

3. その他

なし

表 1. ニホンザルの *Bartonella* 陽性数と血中菌数

捕獲地域	検体数	陽性数 (%)	血中菌数 (CFU/ml)
湯浅町	11	2 (18.1)	No.10 1.2×10^4
			No.11 6.0×10^3
白浜市	3	1 (33.3)	No.3 0.5×10^2
田辺市	1	1 (100)	No.1 3.4×10^2

表 2. ニホンザル分離株と *B. quintana* Fuller^T 株および RM-11 株の *gltA*, *rpoB* および ITS 領域における塩基配列の相同性

ニホンザル分離株*	<i>B. quintana</i> Fuller ^T 株 (ヒト由来)			<i>B. quintana</i> RM-11 株 (飼育アカゲザル由来)		
	<i>gltA</i>	<i>rpoB</i>	ITS	<i>gltA</i>	<i>rpoB</i>	ITS
MF1.1	98.0	99.0	98.0	100	100	100
MF3.1	98.0	97.0	98.0	100	98.0	99.0
MF10.1	98.0	99.0	98.0	100	100	99.0
MF11.1	98.0	99.0	98.0	100	100	99.0

* 同一個体から選択した 3 株は, 3 領域全てにおいて同一配列であった。

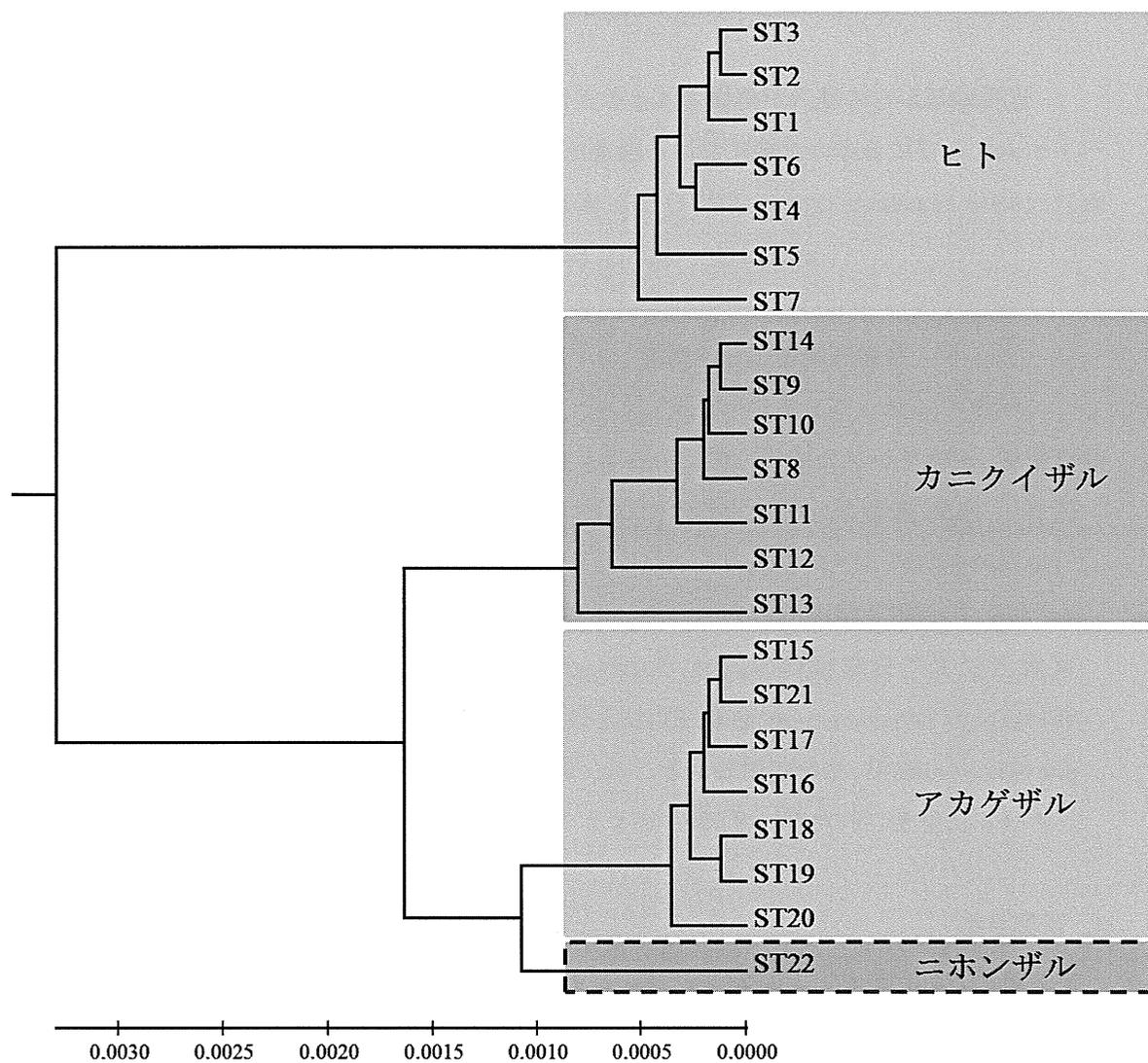


図 1. MLST 法に用いた 9 遺伝子領域の連結配列に基づく系統解析

各 ST の 9 つのハウスキーピング遺伝子領域の塩基配列を連結させ、UPGMA, Kimura 2-parameter モデルを用いて系統解析を行った。

厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）

分担研究報告書

近隣地域からの侵入が危惧されるわが国にない感染症の発生予防に関する研究
ベトナムならびに日本の土壌における類鼻疽菌の分布ならびに *Salmonella* Weltevreden の
Multilocus sequence typing (MLST) 法と Multiple-locus variable number tandem repeat
analysis (MLVA) 法による分子遺伝子型別

