDP-bound Rac1 than with GTP-bound Rac1 (Sasaki et al., 1993) and the conversion of GTP-bound Rac1 to GDP-bound Rac1 was the rate-limiting step in the dissociation from membrane (Moissoglu et al., 2006). On the other hand, RhoGDIα can bind GTP-bound Rac1 and can inhibit GTP hydrolysis by Rac1. This interaction is suggested to be a mechanism that maintains Rac1 in an active form (Chuang et al., 1993). According to these findings, the function of ΔC-RhoGDIβ with the defective isoprenyl-binding pocket in our experiment should act as depicted in Fig. 3. GTP-bound Rac1 is converted to the GDP-bound form and then wild type RhoGDIβ binds GDP-bound Rac1 by an N-terminal regulatory domain and, through extraction of the hydrophobic,

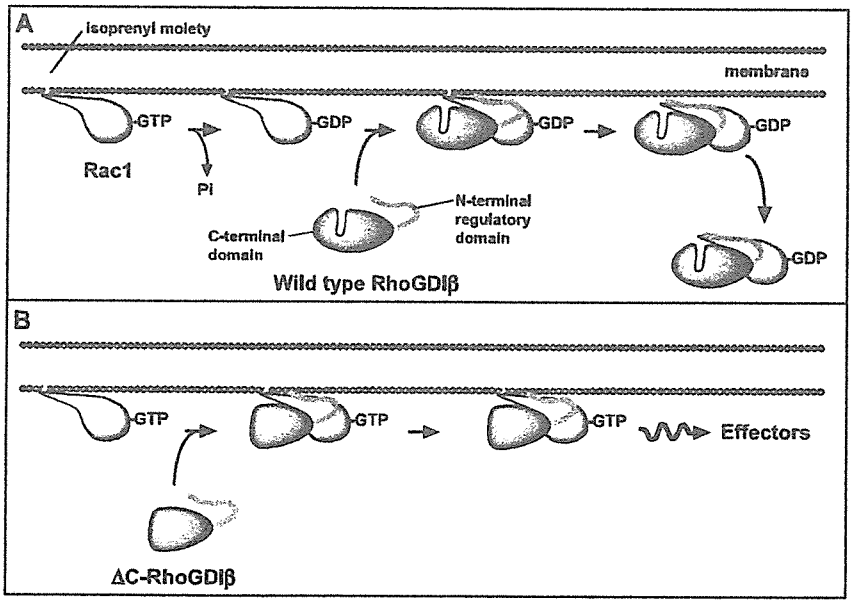

Fig. 3. Schematic drawing of proposed function of ΔC-RhoGDIβ. (A) GTP-bound Rac1 is converted to GDP-bound form and then the N-terminal regulatory domain of wild type RhoGDIβ binds GDP-bound Rac1 and the C-terminal isoprenyl-binding pocket of RhoGDIβ extracts the membrane-buried isoprenyl moiety of Rac1 from membrane, thereby releasing Rac1 from membrane. (B) ΔC-RhoGDIβ binds GTP-bound Rac1 by the N-terminal regulatory domain, but can not remove Rac1 from membrane due to the defect of the isoprenyl-binding pocket. Therefore, ΔC-RhoGDIβ remains attached to GTP-bound Rac1 on membrane and supports Rac1 activation of effectors.