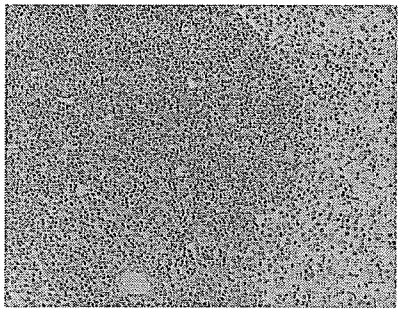
布する大型の細胞が稀に陽性であったが、KOではほぼ同じ領域に非常に多数のウイルス抗原強陽性の大型の細胞が認められた。抗原は肝細胞同様に細胞質が陽性となっていた。組織学的にはKOマウスでは白脾髄が萎縮していた。

A. WT



B. KO

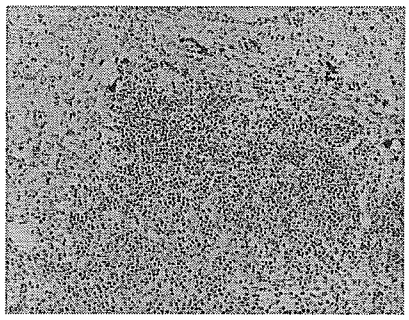
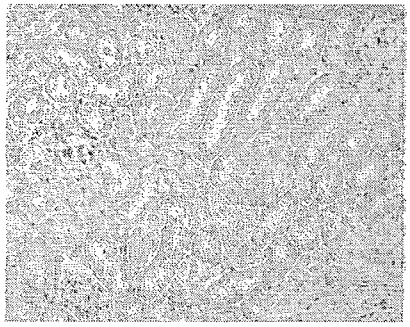


図2 ポリオウイルス経静脈性接種後の脾

A. WT



B. KO

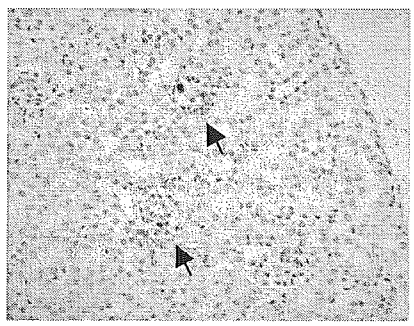


図3 ポリオウイルス経静脈性接種後の腎

脾の濾胞周辺帯に認められた抗原陽性の大型細胞の性状phenotypeについては現在検討を行っている。

腎においてはWTマウスでは抗原陽性細胞は認められず、またKOマウスでもウイルス接種量等が同条件では観察できなかったが、高ウイルス量を接種すると、KOマウスのみで糸球体内に抗原陽性細胞が確認された。

4. 肝細胞逸脱酵素の生化学的解析：上記の肝の組織学的変化を再確認するため、血清トランスアミナーゼを静脈内接種後3日目に測定した。 その結果、KOマウスでは他のマウスに比較し、酵素活性は3倍以上に増加しており、明らかな肝細胞の破壊が生じていることが確認された。

5. IFN-βならびに種々のISGの発現レベル：ウイルス感染後真っ先に発現してくるIFN-βとIFN応答の結果発現するOAS遺伝子群の発現を解析したところ、非感染マウスではいずれの組織でも殆ど発現していないが、ウイルスを接種したWTでは肝・脾等の非標的組織では接種後1日目にmRNA量が高値となるのに対し、標的組織のCNS（脳・脊髄）では接種後1日目では未だに低値であった。

6, 細胞内dsRNAセンサーRIG-I, MDA-5の発現レベル：2004年に米山・藤田らにより細胞質内に局在し、dsRNAを認識するRIG-Iが同定された。MDA-5も同様の機能を有し、さらにこの両遺伝子産物がdsRNAを認識するとIPSを介して、IRF-7等のISGが誘導され、最終的にI型IFNが産生される。そこで、このRIG-IとMDA-5の発現レベルを、非感染、接種1日、接種3日のWTマウス

で解析した。その結果、脾と腎、肺、心、肝では迅速な応答が接種後1日目で生じているのに対し、脳と脊髄では殆ど上昇が認められなかった。この結果はCNSでは感染初期にこのdsRNAセンサーが十分量発現しないことを明らかにしており、このことがCNSにおいて特定のRNAウイルスの増殖を拘束できない理由であることが示唆された。

C.考察

宿主体内に侵入したウイルスは自然免疫と獲得免疫の両者により対処される。これまでは獲得免疫が主として解析されてきたが、遺伝子組換えマウスの作出が容易となり、かつ自然免疫に関与する遺伝子が同定されるとともに、自然免疫が体内でウイルスの増殖にどのように関与しているかの解析が可能となった。

1994年ごろよりIfn-gamnaレセプター遺伝子あるいはIfn-alpha/betaレセプター遺伝子を欠損したマウスが作出され、これらのマウスを用いたウイルス感染実験が行われるようになった(Muller et al. 1994, Fiette et al. 1995)。これまでに複数のアルファウイルス、水疱性口内炎ウイルス、狂犬病ウイルス、フラビウイルス属のデングウイルスやウェストナイルウイルス、ピコルナウイルス科のタイラーウイルスが検討され、I型IFNが機能しないマウスにおいては本来病原性を発揮できない組織においてもウイルスが増殖できることが明らかとされた。

今回のわれわれの成果はこれらの結果と意味合いは違い、なぜ特定のRNAウイルスが神経系組織を標的とできるかを明らかにする目的でI型IFNが機能しないマウスの解析を中心に行った。その結果、これまで非標的と考えられていた組織においても微小の感染が成立するもののI型IFNが機能することにより感染は直ちに阻止される。しかし、ウイルスを産出する感染細胞はこれらの組織に存在するため、血中に放出されるウイルス量は増加してくる。これが臨床的にウイルス血症として把握される。最終的ウイルスがCNSに侵入し、ニューロンに感染する。しかし、ニューロンは非標的組織のような感染初期の迅速なIFN応答をすることができず、かつウイルス増殖が非常に早い時間で終了するため、殆ど対応できない。このようなことが可能なRNAウイルスが神経ウイルスの一つの範疇であると推察する。

E. 結論

CNSに感染するRNAウイルスの多くは罹患者の大部分は非顕性で、ごく少数の宿主において神経病変を形成し、脳炎・脊髄炎を引き起こす。この発症までにいたる潜伏期に宿主が応答できる免疫としては獲得性免疫よりも自然免疫が重要な役割を果たしていると想定されてきたが、今回の解析でI型インターフェロンが重要な役割を果たしていることが明らかにされた。このI型インターフェロン応答システムのどの遺伝子の脆弱性がヒト宿主における神経病変発症に関与しているか今後の解析が必要と考える。

F. 健康危機情報

なし

G. 研究発表

論文発表

1. Nishimura N, Yoshikawa T, Ozaki T, Sun H, Goshima F, Nishiyama Y, Asano Y, Kurata T, Iwasaki T. in vitro and in vivo analysis of human herpesvirus-6 U90 protein expression. J Med Virol 2005 Jan; 75:86-92
2. Miyachi S, Lu X, Inoue S, Iwasaki T, Koike S, Nambu A, Takada M. Organization of multisynaptic inputs from prefrontal cortex to primary motor cortex as revealed by retrograde transneuronal transport of rabies virus. J Neuroscience 2005

Mar 9; 25(10): 2547-56.
3. Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, Moriishi K, Iwasaki T, Mizumoto K, Matsuura Y, Miyamura T, Suzuki T. Molecular determinants for subcellular localization of hepatitis C virus core protein. J Virol 2005 Jan; 79 (2): 1271-1281
4. Ida-Hosonuma M, Iwasaki T, Yoshikawa T, Nagata N, Sato Y, Sata T, Yoneyama M, Fujita T, Taya C, Yonekawa H, Koike S. The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus. J Virol 2005; April 79 (7): 4460-9
5. Arita M, Shimizu H, Nagata N, Ami Y, Suzaki Y, Sata T, Iwasaki T, Miyamura T. Temperature sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys: Implication of temperature sensitivity as a determinant of attenuation. J Gen Virol. 2005 May; 86(Pt 5): 1391-401.
6. Jongwutiwes S ,Putaporntip C, Iwasaki T, Ferreira MU ,Kanbara H ,Hughes AL . Mitochondrial genome sequences support ancient population expansion in Plasmodium vivax. Mol Biol Evol. 2005 Aug;22(8):1733-9. Epub 2005 May 18.
7. Honda R, Ohno Y, Iwasaki T, Okudaira S, Okada M, Kamitamari A, Ohshima K, Moriuchi H, Kanematsu T. Langerhans' cell histiocytosis after living donor liver transplantation: report of a case. Liver Transpl. 2005 Nov;11(11):1435-8.