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研究要旨：東南アジアの環境に広く分布し、人獣共通感染症の原因菌の一つとして知られ、近年、海外感染症として我が国に持ち込まれる事例が報告されている類鼻疽 (*Melioidosis*) について、ベトナムならびに沖縄の土壌から類鼻疽菌 (*Burkholderia mallei*) の分離を試みた。その結果、培養法では類鼻疽菌は分離されなかった。また、東南アジアで分離頻度の高い *Salmonella* Weltevreden に分子遺伝子型別法である Multilocus sequence typing (MLST) 法と Multiple-locus variable number tandem repeat analysis (MLVA) 法を応用したところ、供試したベトナム由来 26 株は MLVA 法では 13 パターンに型別されたが、MLST 法では 1 パターンにしか型別できなかった。

A. 研究目的

類鼻疽菌 (*Burkholderia mallei*) は、東南アジアやオーストラリア北部の環境に広く分布し、人獣共通感染症の原因菌の一つとして知られており、我が国では 4 類感染症に指定されている。国内感染例は報告されていないが、近年、主に東南アジアで感染し帰国後発症した患者が、2010 年に 2 例、2012 年に 3 例、2013 年に 4 例報告されている。本研究では日本に侵入・定着することが危惧される類鼻疽の基礎研究の一環として、まず、我が国の本菌感染患者の渡航先として報告事例の多いベトナムならびに亜熱帯に属し、本菌の侵入

が危惧される沖縄県において、土壌から本菌の分離を試みた。また、東南アジアに広く分布し、これらの地域の人のサルモネラ感染症の大きな割合を占めており、近年、我が国の沖縄県に侵入し、その定着・拡大が問題となっている *Salmonella* Weltevreden について、病原微生物の菌株間の遺伝的異同を識別して、その感染源および感染経路を明らかにするための手法として開発されたシーケンス解析法である Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) 法ならびに Multilocus sequence typing (MLST) 法を用いて分子遺伝子型別を行い、その有

用性をフラグメント解析法である PFGE 法と比較しながら検討した。なお、MLVA 法は、ゲノム中の単純反復配列 (Tandem Repeat、以下 TR) を含む複数の遺伝子座を PCR で増幅し、各座位における TR の繰り返し数 (Repeat Number、以下 RN) の違いによって菌株の型別を行う手法である。また、MLST 法は 菌株ごとの複数遺伝子の配列の差異をパターン化し、それらを統合的に解析することにより株の型別を行う手法である。

B. 材料と方法

1. ベトナムならびに沖縄の土壌からの類鼻疽菌の分離

1) 供試検体

供試検体として、2013 年 5 月と 11 月にベトナム・メコンデルタで採取した水田の表土 40 検体ならびに 11 月に沖縄中部で採取した畑の表土 20 検体、計 60 検体を用いた。

2) 類鼻疽菌の分離・同定

供試検体 10 g を、5 倍量のリン酸緩衝生理食塩水 (PBS, pH7.2) とよく混和し、その上清を Ashdown's Medium 寒天培地に接種し、37°C で 48 時間培養した。培養後、培地上に発育してきた類鼻疽菌が疑われるコロニーを釣菌し、純培養後、生化学的検査を行い、類鼻疽菌を同定した。

2. *S. weltevreden* の MLVA ならびに MLST による遺伝子型別

1) 供試菌株

供試菌株として、これまでにベトナム・メコンデルタにおいてさまざまな検体から分離された *S. Weltevreden* 26 株を用いた。これらの供試菌株 26 株は、これまでの研究で、19 の PFGE パターンならびに 7 のリボタイプに型別され、さらに PFGE とリボタイピングの解析結果を組み合わせ、26 の遺伝子タイプに分類されることが明らかになっている。供試菌株からは、ボイル法により DNA を抽出し供試検体とした。

2) MLVA 法

MLVA は、Lindstedt らの方法に準じて、以下のように行った。使用する TR 領域は、過去に *Salmonella enterica* の MLVA で用いられた領域から 8 領域 (Sa102, Sa116, Sa120, TR1, TR2, STTR8, SE-4, SE-8) を選択した。供試検体を鋳型 DNA として、それぞれの領域ごとに所定の条件のもとに PCR を行い、得られた PCR 産物を鋳型として Applied Biosystems 3130 Genetic Analyzer (ABI) を用いて DNA シーケンシングを行い、各フラグメントに含まれる反復配列の RN を求めた。得られた配列データは、遺伝子解析ソフトウェア SeqScanner 2 (ABI) ならびに Molecular Evolutionary Genetics Analysis version 5.2 を用いて解析した。