G. 知的所有権の取得状況

なし

厚生労働科学研究費補助金（新興・再興感染症研究事業）
分担研究報告書

野生げっ歯類及び節足動物に由来する感染症の診断、疫学及び予防に関する研究

分担研究項目：回帰熱の診断と疫学－回帰熱ボレリアの抗原解析

分担研究者　福長　将仁　福山大学薬学部　教授

研究要旨：回帰熱ボレリア抗原変換の主体を成す菌体表層蛋白（Vmp）遺伝子をクローン化、血清抗体による反応性を指標としてエピトープ解析を行った。抗原蛋白のアミノ酸一次配列に対応する５カ所のエピトープ領域を推定し、組換え遺伝子断片から発現した蛋白を作成して、それぞれ15アミノ酸にまで限定した。ついでペプチドを合成して抗原抗体反応を行うことによって、抗原エピトープをさらに６アミノ酸まで絞り込んだ。５カ所の抗原エピトープのうち、AGGIELの配列をもつ領域はほかのVmp蛋白でも高度に保存されており、共通抗原の候補としての可能性が示唆された。反応性がもっとも強いC末端の配列KAADQQではそれぞれの抗原蛋白において多様性が高い領域であった。

A. 研究目的

ダニ媒介性回帰熱（tick-borne relapsing fever, TBRF）は４類感染症に分類される人畜共通感染症で、ヒトのほかウシやトリに病原性を示す種もある。媒介節足動物は主にヒメダニ属ダニ（*Ornithodoros*）で、比較的乾燥した衛生状態不良な地域に流行する。感染はダニ刺咬により病原体ボレリア（*Borrelia duttonii*）が宿主体内に侵入しておこる。感染後ボレリアは血液中で増殖し、頭痛、筋肉痛、関節痛、咳などを伴って発熱がおこることが多い。発熱期は数日間続き、このときボレリアが血液中に検出されるが、発症に伴い病原体表層抗原に対する体液性免疫が働き、ボレリアは血中から消失、無熱期に入る。しかし４～10日後には再びボレリアが増殖して発熱期を迎える。この特徴的な発熱と解熱の回帰は、宿主の免疫機構による攻撃から逃れた一部のボレリアが抗原変換を起こし、再び血液中で増殖するためである。

ダニ媒介性回帰熱は、北米、中東アジア、ヨーロッパ南部やアフリカに分布している。我が国でも最近疑似患者が発生したが詳細については不明である。我々の調査地域であるタンザニア中部では、患者数はマラリアに次いで多く、特に感染者のほとんどは５歳以下の乳幼児である。発生動向としては、マラリアが雨期に集中しているのに対して、TBRFは年間を通して平均している。我々が行った

361名の乳幼児の血液検査では、有熱者の11%、健常者の4%がボレリアを保有していた。住居調査ではほとんどの住宅の土間、壁にダニが生息しており、夜間牛皮を敷いて床で就寝する住民から吸血している。これらのダニのボレリア保有率は平均して40%以上である。またこれまで、殺虫剤によるダニの駆除、就寝時ネットを使用してダニ刺咬防止などが試みられたがいずれも効果を上げるに至っていない。

これらの調査結果を総合すると、夜間のダニ刺咬によりボレリアに感染し、成人は抗体を保有するために発症することなく、乳幼児のみが感染して発熱、栄養不良による抵抗力不足から死亡すると考えられる。したがって、乳幼児に対してボレリア抗原による免疫付与ができればTBRFによる感染死は防ぐことが出来るものと考えられる。しかしボレリアの多様な抗原性に対応できることとあわせ、乳幼児を対象とするために安全性の高いものでなくてはならない。本研究目的はボレリアの病原性すなわち抗原変換メカニズムにおける菌体表層蛋白の抗原構造を解明することと、初期発現蛋白のエピトープを持つ感染防御ワクチンを創製することである。

B. 研究方法

1. 抗ボレリア抗原VmpP抗体作成

ウサギに、精製蛋白をcompelete freund adjuvant (DIFCO)と混合したものを皮内注射、2週間後背部に再接種して追加免疫を行った。さらに2週間後、前回と同様に皮下注射、1週間後ウサギの耳深静脈から血液を採取、抗血清を得た。抗VmpP抗体はプロテインGカラムで精製してエピトープ解析に用いた。

2. ペプチド合成

昨年の実験の結果明らかになった、抗原蛋白VmpPの5カ所のエピトープ領域のうちのf3-b (73-87aa)、f6-c (155-169aa)、f9-b (223-237aa)、f11-c (280-294aa)、f13-d (342-355aa)のアミノ酸配列にもとづいて7～8アミノ酸から成る、互いに重なりを持つペプチドをそれぞれ合成した。

3. 合成ペプチドのELISA

ペプチドは10μMの濃度で用い、96穴ELISAプレートに100μlでコーティングしたのち、1%BSAを含むPBSで3回洗浄、最後にTween溶液(PBS-T)でブロックした。前述の方法で作成した抗VmpPポリクローナル抗体との反応は37℃で2時間行い、未反応の抗体はPBS-Tで洗い、定法にしたがって抗体の検出をおこなった。

C. 研究結果

ボレリア抗原蛋白VmpPの5カ所のエピトープ推定領域[f3 (65-94aa)、f6 (140-169aa)、f9 (215-244aa)、f11 (265-294aa)、f13 (315-355aa)]の7～8アミノ酸残基からなるペプチドのそれぞれについて抗体反応性を確認した。

その結果、有意に反応性の高かった領域はf9-b, f11-cとf13-dであった。さらにこれらの領域の中の抗原決定基はLASIVD(f9-bR, aa 232-237), AGGIAL

(f11-cL, aa 280-285) と KAADQQ (f13-dM, aa 350-355) のそれぞれ6アミノ酸残基であることが明らかになった。さらにこれらの領域の変化率（多様性）を調べたところ、f9-bR と f11-cL は比較的保存性が高く、f13-dR はもっとも可変な領域であった。この f13-dR は抗体反応性がもっとも高く VmpP 蛋白のC末端に位置していた。

D. 考察

本研究で、ボレリア抗原蛋白 VmpP の2カ所のαヘリックスを形成すると考えられる領域と、C末端に位置する可変領域において高い抗体反応性が確認でき、これらの部分はエピトープである可能性が高い。アミノ酸配列から推定されるふたつのαらせん構造領域は、他のボレリア抗原蛋白と比較して保存性が高いので、共通抗原として利用できる可能性がある。この点を確かめるために、このペプチドを用いて免役した動物が、ボレリア感染に抵抗性を持つかという確認実験を行う必要がある。

一方、C末端に位置する可変領域は特定の構造をとらず、菌体表層でフリーな状態にあると推定される。そのため抗原として認識されやすいものと考えられる。最も高い抗体反応性はワクチンとして有望であるが、その可変性から免疫応答を惹起するには他種類の抗原蛋白のC末端領域をまとめて用いなければならない。

一般にエピトープは5-8個のアミノ酸で構成されるので、本実験で確かめられた3カ所の抗体反応陽性断片はこの蛋白のエピトープと考えられる。すでに立体構造が報告されているライム病ボレリアの抗原蛋白 OspA は、ダニ体内で発現、宿主感染時には発現量が低下、代わって OspC が発現されるように変化する。OspA は主にアンチパラレルなβ鎖構造で、C末端に位置する可変領域がエピトープと考えられている。一方、OspC の立体構造は逆にαらせん構造で、回帰熱ボレリア菌体表層蛋白と似た立体構造をとると考えられる。OspC の C 末端領域 (PVVAESPKKP) も細胞膜から露出して抗原エピトープになっていると考えられている。

ライム病ボレリアは感染時、組織や関節にとどまり、血液中に入ることはまれで、回帰熱ボレリアとは異なった挙動を示す。これは媒介ダニの吸血行動の違いによると考えられる。ライム病ボレリア媒介ダニの吸血は3～7日間で、一生のうちに雄は2回、雌は3回しか吸血しない。回帰熱ボレリア媒介ダニの吸血時間は通常30分程度で、胞血後数日～数週間かけて脱皮、7回前後の吸血を経て成虫となる。つまり回帰熱ボレリアが吸血時媒介ダニに移行するためには血液中で一定の菌数を保っている必要がある。

我々の調査した地域において、ダニのボレリア保有率が40%以上と高いことは、頻回の吸血によるものと考えられる。現在、回帰熱ボレリアに有効なワクチンはない。貧困と衛生教育の普及の遅れから、ダニの住居からの駆逐や、刺咬防止のために就寝時ネットを使用することも難しい。回帰熱ボレリアの特異抗原や有

効な共通抗原領域を探索して、ボレリアの抗原変換メカニズムを解明、回帰熱に有効なワクチンの開発にもつなげていきたい。

E. 結論

回帰熱ボレリア抗原蛋白 VmpP のエピトープ解析を行って、3カ所6アミノ酸残基まで絞り込んだ。ふたつの領域はαヘリックス構造の保存領域であった。もうひとつの領域は外部ループに相当するC末端の可変部分であった。

F. 健康危険情報

1　国内でも回帰熱疑似患者発生があるので、精査する必要がある。

2　近年都市部の路上生活者にコロモジラミが増加しているので、致死性の高いシラミ媒介性回帰熱ボレリアが国内に流行する素地がある。このため回帰熱流行地域からの帰国者やげっ歯類、貨物に紛れての病原体保有ダニの持ち込みを防止しなければならない。

G. 研究発表

1. 論文発表

1) Shao, R., Barker, S. C., Mitani, H., Aoki, Y. and Fukunaga, M. 2005. Evolution of duplicate control regions in the mitochondrial genomes of metazoa: a case study with Australasian *Ixodes* ticks. Mol. Biol. Evol., 22 (3) : 620-629.

2) Shao, R., Mitani, H., Barker, S. C., Takahashi, M. and Fukunaga, M. 2005. Novel mitocjndrial gene content and gene arrangement indicate illegitimate inter-mtDNA recombination in the chigger mite, *Leptotrombidium pallidum*. J. Mol. Evol., 60 (6) : 764- 773.

3) Shao, R., Barker, S. C., Mitani, H., Takahashi, M. and Fukunaga, M. 2006. Multiple mechanisms acted in the evolution of gene-arrangement in the mitochondrial genomes of chgger mites. J. Mol. Evol., 62, in press.

4) Tabuchi, N., Tomoda, K., Kawaguchi, H., Iwamoto, H., and Fukunaga, M. 2006. Immuno-dominant epitope in the C-terminus of a variable major protein in *Borrelia duttonii*, an agent of tick-borne relapasing fever. Microbiol. Immunol. 50, in press.

2. 学会発表

1) 田淵紀彦、友田幸一郎、福長将仁. *Borrelia duttonii* Ly 株菌体表層蛋白抗原 VmpP のエピトープ解析、第78回日本細菌学会総会、東京、2005年4月4日～6日.

2) 三谷春美、高橋守、福長将仁. ミトコンドリア遺伝子による *Ixodes philipi* の位置づけ、第14回日本ダニ学会大会、帯広、2005年9月20日～22日.

3) 福長将仁　マダニの生物学：ダニミトコンドリア遺伝子構成とダニ系統解析　第13回ダニと疾患のインターフェ

イスに関するセミナー、伊豆、2005年9月22日～24日.

4) 田淵紀彦、友田幸一郎、福長 将仁、ダニ媒介性回帰熱ボレリア *Borrelia duttonii* Ly 株菌体表層蛋白のエピトープ解析、第58回日本細菌学会中国・四国支部総会、高知、2005年10月6日～7日.

5) 福長 将仁、ボレリア感染症の生態：ライム病と回帰熱の昨日、今日、明日、人獣共通感染症セミナー、宮崎、2005年10月21日.

6) Shao, R., Barker, S. C. and Fukunaga, M. 2005. The mitochondrial genomes of ticks and their kin: a review, plus the description of the mitochondrial genomes of *Amblyomma triguttatum* and *Ornithodoros porcinus*. Vth International Meeting of Ticks and Tick-Borne Pathogens, New Chatel, Swizerland, Augst 29-September 3,

H. 知的財産権の出願・登録状況

なし。

(別添5-1)
Ⅲ. 研究成果の刊行に関する一覧表
雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshii, K., Hayasaka, D., Goto, A., Kawakami, K., Kariwa, H. Takashima, I.	Packaging the replicon RNA of the Far-Eastern subtype of tick-borne encephalitis virus into single-round infectious particles: development of a heterologous gene delivery system.	Vaccine	23	3946-3956	2005
Goto, A., Yoshii, K., Obara, M., Ueki, T., Mizutani, T., Kariwa, H. Takashima, I.	Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion.	Vaccine	23	3043-3052	2005
Scmidt, J., Jandrig, B. , Klempa, B. , Yoshimatsu, K., Arikawa, J., Meisel, H., Niedrig, M., Pitra, C., Kruger, D.H. Ulrich. R.	Nucleocapsid protein of cell culture-adapted Seoulvirus strain 80-39: analysis of its encoding sequence, expression in yeast and immuno-reactivity.	Virus Gene	30	37-48	2005
Chandy, S., Mitra, S. , Sathish, N., Vijayakumar, T.S., Abraham, O.C., Jesudason, M.V. , Abraham, P., Yoshimatsu, K., Arikawa, J., Sridharan, G	A pilot study for serological evidence of hantavirus infection in human population in south India.	Indian J Med Res	122	211-215	2005
Zhang, G., To, H., Russell, K. E., Hendrix, L.R., Yamaguchi, T., Fukushi, H., Hirai, K., Samnel, J. E.	Identification and characterization of an immunodominant 28-kilodalton *Coxiella kurnetii* outer membrane protein specific to isolates associated with acute disease.	Infect. Immun	73	1561-1567	2005
Iwata T., Une Y., Okatani A.T., Kaneko S., Namai S., Yoshida S., Horisaka T., Horikita T, Nakadai A. Hayashidani H.	*Yersinia enterocolitica* serovar O:8 infection in breeding monkeys in Japan.	Microbiol.Immunol	49		2005
Kawabuchi, T., Tsuji, M., Kuwahara, S., Nishida, A., Shimofurutachi, T., Oka, H. Ishihara, C	Isolation of a human erythrocyte-adapted substrain of Babesia rodhainiand analysis of the merozoite surface protein gene sequences.	J. Vet. Med. Sci	67	901-907	2005
Kawabuchi, T., Tsuji, M., Sado, A., Matoba, Y., Asakawa, M. Ishihara, C	Babesia microti-like parasites detected in feral raccoons (Procyon lotor) captured in Hokkaido, Japan.	J. Vet. Med. Sci.	67	825-827	2005

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shao, R., Mitani, H., Barker, SC., Takahashi, M., and Fukunaga, M	Novel mitocjndrial gene content and gene arrangement indicate illegitimate inter-mtDNA recombination in the chigger mite, Leptotrombidium pallidum.	J. Mol. Evol	60	764-773	2005
Shao, R., Barker SC., Mitani, H., Aoki, Y., Fukunaga, M	Evolution of duplicate control regions in the mitochondrial genomes of metazoa: a case study with Australasian Ixodes ticks.	Mol.Biol. Evol	22	620- 629	2005
林谷秀樹	Yersina pseudotuberculosis感染症(仮性結核)	モダンメディア	51	211-216	2005
苅和宏明	ハンタウイルス感染症	Virus Report	2(2)	27-34	2005
高島郁夫、早坂大輔、後藤明子、好井健太朗、苅和宏明	日本と極東ロシアのダニ媒介性脳炎ウイルスの系統解析と病原性	ウイルス	55(1)	35-44.	2005
高島郁夫	8)ダニ媒介性脳炎	Progress in Media	26(1)	61-63	2006
林谷秀樹、岩田剛敏	Yersina pseudotuberculosis感染症(仮性結核)	モダンメディア	51 (9)	1-6	2005

Ⅳ. 研究成果の刊行物・別刷

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 23 (2005) 3946–3956
Vaccine
www.elsevier.com/locate/vaccine

Packaging the replicon RNA of the Far-Eastern subtype of tick-borne encephalitis virus into single-round infectious particles: development of a heterologous gene delivery system

Kentarou Yoshii [a], Daisuke Hayasaka [b], Akiko Goto [a], Kazue Kawakami [a], Hiroaki Kariwa [a], Ikuo Takashima [a,*]

[a] *Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan*
[b] *Department of Pathology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*

Received 15 September 2004; received in revised form 31 January 2005; accepted 2 March 2005
Available online 25 March 2005

Abstract

The sub-genomic replicon of tick-borne encephalitis (TBE) virus (Far-Eastern subtype) was packaged into infectious particles by providing the viral structural proteins in *trans*. Sequential transfection of TBE replicon RNA and a plasmid that expressed the structural proteins led to the secretion of infectious particles that contained TBE replicon RNA. The secreted particles had single-round infectivity, which was inhibited by TBE virus-neutralizing antibody. The physical structure of the particles was almost identical to that of infectious virions, and the packaged replicon RNA showed no recombination with the mRNAs of the viral structural proteins. Furthermore, heterologous genes were successfully delivered and expressed by packaging TBE replicon RNA with inserted GFP and Neo genes. This replicon packaging system may be a useful tool for the molecular study of the TBE virus genome packaging mechanism, and for the development of vaccine delivery systems.

Keywords: Tick-borne encephalitis virus; Replicon; Virus-like particles

1. Introduction

The genus *Flavivirus* (family *Flaviviridae*) contains important human pathogens, which include the tick-borne encephalitis (TBE) virus, Japanese encephalitis virus, yellow fever virus, Dengue virus, and West Nile virus. Flaviviruses can be divided into three phylogenic and ecological groups: a tick-borne group, a mosquito-borne group, and a vector-unknown group [1–3]. The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e. the core (C), premembrane (prM), and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), within a single long open reading frame, which is co-translated and cleaved post-translationally [4]. The 5′- and 3′-UTRs have predicted secondary structures that are implicated in viral replication, translation, and packaging of the genomes [5–8].