3) MLST

MLST による解析では、ハウスキーピン

グ遺伝子 7 種と病原性遺伝子 2 種の計 9 種を用いた。ハウスキーピング遺伝子としては、コリスミ酸シンターゼをコードしている *aroC*、DNA ポリメラーゼ β サブユニットをコードしている *dnaN*、ウロポルフィリノーゲン III シンターゼをコードしている *hemD*、ヒスチジノールデヒドロゲナーゼをコードしている *hisD*、ホスホリボシルアミノイミダゾールカルボキシラーゼをコードしている *purE*、 α ケトグルタル酸デヒドロゲナーゼをコードしている *sucA*、ならびにアスパルトキナーゼおよびホロセリンデヒドロゲナーゼをコードしている *thrA* の計 7 種を、病原性遺伝子としては 宿主細胞の認識に関わる繊毛抗原をコードしている *fimH*、炎症反応などに関わるデユビキチナーゼをコードしている *sseL*、ならびにファージやプラスミド由来のスペーサー配列が挿入されたタンデムリピート配列であり、細菌自身の獲得免疫として機能する Cluster regularly interspaced short palindromic repeat (CRISPR) 2 領域を選んだ。PCR は、ハウスキーピング遺伝子については遺伝子増幅条件として、いずれも 94°C で 2 分維持した後、94°C 1 分、55°C 1 分および 72°C 1 分のサイクルを 34 回反復し、最後に 72°C で 2 分伸長反応を行った。病原性遺伝子については、遺伝子増幅条件として、94°C で 10 分維持した後、94°C 1 分、55°C 1 分および 72°C 1 分のサイクルを 28 回反復し、最後に 72°C で 10 分伸長反応を行った。増幅反応終了後、増幅産物を電気泳動で確

認した後、BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI) および Applied Biosystems 3130 Genetic Analyzer (ABI) を用いてシーケンシングを行った。シーケンシングにより得られた配列データは Sequence scanner v1.0 (ABI) および MEGA5 (27) を用いて解析し、それぞれの菌株における各ハウスキーピング遺伝子の塩基配列を決定した。決定されたそれぞれの菌株における各ハウスキーピング遺伝子の塩基配列を MLST データベースに提出し、MLST データベースにおいて定められた、それぞれの塩基配列に対応する番号を得た後、得られた各株の 7 種のハウスキーピング遺伝子全ての番号を用いて、MLST データベースにおいて Sequence type (ST) の検索を行った。なお、本研究における ST とは、*aroC*、*dnaN*、*hemD*、*hisD*、*purE*、*sucA*、および *thrA* の 7 つのハウスキーピング遺伝子の塩基配列を統合的に解析して得られる MLST データベースにおいて定められた番号である。得られた ST について、eBurst v3 を用い、MLST データベースに登録されている全 *S. enterica* 株の ST との比較を行った。また、病原性遺伝子については、遺伝子増幅条件として、いずれも 94°C で 10 分維持した後、94°C 1 分、55°C 1 分および 72°C 1 分のサイクルを 28 回反復し、最後に 72°C で 10 分伸長反応を行った。増幅反応終了後、増幅産物を電気泳動で確認した。なお、*fimH* および *sseL* については増幅が確認されたが、CRISPR1 および CRISPR2 について

は増幅が確認されなかったため、*fimH* および *sseL* の PCR 産物についてのみハウスキーピング遺伝子と同様にシーケンシングおよび配列解析に供した。

C. 研究結果

1. ベトナムならびに沖縄の土壌からの類鼻疽菌の分離結果

今回、ベトナム・メコンデルタで採取した水田の土 40 検体ならびに沖縄で採取した畑の土 20 検体から、培養法により類鼻疽菌の分離を行ったが、いずれの検体からも分離されなかった。

2. *S. weltevreden* の MVLA と MLST による遺伝子型別結果

1) MLVA 法

供試された *S. Weltevreden* 26 株について MVLA を実施した結果、13 の MLVA タイプに分類された。しかし、解析を行った 7 つの RN 領域においてが菌株間で異なっていた領域は、Sal16 と SE4 の 2 領域のみであり、特に Sal16 領域において RN のばらつきが顕著であった。MST をみると、*S. Weltevreden* の MLVA タイプ間の遺伝的差異は 1 遺伝子座であることが示された。

2) MLST 法

供試された *S. Weltevreden* 26 株について MLST を実施した結果、ハウスキーピング遺伝子は 26 株すべてで一致し、すべての株が ST365 に型別された。また、病原

性遺伝子 *fimH* と *sseL* については、いずれの遺伝子も供試した 26 株全ての配列が一致した。

D. 考察

1. ベトナムならびに沖縄の土壌からの類鼻疽菌の分離

今回、我が国の類鼻疽の海外での感染国として報告の多いベトナムならびに我が国で亜熱帯に属する沖縄で、水田ならびに畑の土を採取し、類鼻疽菌の分離を試みたが、培養法では分離されなかった。今回、土の中の類鼻疽菌の定量をする意味もあり、検体から選択培地に直接塗抹する方法で分離を試みたが、分離されなかった。ベトナム南部での類鼻疽菌の分布を明らかにした報告はみられないが、東南アジアや北オーストラリアなどで、土や河川水から PCR 法により類鼻疽菌の分離を行った報告では、比較的高率に本菌が分離されており、ベトナム・メコンデルタでも汚染菌量は少ないながらも土に本菌が分布している可能性は高いと思われ、次は増菌または PCR 法による検出を試みる予定である。

2. *S. weltevreden* の MLVA ならびに MLST による遺伝子型別

MLVA 法ならびに MLST 法のような遺伝子のシーケンス解析法は、PFGE 法などの遺伝子のフラグメント解析法に比べ、データベースを構築しやすく、他の研究施設との

間でデータの共有や比較がしやすい。

本研究で供試した *S. Weltevreden* 26 菌株は、過去の研究で 19 の PFGE パターンならびに 7 のリボタイプに型別され、それらの結果の組み合わせによって 26 の遺伝子タイプに分類されることが明らかになっている。本研究により、供試菌株 26 株は 13 の MLVA タイプに型別された。これらの成績から、今回用いた MLVA の *S. Weltevreden* についての菌株型別は、リボタイプングよりは高いものの、PFGE よりは低かった。しかしながら、Chiou らは、*S. Typhimurium* を PFGE ならびに MLVA で解析した結果、PFGE では 8 パターン、MLVA では 108 パターンに型別され、菌株型別能は MLVA のほうが高かったことを報告しているように、MLVA のほうが PFGE より高い識別能を示すことを指摘する研究者は多い。今回、RN のばらつきが確認された領域は、8 領域中、Sal16 領域と SE4 領域の 2 領域だけであったが、MLVA においては、RN のばらつきが多いほど、その領域の多型性が高いことになるので、今回使用した TR 領域のうち、多型性がみられたのは 2 領域だけであったことになる。したがって、今後 Sal16 領域と SE4 領域以外にも多型性がみられる領域をより多く含むプライマーセットを見いだして MLVA を行うことができれば、PFGE と同等かそれ以上の識別能力を発揮する可能性も考えられる。今回使用したプライマーは、*S. Typhi*、*S. Typhimurium* および *S. Enteritidis* を基に設計されたものであるため、今後は、

Boxrud らの研究でおこなわれているように、Tandem Repeat Finder software を用いて、*S. Weltevreden* のシーケンスデータから MLVA マーカーになりうる領域を探し出し、*S. Weltevreden* においてより菌株識別能の高いプライマーセットの開発を試みる必要があるだろう。また、MLST ではハウスキーピング遺伝子および病原性遺伝子を用いて MLST 解析を行った結果、いずれの場合も 1 種類に型別された。これらの結果より、*S. Weltevreden* において、ハウスキーピング遺伝子 *aroC*、*dnaN*、*hemD*、*hisD*、*purE*、*sucA*、および *thrA* を用いた MLST 法ならびに病原遺伝子 *fimH* および *sseL* を用いた MLST 法は、PFGE 法およびリボタイプング法と比較して菌株型別能が低いことが明らかとなった。

E. 結論

1. ベトナム・メコンデルタならびに沖縄では、培養法では土から類鼻祖菌は検出されなかった。
2. *S. weltevreden* 26 株は MLVA 法では 13 パターンに型別されたが、MLST 法では 1 パターンにしか型別できなかった。

F 健康危険情報

なし

G. 研究発表

なし

なし

2. 実用新案登録

なし

H. 知的財産権の出願・登録状況

3. その他

1. 特許取得

なし

III. 研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・印刷

N-linked glycan in tick-borne encephalitis virus envelope protein affects viral secretion in mammalian cells, but not in tick cells

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Tick-borne encephalitis virus (TBEV) is a zoonotic disease agent that causes severe encephalitis in humans. The envelope protein E of TBEV has one *N*-linked glycosylation consensus sequence, but little is known about the biological function of the *N*-linked glycan. In this study, the function of protein E glycosylation was investigated using recombinant TBEV with or without the protein E *N*-linked glycan. Virion infectivity was not affected after removing the *N*-linked glycans using *N*-glycosidase F. In mammalian cells, loss of glycosylation affected the conformation of protein E during secretion, reducing the infectivity of secreted virions. Mice subcutaneously infected with TBEV lacking protein E glycosylation showed no signs of disease, and viral multiplication in peripheral organs was reduced relative to that with the parental virus. In contrast, loss of glycosylation did not affect the secretory process of infectious virions in tick cells. Furthermore, inhibition of transport to the Golgi apparatus affected TBEV secretion in mammalian cells, but not in tick cells, indicating that TBEV was secreted through an unidentified pathway after synthesis in endoplasmic reticulum in tick cells. These results increase our understanding of the molecular mechanisms of TBEV maturation.

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INTRODUCTION

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* within the family *Flaviviridae*, causes tick-borne encephalitis (TBE) in humans. TBE is endemic in Europe, Russia and Far-Eastern Asia, including Japan (Blaskovic *et al.*, 1967; Korenberg & Kovalevskii, 1999; Süß, 2011). TBEV can be divided into three subtypes: the Far-Eastern subtype (known as Russian spring–summer encephalitis virus), the European subtype and the Siberian subtype (Ecker *et al.*, 1999; Gritsun *et al.*, 1993, 1997; Wallner *et al.*, 1995). TBE remains a significant public health problem in these endemic regions.