The TBE virus is a species of mammalian tick-borne flavivirus that is prevalent over wide areas of Europe and Asia [9,10]. It causes severe encephalitis in humans with serious sequela and has a significant impact on public health in these endemic regions [11,12]. The virus has been subdivided into Far-Eastern, Siberian, and European subtypes [13–15]. The Far-Eastern subtype, which was previously known as Russian spring-summer encephalitis (RSSE) virus, causes severe clinical manifestations and has a higher mortality rate (5–20%) than the other TBE virus subtypes [9,16].

The subgenomic replicons of positive-stranded RNA viruses contain genes that are necessary for viral amplification in host cells, but they lack the genes for the viral structural

* Corresponding author. Tel.: +81 11 7065211; fax: +81 11 7065211.
E-mail address: takasima@vetmed.hokudai.ac.jp (I. Takashima).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.03.004

proteins. Thus, the replicon RNAs can replicate in cells but cannot be incorporated into virus particles. Therefore, these replicons have been used as a tool for the study of genome replication in various viruses [17–20]. Moreover, little is known about the flavivirus genome packaging mechanism, packaging signal(s), and nucleocapsid formation, mainly because it is difficult to reproduce the packaging of flavivirus genome RNA into nucleocapsids. Therefore, the construction of a packaging system that uses these replicons could be a useful tool for studying the flavivirus genome packaging mechanism.

Another application of these replicon systems is the development of vectors for gene expression and vaccines [20–22]. Replicon-based vectors of positive-strand RNA viruses are becoming more popular for development of antiviral and anticancer vaccines. By using replicon systems, a high level of expression of heterologous genes can be expected, and the inability of replicon RNA to spread from transfected cells gives biological safety for the vaccine application. In several replicon studies of the mosquito-borne flaviviruses, heterologous gene expression following the insertion of genes into the deleted structural protein gene region or 3′-UTR has been reported [23–25]. In a study of the Kunjin virus, the packaging of replicon RNA into particles by providing the structural protein in *trans* was developed and applied for heterologous gene delivery vector and as a vaccine [26–30].

In our previous study, a sub-genomic replicon of the Far-Eastern subtype TBE virus strain Oshima 5–10, which was isolated in Japan [31], was constructed, and heterologous genes were inserted into the 3′-UTR [32]. The heterologous genes were expressed appropriately, and the host cells that were transfected with the replicon RNA showed no cytopathic effect (CPE). In this study, a TBE virus replicon packaging system was developed using the expression of the viral C/prM/E proteins in *trans*. C-prM-E processing was carried out correctly by NS proteins that were produced from the replicon, and replicon RNAs were incorporated into single-round infectious particles without recombination between the replicon RNA and the mRNAs for the structural proteins. Furthermore, heterologous genes were delivered and expressed using this packaging system, whereby, for the first time with a tick-borne flavivirus TBE virus, replicons were inserted with heterologous genes. These data suggest that this newly developed packaging system is a useful tool for the molecular study of the TBE virus genome packaging mechanism, and for the development of gene delivery vectors using the TBE virus replicon.

2. Materials and methods

2.1. Cells and viruses

The baby hamster kidney (BHK)-21 cell line was grown at 37 °C in Eagle's minimum essential medium (MEM) that was supplemented with 8% FCS and L-glutamine. For the selection of cells that expressed the neomycin resistance gene, G418 (1 mg/ml) was added to medium. TBE virus was prepared from the infectious virus recovered from TBEV infectious cDNA O-IC-pt, in which the full-length cDNA of TBE virus Oshima strain (Far-Eastern subtype) was cloned, as described previously [33].

2.2. Antibodies

For the detection of TBE virus E proteins in the ELISA, the mouse monoclonal anti-E antibodies 1H4 and 4H8 [34] were used. Rabbit polyclonal anti-prM, anti-E, and anti-NS3 antibodies were prepared by immunization with recombinant prM, E, and NS3 proteins, respectively, which were expressed in the pET43 system (Novagen, Madison, WI). FITC-conjugated anti-mouse IgG antibodies and anti-rabbit IgG antibodies (Jackson Immunoresearch, West Grove, PA) were used as secondary antibodies in immunofluorescence assay.

2.3. Plasmid construction

The derivation of the recombinant plasmid pCAGprME, which expresses both prM and full-length protein E derived from the Oshima 5–10 strain of TBE virus (GenBank accession no. AB062063), has been described previously (Fig. 1A) [35]. For the construction of pTBECprME, TBE viral RNA was extracted from a virus-inoculated suckling mouse brain, and RT-PCR was performed as described previously [31]. PCR for the amplification of the DNA fragment that contains the gene for protein C and part of the *prM* gene was carried out with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), using the following primers: forward, 5′-AGC*GCTAGC*AGATTTTCTTGCACGTGCAT-3′; and reverse, 5′-GTGTCCCCTTCCTGTGAGAT-3′. The PCR products were digested with *Nhe*I and *Age*I, and inserted into the pCAGprME plasmid, which was predigested with *Nhe*I and *Age*I.

TBE replicon RNA transcripts were prepared form Oshima REPpt, Oshima REP-GFP, and Oshima REP-NEO plasmids, as described previously [32]. For the construction of Oshima REPpt, the NS5 region spanning residues 7881 to 9829 (between the restriction sites for *Afl*II to *Asc*I) in Oshima REP, which has two amino acid mutations in the NS5 gene, was replaced with the parental NS5 gene. The Oshima REP-GFP and Oshima REP-NEO plasmids, in which the GFP and neomycin-resistance genes were inserted into the 3′-UTR, have been described in our previous study [32].

2.4. Cell transfection

Cells that were grown to 60–70% confluence in 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) were transfected with 2 μg of pCAGprME or pTBECprME that was complexed with the *Trans*IT-LT1 reagent (Pan-Vera Corp., Madison, WI) in Opti-MEM (Invitrogen) without

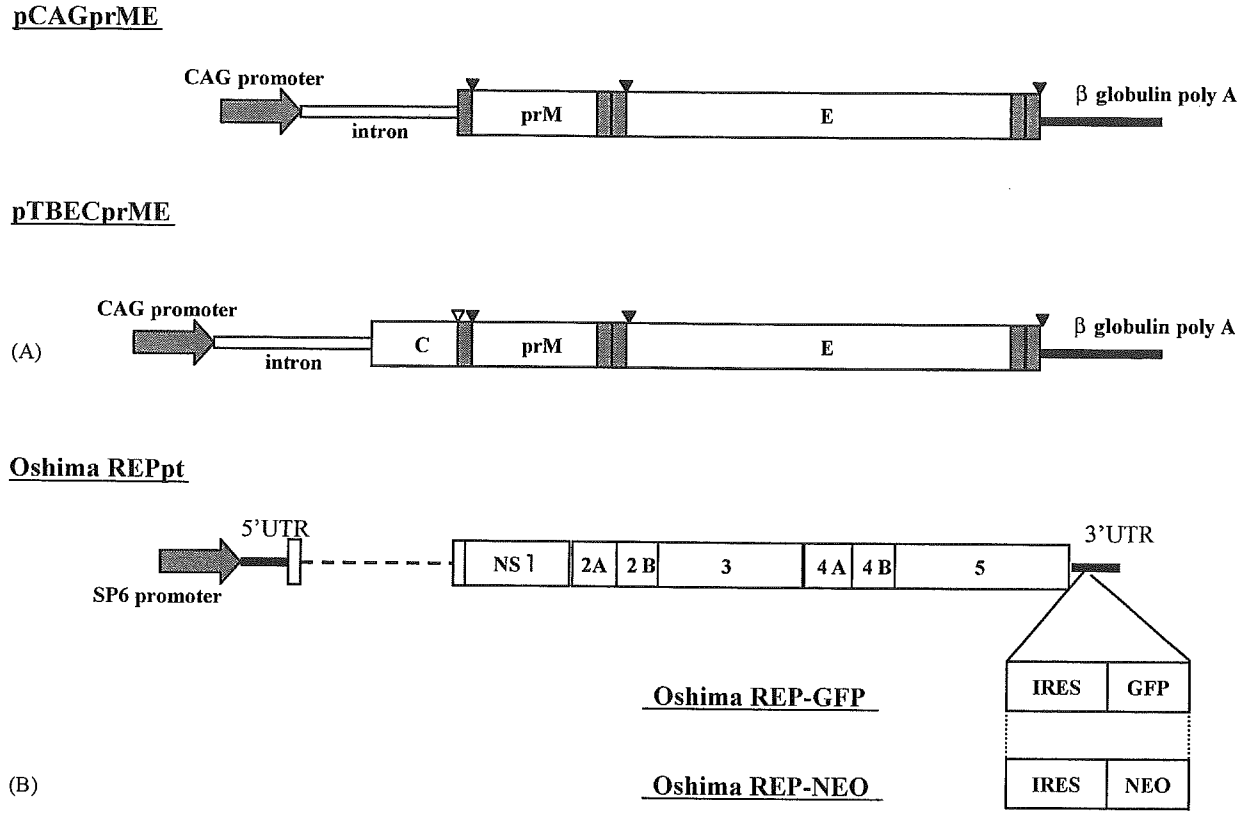


Fig. 1. Schematic representation of the plasmids used in this study. (A) Mammalian expression vector that encodes the TBE virus structural proteins. The TBE virus prM/E and C/prM/E regions were cloned into the pCAGGS vector [52] and designated pCAGprME and pTBECprME, respectively. The transmembrane domain and signal sequence are indicated by a gray box. The intracellular protease cleavage sites are indicated by black triangles, and the NS2B-3 protease site is indicated by an open triangles. (B) Constructs of TBE replicon. TBE replicon regions with or without the IRES GFP/NEO gene in the 3′-UTR were inserted under the control of the SP6 promoter, as described previously for the Oshima REP plasmid [32].

serum or antibiotics. Twenty-four hours post-transfection (unless stated otherwise), the cells and supernatants were harvested and used for further experiments.