Flavivirus virions are spherical with diameters of 40–50 nm and contain a nucleocapsid and envelope. The envelope has two viral proteins: the major envelope protein E and the small membrane protein prM/M. Both proteins are synthesized as part of a polyprotein precursor, which is co- and post-translationally cleaved into the individual proteins (Lindenbach *et al.*, 2007). Protein E has been well characterized and mediates viral entry via receptor-mediated endocytosis; in addition, it contains the major antigenic epitopes that generate protective immune

responses (Heinz & Allison, 2003). The X-ray crystallographic structure of TBEV protein E ectodomain revealed that protein E forms head-to-tail homodimers that lie parallel to the viral envelope (Rey *et al.*, 1995). In low-pH conditions, as in endocytic vesicles, the homodimers dissociate, followed by the irreversible formation of homotrimers (Allison *et al.*, 1995; Stiasny *et al.*, 2001, 2002).

In the majority of TBEV strains, as in other flaviviruses, protein E contains a conserved *N*-linked glycosylation site. It has been reported that the deglycosylation of TBEV by endoglycosidase F does not impair infectivity (Winkler *et al.*, 1987), but the inhibition of *N*-linked glycosylation reduces the secretion of subviral particles from cells expressing the viral proteins prM and E (Goto *et al.*, 2005; Lorenz *et al.*, 2003). However, as functional analyses of the *N*-linked glycan on protein E have been limited to inhibitor treatment or subviral particle systems, little is known regarding the effects of glycosylation on the biological properties of infectious virions, including replication in the tick vector and pathogenicity in mammals.

In this study, we used an infectious TBEV cDNA clone to generate infectious virus containing protein E with or without the *N*-linked glycan and directly examined specific

One supplementary figure is available with the online version of this paper.

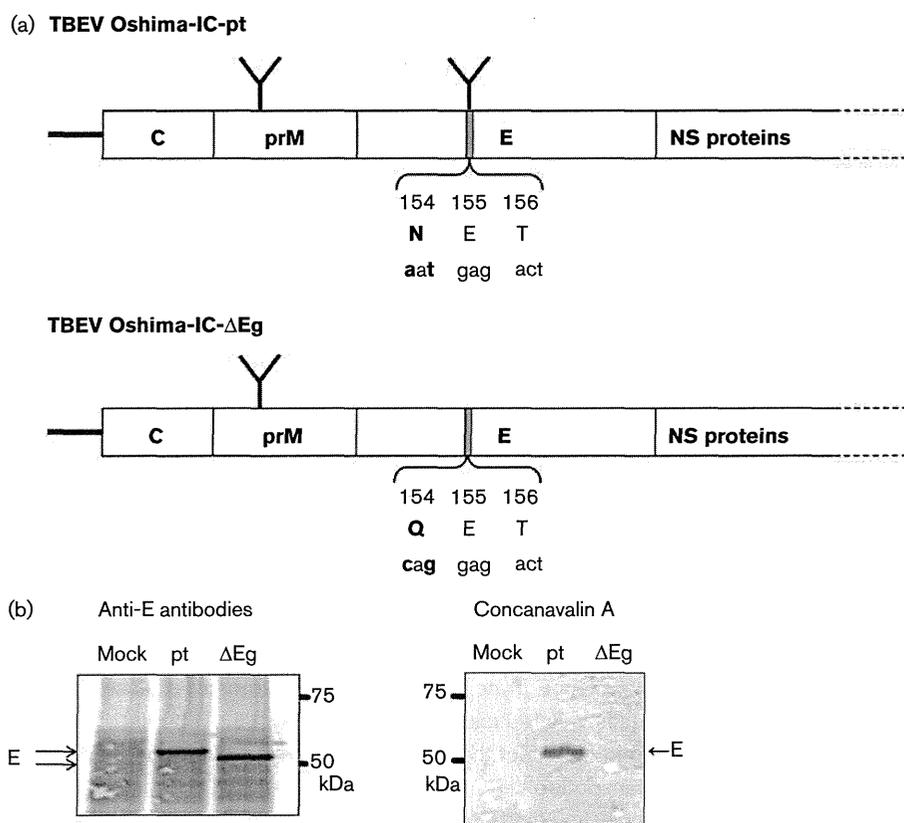


Fig. 1. Construction of recombinant TBEV containing protein E with or without its *N*-linked glycan. (a) Schematic of recombinant TBEV. The symbol Y shows the predicted glycans on TBEV envelope proteins. The amino acid sequence of the protein E glycosylation site is expanded at the bottom of the figure for Oshima-IC-pt. In Oshima-IC-ΔEg, the glycosylation site of protein E contains a mutation (bold). (b) Confirmation of protein E glycosylation in recombinant viruses. BHK cells were infected with Oshima-IC-pt (pt) or Oshima-IC-ΔEg (ΔEg). At 48 h p.i., intracellular protein E was immunoprecipitated using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (left panel) or concanavalin A (right panel).

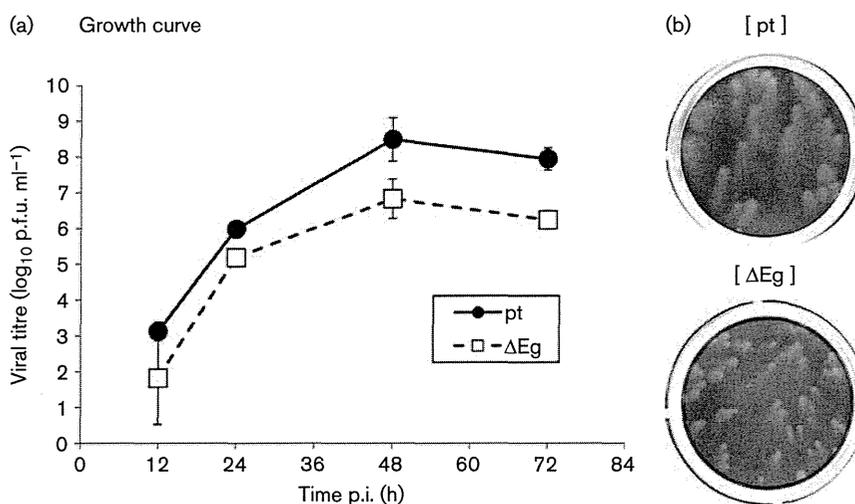


Fig. 2. Effect of protein E glycosylation on viral replication in BHK cells. (a) BHK cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at an m.o.i. of 0.01. At each time point, medium was harvested and virus titres were measured using plaque assays in BHK cells. (b) Plaques of Oshima-IC-pt and Oshima-IC-ΔEg in BHK cells at 4 days p.i.

phenotypic changes. Recombinant virus characteristics were examined in mammalian and tick cells as well as in a mouse model. The results suggest that glycosylation is critical for virus activity in mammals, but not in tick vectors.

RESULTS

Generation of glycosylation-deficient TBEV

TBEV protein E has one *N*-linked glycosylation site at amino acids 154–156. To examine the role of the *N*-linked glycan, recombinant TBEV expressing protein E that lacks the *N*-linked glycan, designated Oshima-IC- Δ Eg, was constructed using an infectious cDNA clone of Oshima 5-10 TBEV strain. The asparagine at position 154 of

protein E was mutated to glutamine to avoid recognition by oligosaccharyltransferase (Fig. 1a).

To confirm the absence of protein E glycosylation, the nucleotide sequences of the recovered Oshima-IC- Δ Eg virus were examined. The introduced mutation was conserved even after 10 passages in baby hamster kidney (BHK) cells and no complementary mutation was found in the coding sequence of any structural protein.

BHK cells were infected with the Oshima-IC-parent (pt) or - Δ Eg virus, and intracellular protein E was immunoprecipitated with anti-E monoclonal antibody and analysed on Western blots and lectin blots. As shown in Fig. 1(b), protein E was detected in immunoprecipitated eluates from cells infected with either Oshima-IC-pt or - Δ Eg, but the band for protein E from Oshima-IC- Δ Eg migrated faster

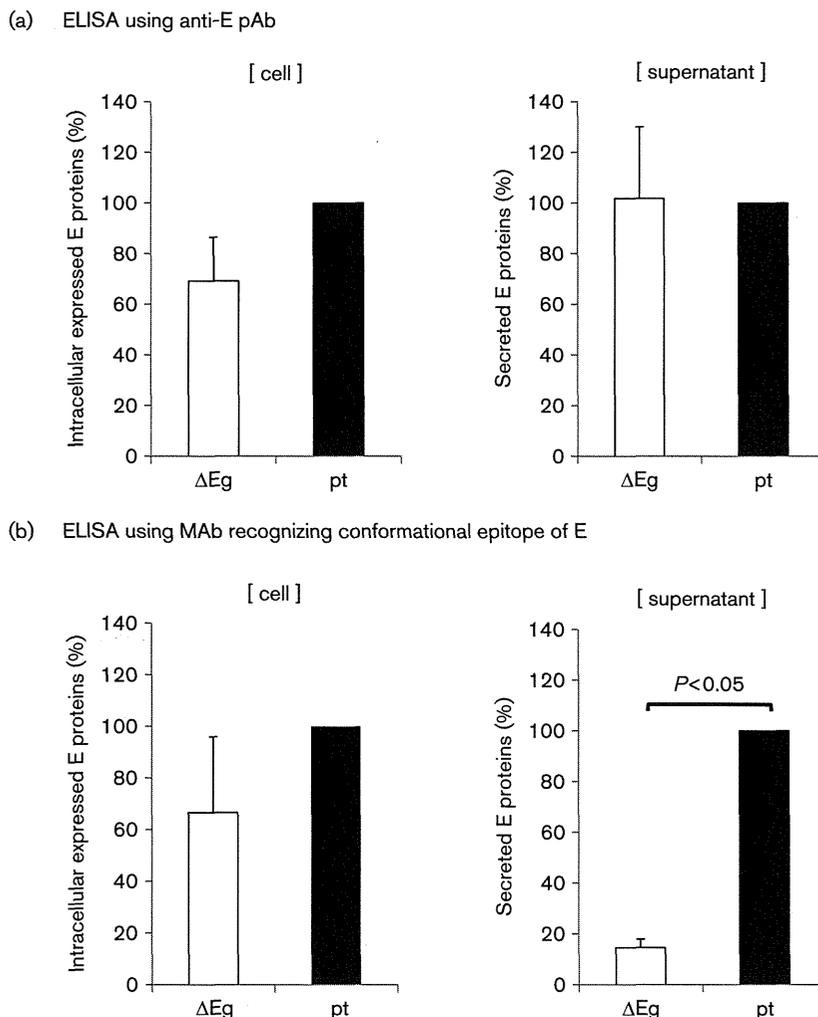


Fig. 3. Effect of protein E glycosylation on synthesis and secretion of protein E in BHK cells. BHK cells were infected with Oshima-IC-pt (pt) or Oshima-IC- Δ Eg (Δ Eg) at an m.o.i. of 0.01. At 48 h p.i., cell lysates and culture supernatants were harvested. Levels of intracellular and secreted protein E were measured by ELISA using (a) anti-E polyclonal antibodies, or (b) monoclonal antibodies recognizing conformational epitopes of protein E. The percentage of protein E was calculated from the calibration curve for the amount of pt in each experiment.