2.5. SDS-PAGE and Western blotting

Transfected cells were lysed with Laemmli buffer [36] and electrophoresed in 8% and 15% polyacrylamide-sodium dodecyl sulfate (SDS) gels under non-reducing condition. The protein bands on the gels after SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with 1% Gelatin in 25 mM TBS that contained 0.01% Tween-20 (TBST), for 30 min at room temperature. After washing with TBST, the membranes were reacted with the anti-E and anti-prM rabbit IgG for 1 h, and then with alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (Promega, Madison, WI) for 30 min at room temperature. The protein bands were visualized with the AP Detection Kit (Novagen).

2.6. Preparation of encapsidated particles

Oshima REPpt was digested with *Spe*I and extracted using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The mMESSAGE mMACHINE SP6 Kit (Ambion Inc., Austin, TX) was used to in vitro-transcribe infectious RNA in a 20-μl reaction mixture that contained an additional 1 μl of GTP solution. After transcription at 37 °C for 2 h, the DNA template was removed by DNase I digestion at 37 °C for 15 min. The RNA was precipitated using lithium chloride, washed with 70% ethanol, resuspended in RNase-free water, quantitated by spectrophotometry, and stored at −80 °C in aliquots.

Approximately 5×10^6 BHK cells in 0.5 ml cold PBS were electroporated with 10 μg of RNA in 0.4-cm cuvettes using the GenePulser apparatus (Bio-Rad Laboratories, Hercules, CA), with two separate pulses, without the pulse controller at settings of 1.3 kV, 25 μF, and at maximum resistance. The transfected cells were aliquoted into 24-well culture plates. After 24 h, the cells were transfected with 2 μg of pTBECprME per well. At 36 h post-transfection, the supernatant was harvested and cleared by low-speed centrifugation at $1000 \times g$ for 10 min. The particles in the cleared supernatant were precipitated with 10% polyethylene glycol (PEG; MW = 8000) and 1.9% NaCl for 2 h at 4 °C, and pelleted at $10{,}000 \times g$ for 20 min. The pellets were resuspended in PBS that was supplemented with RNase A (20 μg/ml),

left to dissolve overnight at 4 °C, and then used in further experiments.

2.7. Infectivity assays

In order to determine the titers of the encapsidated particles, BHK cells were grown on 16-well Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) and infected with serially diluted encapsidated particle solutions for 1.5 h at 37 °C. In the particle neutralization test, 1000 infectious units of encapsidated particles were incubated with at a 1:100 dilution of the anti-TBE E protein monoclonal antibody 1H4, which has neutralizing activity against the TBE virus, prior to infection of BHK cells. The culture supernatant was replaced with fresh medium and incubated for 24 h at 37 °C. The cells were rinsed with phosphate-buffered saline (PBS), subjected to fixation with 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 for 4 min at room temperature. After blocking with 2% BSA for 30 min, the cells were incubated at room temperature for 1 h with anti-NS3 rabbit IgG antibodies, at a dilution of 1:100, in antibody-dilution buffer (PBS that contained 0.1% Triton X-100 and 2 mg/ml BSA). After extensive washing with PBS, the cells were incubated at room temperature in the dark for 1 h with fluorescence-labeled secondary antibodies, which were diluted 1:200. The cells were washed three times with PBS, followed by mounting of the coverslips on glass slides. The images were viewed and recorded using confocal microscopy.

The infectious titer of the packaged particles was calculated using the following formula: titer (infectious unit [IU] per ml) $= N \times (\mathrm{SW/SIA}) \times 1/V \times 10^n$, where N is the average number of anti-NS3 positive cells in the image area, as calculated from 12 image areas in different regions of the coverslips; SW is the surface of the well in a 16-well plate (70 mm^2); SIA is the surface of the image area (3.6 mm^2); V is the total volume of the diluted particle solutions; and 10^n is the dilution factor.

2.8. Particle analysis

Recombinant subviral particles (RSPs) were prepared from pCAGprME-transfected cells, and encapsidated particles were precipitated with PEG (as described above), and then subjected to equilibrium density centrifugation at 35,000 rpm in a 10–50% sucrose gradient at 4 °C for 14 h (P45AT rotor; Hitachi Koki Co., Ltd., Tokyo, Japan). Fractions of 0.5 ml were collected from the tops of the tubes. Aliquots of the fractions were treated with 1% Triton X-100, and added to MAb 1H4-coated wells of 96-well microtiter ELISA plates, which were blocked with 3% BSA. TBE virus-specific antigen in the fractions was detected using the biotinylated MAb 4H8 and horseradish peroxidase (HRP)-conjugated streptavidin (Sigma Chemical Co., St. Louis, MO). HRP activity was detected by adding 100 μl of *O*-phenylenediamine dihydrochloride (Sigma) in the presence of 0.03% H_2O_2.

2.9. RT-PCR

RNA was extracted from the encapsidated particles, which were purified by centrifugation in sucrose gradients, using the Isogen Kit (Nippon Gene, Toyama, Japan). The reverse transcription (RT) reaction was carried out with Superscript II reverse transcriptase (Invitrogen) and the primer HO_2 (5′-AGCGGGTGTTTTCCGAGTC-3′). PCR was carried out as described previously [31] using the following primers: for amplification of the region between the 5′-UTR and NS1, (forward) 5′-AGATTTTCTTGCACGTGCAT-3′ and (reverse) 5′-GTATGCATAATTGTCATACC-3′; and for amplification of the partial prM gene and E gene, (forward) 5′-TCTGCAAAGACGTGGCAAAA-3′ and (reverse) 5′-CATTGAGGGCTTCCCCTCAG-3′.

3. Results

3.1. Expression of the TBE virus structural proteins by the pTBECprME plasmid

For the expression of the TBE virus structural proteins that were used to package the TBE replicon RNA, the TBE C-prM-E region was cloned into the pCAGGS expression vector, thereby generating pTBECprME (Fig. 1A). To examine the expression of the viral structural proteins and cleavage by the signal peptidase, BHK cells were transfected with pTBECprME, and viral structural protein expression was detected by specific immune sera in Western blots (Fig. 2). In the pTBECprME-transfected cells, E proteins with the same molecular size as those expressed in cells that were transfected with pCAGprME, which encodes the TBE prM-E gene, were detected. On the other hand, a band larger than that in prM was detected by the TBE prM-specific antibodies, while the prM band was detected in pCAGprME-transfected cells. This band corresponds to the C-prM polyprotein, which is not cleaved by the ER signal peptidase. It has been reported previously that the C-prM junction is first cleaved by the NS3 protein on the cytoplasmic side, and then cleaved continuously by the ER signal peptidase on the ER luminal side [37–39]. Therefore, in this case, the C-prM polyprotein was not cleaved due to the absence of the NS3 protease.

It is known that the expression of the flavivirus envelope proteins prM and E leads to the secretion of recombinant subviral particles (RSPs). E protein that was derived from secreted RSPs was detected in the supernatants of pCAGprME-transfected cell cultures, as described previously [35]. However, E protein was not detected in the culture supernatants of cells that were transfected with pTBECprME. This was due to a failure to digest the C-prM junctions. Thus, the viral envelope proteins were unable to assemble into RSPs.