than that of protein E from Oshima-IC-pt (left panel). Protein E from Oshima-IC-pt was detected by concanavalin A, which binds specifically to high-mannose type *N*-linked glycans, whereas no band from cells infected with Oshima-IC- Δ Eg was detected by concanavalin (Fig. 1b, right panel). These data indicate that, as expected, mutated protein E encoded by Oshima-IC- Δ Eg virus was not glycosylated.

Characteristics of glycosylation-deficient TBEV in mammalian cells

To examine the effect of glycosylation of protein E on viral multiplication, BHK cells were infected with Oshima-IC-pt or Δ Eg at an m.o.i. of 0.01. Virus was harvested 12–72 h post-infection (p.i.) and the yield was quantified using a plaque assay. As shown in Fig. 2(a), a lower titre of infectious virus was secreted from cells infected with Oshima-IC- Δ Eg compared with -pt. The plaque size in BHK cells was also smaller for Oshima-IC- Δ Eg than for -pt. These data indicate that glycosylation of protein E affects viral multiplication in BHK cells.

To further characterize the role of glycosylated protein E, the secretion of viral particles was analysed. BHK cells were infected with Oshima-IC-pt or Δ Eg at an m.o.i. of 0.01. At 48 h p.i., cell lysates and culture supernatants were prepared and the levels of intracellular and secreted protein E were quantified. With the ELISA using polyclonal anti-E antibodies, the levels of intracellular and secreted protein E were similar between cells infected with Oshima-IC-pt and with Δ Eg (Fig. 3a). However, based on the ELISA using monoclonal antibodies specific for protein E conformational epitopes, low levels of protein E were detected in the culture supernatant of cells infected with Oshima-IC- Δ Eg compared with -pt, while the levels of protein E in cell lysates were similar between the cells infected with Oshima-IC-pt and with Δ Eg (Fig. 3b). These data show that the lack of protein E glycosylation did not affect the production or secretion of protein E, but did affect the conformation of secreted protein E.

Next, we examined whether the *N*-linked glycan on protein E of secreted TBEV was involved in viral entry. A total of 100 p.f.u. of Oshima-IC-pt or Δ Eg was treated with serially diluted *N*-glycosidase F, which cleaves all types of asparagine-bound glycans, and the infectivity of the resultant virus was analysed using a plaque assay (Fig. 4). There was no reduction of the virus titre of Oshima-IC-pt or Δ Eg even after treatment with 1 U ml⁻¹ of *N*-glycosidase F, indicating that the cleavage of *N*-linked glycans on secreted TBEV does not directly affect viral entry processes such as receptor binding and membrane fusion.

These results demonstrate that the conformational structure of protein E during secretion was affected by the lack of *N*-linked glycosylation and this reduced virion infectivity,

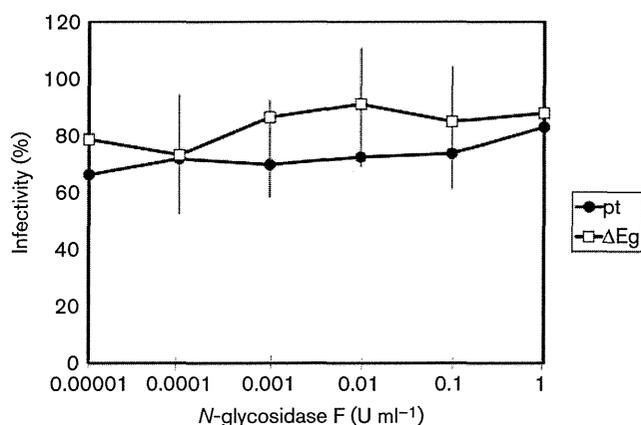


Fig. 4. Infectivity of TBEV after cleavage of the *N*-linked glycan on the virion. A total of 100 p.f.u. of Oshima-IC-pt (pt) or Oshima-IC- Δ Eg (Δ Eg) was treated with serially diluted *N*-glycosidase F and the virus titres were determined using plaque assays in BHK cells. The p.f.u. of mock-treated virus was set at 100%.

although the *N*-linked glycan was not required for viral entry.

Characteristics of glycosylation-deficient TBEV in tick cells

The effect of the absence of protein E glycosylation was examined in the tick cell line ISE6. As was observed with BHK cells, protein E of Oshima-IC-pt was glycosylated but protein E of Δ Eg was not (Fig. 5a). In contrast to BHK cells, ISE6 cells infected with Oshima-IC-pt and with Δ Eg showed no difference in viral multiplication (Fig. 5b) or the amount of secreted protein E detected using anti-E monoclonal antibodies recognizing protein E conformational epitopes (Fig. 5c). To investigate whether the different incubation temperature between mammalian cells (37 °C) and tick cells (34 °C) affected the stability of the unglycosylated E protein, the virus multiplication was examined in BHK cells at 34 °C. As was observed with the incubation at 37 °C (Fig. 2a), a lower titre of infectious virus was secreted from cells infected with Oshima-IC- Δ Eg compared with -pt in BHK cells at 34 °C (Fig. S1, available in JGV Online). These results indicate that the glycosylation of protein E was not important for viral multiplication or secretion in tick cells.