These data suggest that the TBE C-prM-E proteins are expressed in an appropriate manner following pTBECprME transfection. These cassettes were used in further studies to

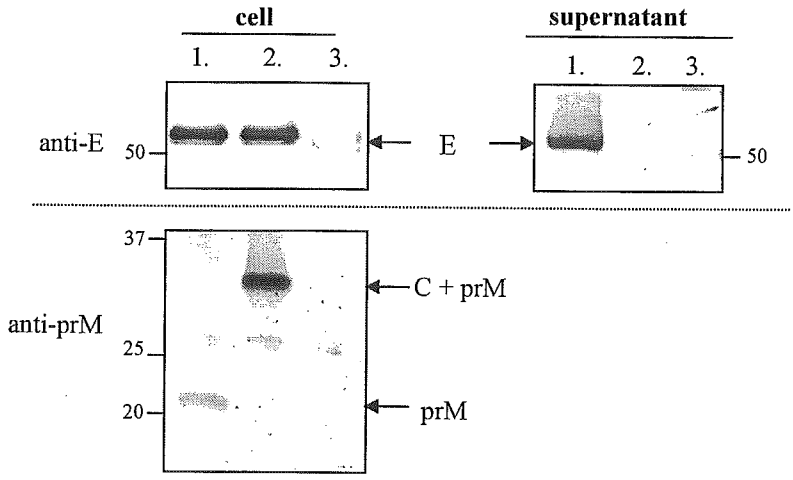


Fig. 2. Expression of TBE virus structural proteins. BHK cells were transfected with pCAGprME (lane 1), pTBECprME (lane 2), or the control pCAGGS plasmid (lane 3). At 24 h post-transfection, the cells and PEG-precipitated supernatant samples were separated by 12% SDS-PAGE under non-reducing condition and subjected to Western blotting. The viral proteins were visualized using anti-E (upper panel) and anti-prM (lower panel) rabbit polyclonal antibodies. The positions of the individual proteins are marked, and the molecular size (in kDa) is indicated at the side.

construct a system for packaging the TBE replicon RNA into infectious particles.

3.2. *Packaging of the TBE replicon RNA into single-round infectious particles by trans-complementation*

For the RNA packaging experiment, TBE virus replicon RNA was prepared from the Oshima REPpt plasmid, which was constructed from the Oshima REP plasmid with two mutations in NS5 region, as described previously [32]. It was necessary to use a TBE replicon with high transfection efficiency for packaging in doubly transfected cells (i.e., cells transfected with the TBE replicon and pTBECprME plasmid that expresses the TBE structural proteins). This replicon construct showed high efficiency of transfection into BHK21 cells, with 90–100% of the cells being positive in the IFA tests (data not shown). Consequently, the Oshima REPpt replicon was used in the subsequent packaging studies.

In preliminary experiments, transfection of pTBECprME, which expresses the TBE structural proteins, prior to Oshima REPpt replicon electroporation gave no secretion of infectious particles. This result was in accordance with the finding of the Kunjin virus study, which showed that the replicon had to accumulate before the structural proteins were expressed [30]. Therefore, in subsequent experiments, the BHK21 cells were first electroporated with TBE replicon Oshima REPpt, and then 12 h or more later, transfected with pTBECprME, thus ensuring the secretion of infectious particles.

The culture supernatants from the BHK21 cells that were transfected with pTBECprME and replicon Oshima REPpt were cleared by low-speed centrifugation and precipitated with PEG 8000. The precipitated pellets were resuspended in PBS that contained RNase A, and used for further study. In the BHK21 cells that were infected with PEG-precipitated supernatant from pTBECprME- and replicon-transfected cells, the production of TBE NS3 proteins was detected by the immunofluorescence assay (Fig. 3A), in contrast to the lack of viral protein production observed in cells that were infected with the supernatant of replicon-transfected cells (Fig. 3B). A second passage, in which the supernatants of the infected BHK21 cell cultures were transferred to fresh BHK21 cells, resulted in infection in the case of the wild-type control of RNA transfection from TBE virus infectious cDNA O-IC (authentic virus particles; Fig. 3F) but not in the case of transfection with pTBECprME and replicon Oshima REPpt (Fig. 3D). These data demonstrate that the particles (virus-like particles; VLPs) secreted by pTBECprME- and TBE replicon Oshima REPpt-transfected cells have only single-round infectivity potential.

To optimize the conditions for efficient packaging of replicon RNA into VLPs, various time-points between the transfections (Fig. 4A), and between the second transfection and harvesting the VLPs (Fig. 4B), were examined. First, the optimal time period between replicon RNA electroporation and pTBECprME transfection was determined. BHK21 cells were electroporated with Oshima REPpt RNA, and seeded into cell culture dishes at 40–50% confluency for the second plasmid transfection. After incubation for 12, 18, 24, or 30 h, the cells were transfected with pTBECprME, and the culture supernatant was recovered from each dish 48 h after the second transfection. The recovered supernatants were subjected to PEG precipitation and used for the titration of VLPs by IFA with anti-NS3 antibodies. As shown in Fig. 4A, the highest titer of VLPs was obtained from cells that were transfected with the 24-h interval (6.2×10^6 IU/ml). This time delay for optimal transfection was also observed in the Kunjin virus study [30]. This increase in VLP titer with time between transfections indicates that viral RNA and/or viral non-structural proteins are required for the efficient packaging of replicon RNA by *trans*-complementation of structural proteins. Second, the optimal time for VLP harvesting after

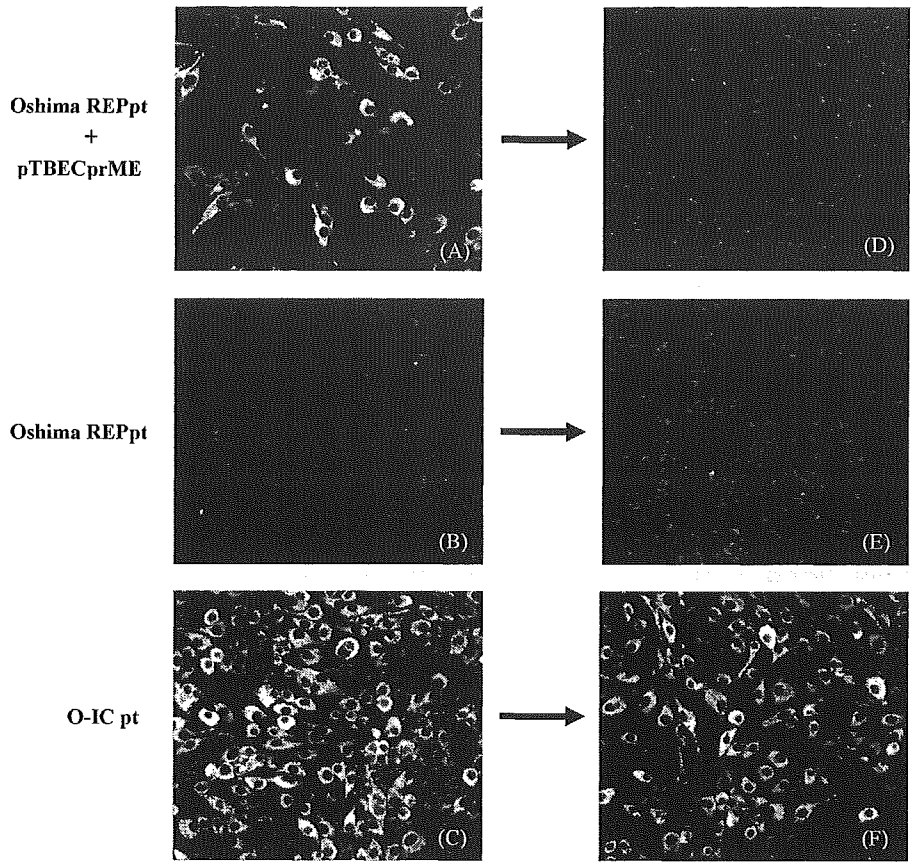


Fig. 3. Secretion of single-round infectious particles. Fresh BHK cells were infected with the culture supernatants of BHK cells transfected with in vitro-synthesized Oshima REPpt RNA and pTBECprME (A); Oshima REPpt alone (B); or O-IC pt RNA (C); and incubated for 24 h (A to C). Panels D to F show re-infection of fresh BHK-21 cells, as indicated by the arrows. Expression of the viral NS3 proteins was visualized by immunofluorescence using anti-NS3 antibodies.

the second transfection was examined. BHK cells were transfected with pTBECprME 24 h after electroporation with the Oshima REPpt RNA. The culture supernatant was harvested 24, 36, 48, or 60 h after the second transfection and used for the titration of VLPs, as described above. The VLP titer increased gradually with time and peaked 48 h after the second transfection. At 60 h after the second transfection, the titer was relatively reduced.

3.3. Characterization of single-round infectious particles

To analyze the secretion of VLPs from the cells that were transfected with pTBECprME and the Oshima REPpt replicon, the transfected cells and supernatant were subjected to Western blot analysis (Fig. 5). In the pTBECprME-transfected cells, E protein bands were detected by the E-specific antibodies, regardless of replicon RNA electroporation. When the C-prM-E viral structural proteins were expressed alone, the C-prM polyprotein bands were detected by prM-specific antibodies, due to the absence of the viral NS3 protease, as shown in Fig. 2. On the other hand, bands corresponding to prM were detected in the cells that expressed the viral structural proteins with the Oshima REPpt replicon RNA (Fig. 5; cell lysate, lane 1), which indicates that the C-prM junctions were partially cleaved by viral NS3 protease that was derived from the expression of the TBE replicon RNA. Moreover, E protein secretion was observed in the culture supernatants of cells that were transfected with pTBECprME and Oshima REPpt replicon, whereas E protein was not secreted from cells that were transfected with pTBECprME alone (Fig. 5; supernatant). This means that viral envelope proteins are incorporated into VLPs and secreted into the culture supernatant following appropriate cleavage of the C-prM junctions.

To confirm that the infectious particles were actually packaged by TBE structural proteins, a virus neutralization test was performed. Prior to infection of the BHK cells, the VLPs (1000 IU) were incubated with the anti-TBE E protein monoclonal antibody 1H4 (at 1:100 dilution), which has neutralizing activity against the TBE virus; this led to loss of infectivity, as revealed by IFA (Fig. 6). To elucidate the physical structures of the VLPs, equilibrium density gradient centrifugation in a 10–50% sucrose gradient was performed (Fig. 7). The VLPs that were secreted from the cells transfected with pTBECprME and Oshima REPpt RNA were precipitated with PEG, subjected to centrifugation in the sucrose gradient, and the fractions were collected and assayed by ELISA for the

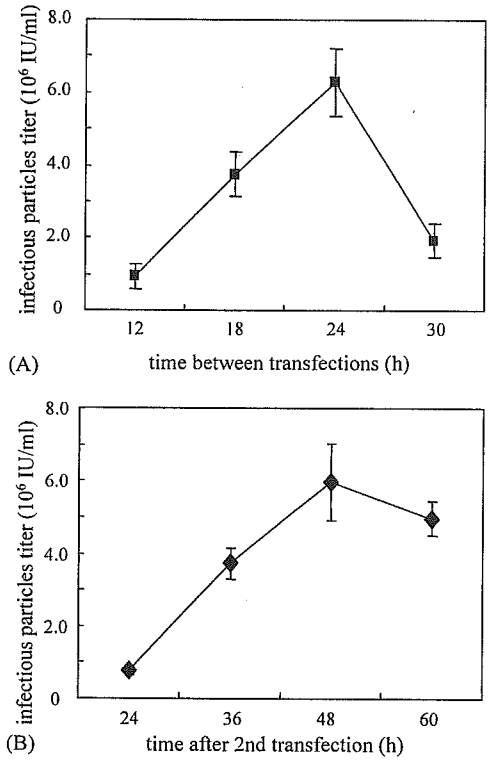


Fig. 4. Optimization of conditions for packaging of TBE replicon RNA. (A) Culture supernatants of cells that were transfected with replicon RNA and pTBECprME were collected 48 h after the second transfection, either at different time intervals between the RNA and plasmid transfections. (B) BHK cells were transfected with replicon RNA and pTBECprME at 24 h interval and the supernatant samples were collected at different times after the second transfection. The collected samples were precipitated with PEG, suspended in PBS that contained RNase A, and then used for the infection of the BHK cells. The infectious titers were calculated by counting the IFA-positive cells using anti-NS3 antibodies, as described in Section 2.

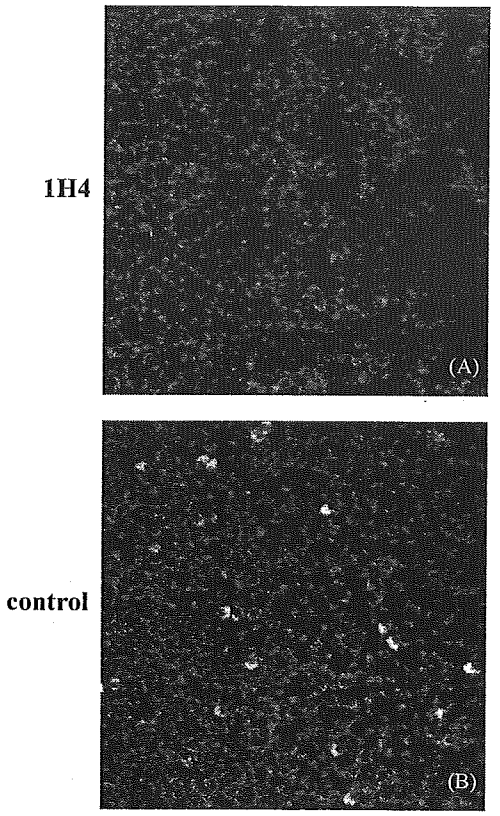


Fig. 6. Inhibition of replicon particle infectivity by virus-neutralizing antibody. Prior to infection, the virus particles were incubated with (A) or without (B) monoclonal antibody 1H4, which has neutralizing activity against the TBE virus. At 24 h post-infection, the cells were stained by IFA using anti-NS3 antibodies.

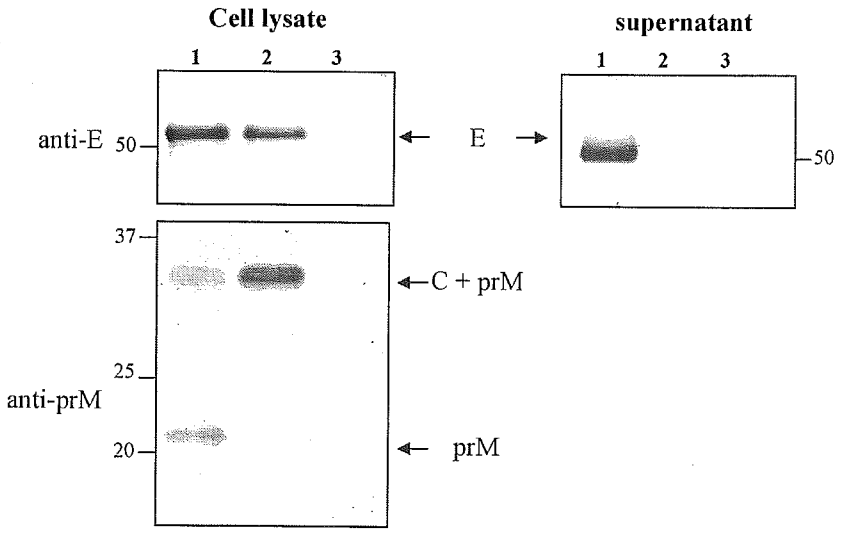


Fig. 5. Cleavage of the C-prM junctions by replicon NS3 protease, and secretion of viral proteins into the culture supernatant. BHK cells were transfected with Oshima REPpt RNA and pTBECprME (lane 1), pTBECprME (lane 2), or Oshima REPpt RNA (lane 3). At 24 h post-transfection with the pTBECprME plasmid, the cells and PEG-precipitated supernatant samples were separated by 12% SDS-PAGE under non-reducing condition and subjected to Western blotting. The viral proteins were visualized using the anti-E (upper panel) and anti-prM (lower panel) rabbit polyclonal antibodies. The positions of the individual proteins are marked, and the molecular size (in kDa) is indicated at the side.

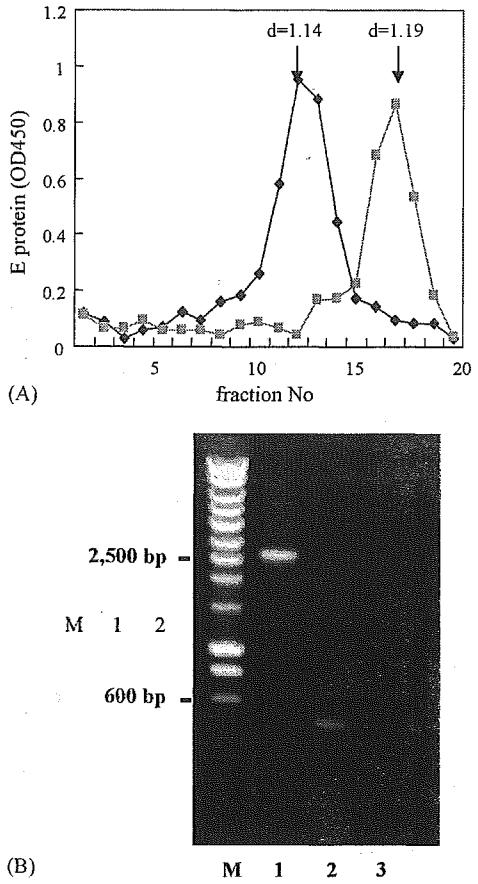


Fig. 7. (A) Sedimentation analysis of particles that were harvested from the culture supernatants of BHK cells that were transfected with in vitro-synthesized Oshima REPpt RNA and pTBECprME. The particles were pelleted with PEG and loaded onto a 10–50% sucrose gradient (squares). The E proteins in each collected fraction were analyzed by ELISA. Control gradients, which were loaded with purified RSPs (diamonds), were analyzed in parallel. The densities of the peak fractions are indicated by arrows. (B) RT-PCR analysis with TBE-specific primers located between the 5′-UTR and the NS1 regions of RNA that was extracted from the infectious particles. RNA was extracted from fractions 16–18 (in panel A), and subjected to RT-PCR (lane 2). PEG-precipitated supernatants of cells that were transfected with O-IC-pt (lane 1) and Oshima REPpt RNA (lane 3) were used as controls.

detection of TBE virus E proteins. The highest OD_{450} values for the E proteins were detected in fractions 16–18 (density of approximately 1.186 g/cm^3), which closely resemble the characteristics of the authentic TBE virus, as compared to the recombinant subviral particles (RSPs) (fractions 11–13; 1.14 g/cm^3), which were secreted from the pCAGprME-transfected cells. Furthermore, infectivity measured by IFA was observed only for fractions 16 to 18 (~6.5×10^6 IU/ml; data not shown). The RNA extracted from the VLPs in fractions 16–18 was reverse-transcribed and PCR-amplified using TBE-specific primers for the region between the 5′-UTR and NS1 gene. As shown in Fig. 7B, while a DNA fragment of 2500 bp (5′-UTR-NS1) was observed as the RT-PCR product of the authentic TBE virus (lane 1), a DNA fragment of the expected size (500 bp) was detected as the RT-PCR product of the VLP (lane 2), which indicates deletion of the region that encodes the viral structural gene and the absence of recombination with the mRNA of the viral structural gene provided by pTBECprME. No RT-PCR product was obtained from RNA that was extracted from the PEG-precipitated supernatant of cells that were transfected with Oshima REPpt RNA alone. In addition, no DNA fragment was amplified from the VLPs by RT-PCR for the partial prM and E genes, which indicates that the mRNA of the viral structural gene from pTBECprME was not incorporated into the particles. Combined with the data about single-round infectivity shown in Fig. 3, these data suggest that the replicon RNA was packaged into single-round infectious particles by *trans*-expressed viral structural proteins, without the regeneration of infectious viruses.

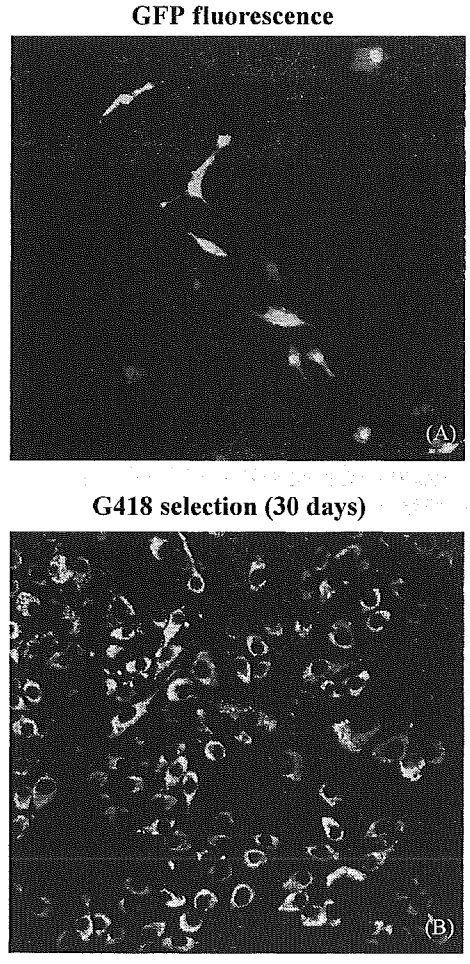


Fig. 8. Packaging of recombinant TBE replicon RNA that expresses a heterologous gene. (A) Culture supernatants from BHK cells that were sequentially transfected with Oshima REP-GFP RNA and pTBECprME were precipitated with PEG and used to infect fresh BHK cells. GFP fluorescence was observed at 60 h post-infection. (B) BHK cells in which the Oshima REP-NEO replicon RNA replicated persistently were transfected with pTBECprME. At 48 h post-transfection, the culture supernatants were precipitated with PEG and used to infect fresh BHK cells. The infected cells were cultured in the presence of G418 for 30 days, after which time the cells were stained by IFA using anti-NS3 antibodies.

3.4. Expression and delivery of heterologous gene by TBE replicon-based pseudoinfectious particles

In our previous study, a heterologous gene was inserted into the 3′-UTR of the TBE virus replicon [32]. Heterologous gene products were expressed in the replicon RNA-transfected cells, and none of the cells exhibited CPE. Therefore, the application of the replicon packaging system for gene delivery was examined. BHK cells were transfected with the Oshima REP-GFP replicon RNA, which expresses the GFP gene, and pTBECprME. At 48 h post-transfection with the pTBECprME plasmid, the culture supernatant was harvested, precipitated by PEG, and used to infect BHK cells. At 60 h post-infection, GFP expression in the infected cells was observed by confocal microscopy (Fig. 8A), which indicated that Oshima REP-GFP RNA is packaged into VLPs by *trans*-complementation of viral structural proteins, and that it acts as a vector for the expression of GFP as a heterologous gene. BHKrepTBE cells, which persistently harbor the TBE virus replicon, have been established previously by transfection of Oshima REP-NEO (containing the Neo gene in the 3′-UTR), followed by G418 selection. The pTBECprME plasmid was transfected into BHKrepTBE cells, and at 48 h post-transfection, the supernatant was precipitated with PEG and used to infect fresh BHK cells. At this point, the infectious titer had increased to 7.2×10^7 IU/ml, and the infected cells grew in the presence of G418 for at least 30 days (five passages), and produced viral non-structural proteins (Fig. 8B). These data indicate for the first time the application of the TBE replicon as a gene delivery vector.

4. Discussion

Subgenomic replicon vectors of positive-stranded RNA viruses have great potential in gene expression and vaccine applications [20,21,40]. Their advantages over other viral vector systems are following: (i) high level expression of encoded heterologous genes due to amplification of replicon RNA; (ii) cytoplasmic replication of replicon, which exclude the possibility of chromosomal integration; (iii) inability of replicon RNA to escape from transfected cells, which limits the spread of vaccine vectors; (iv) easy manipulations due to the relatively small genome size of replicon. In our previous study, we showed that the heterologous GFP gene and Neo gene with the IRES sequence could be inserted into the 3′-UTR of the TBE virus replicon and expressed without any apparent CPE [32]. To apply this TBE replicon to a gene delivery vector, we examined the packaging of replicon RNA into particles by sequential transfection with a plasmid that expresses the TBE virus structural proteins (C-prM-E). We then applied this packaging system to heterologous gene delivery vectors by packaging replicons that carried inserted heterologous genes, such as GFP and Neo. This is the first report about the application of a tick-borne flavivirus replicon packaging system to gene delivery. By using replicon encoding viral antigen and tumor specific antigen, TBE VLPs can be expected to induce protective antiviral and anticancer immunity.

Currently available flavivirus replicon-based vectors have certain advantages, such as their ability to replicate efficiently in a broad range of host cells without any apparent CPE and their genetic stability without spontaneous recombination [20,21]. It has been reported that alphavirus expression vectors have potential problems in terms of their cytopathic effects on host cells. In contrast, TBE replicons have no significant cytopathic effects. Thus, TBE replicon-based vectors facilitate longer-lasting expression of heterologous genes. Furthermore, no spontaneous regeneration of infectious viruses has been observed in the TBE replicon packaging system. In studies with alphaviruses, such as SFV and SIN, co-packaging of replicon RNA with helper RNA produced homologous recombination between the two RNA species, which led to regeneration of the infectious virus [41,42]. In our system, although the C/prM/E mRNA and replicon RNA contain overlapping elements of the viral genome, which raises the possibility of homologous recombination, neither co-packaging nor recombination between the two RNAs was observed in any of the experiments. These features of non-cytopathogenicity and genetic stability are important for the applicability of this tool to more-sensitive host systems.

In our packaging system, the titers of infectious particles reached 6.2×10^6 IU/ml following sequential transfection of pTBECprME and Oshima REPpt replicon RNA into BHK cells; 7.2×10^7 IU/ml VLPs were obtained by transfection of pTBECprME into BHKrepTBE cells when the cells were seeded into 24-well tissue culture plates. These titers are higher than those observed in the Gehrke study, in which particles were prepared by electroporation of the replicon into CHO-ME cells that continuously expressed the TBE virus prM/E proteins [43]. These titers are still below the maximum titers of alphavirus vectors [42,44], but are almost equivalent to those obtained with three-component alphavirus systems, using alphavirus packaging cell lines that have been designed to reduce the regeneration of infectious particles [45–48].

In addition to the vaccine and gene delivery systems, this TBE replicon packaging system can be applied to the study of genome RNA packaging and the rapid mapping of packaging signal(s) in TBE virus RNA and genomic RNA-binding domain(s) in the C protein. Compared to a full-length infectious clone, this packaging system has the advantage that it can separate replication events from assembly and genome RNA packaging events. Furthermore, while C and prM/E have been expressed separately in another packaging system [43], in our system, C/prM/E are provided from a single polyprotein, which is expressed by a plasmid that encodes all the viral structural protein genes. Thus, processing of the viral structural proteins is carried out as for the native virus, followed by the sequential events of genome packaging and particle assembly. Another advantage of this system is that particles cannot be secreted unless the viral structural proteins