Flaviviruses are generally thought to bud into the endoplasmic reticulum of virus-infected cells, followed by transport in vesicles to the Golgi complex and release by exocytosis via the *trans*-Golgi network (Lindenbach *et al.*, 2007; Mackenzie & Westaway, 2001). To analyse the differences in the roles of TBEV glycosylation in maturation and secretory processes between BHK and ISE6 cells, the effects of inhibitors of cellular secretory mechanisms were investigated in virus-infected cells. Tunicamycin was used to inhibit the glycosylation of newly synthesized

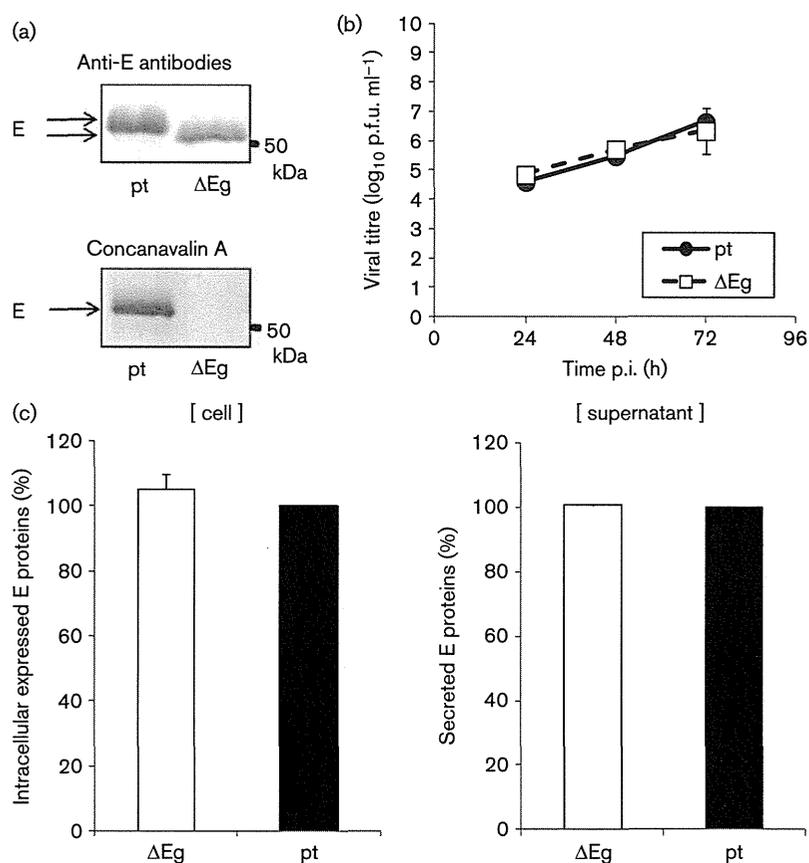


Fig. 5. Effect of protein E glycosylation on viral replication in ISE6 cells. ISE6 cells were infected with Oshima-IC-pt or Oshima-IC- Δ Eg at an m.o.i. of 0.01. (a) Intracellular protein E was immunoprecipitated using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (upper panel) or concanavalin A (lower panel). (b) At each time point, the medium was harvested and virus titres were determined using plaque assays in BHK cells. (c) At 48 h p.i., cell lysates and culture supernatants were harvested. The levels of intracellular and secreted protein E were measured by ELISA using an anti-E monoclonal antibody recognizing conformational epitopes of protein E. The percentage of protein E was calculated from the calibration curve for the amount of pt in each experiment.

glycoproteins (Elbein, 1987). Tunicamycin treatment of BHK cells infected with Oshima-IC-pt or Δ Eg reduced the secreted virus titre; the reduction was the same for Oshima-IC-pt and Δ Eg (Fig. 6a). In ISE6 cells infected with Oshima-IC-pt or Δ Eg, tunicamycin treatment did not reduce the virus titre (Fig. 6b). This suggests that the glycosylation of newly synthesized glycoproteins, including protein E and other glycoproteins such as protein prM or NS1, is important for the maturation and secretion of TBEV in mammalian BHK cells, but not in tick ISE6 cells. The secretion of infectious virions was further analysed using brefeldin A (BFA), which interferes with anterograde transport from the endoplasmic reticulum to the Golgi apparatus (Fujiwara *et al.*, 1988). BFA treatment of infected BHK cells significantly reduced the titres of secreted Oshima-IC-pt and Δ Eg, whereas BFA treatment of infected ISE6 cells did not reduce the virus titres (Fig. 6a, b). The glycosylation of the E proteins of Oshima-IC-pt was examined in ISE6 cells treated with tunicamycin or

BFA (Fig. 6c). Tunicamycin treatment inhibited the glycosylation of the E proteins, whereas the E proteins were still glycosylated after BFA treatment. Thus, the E proteins were naturally glycosylated in ER after synthesis in ISE6 cells, but this was not necessary for virus secretion. Furthermore, the secretion of TBEV in tick ISE6 cells was independent of the traditional secretory pathway through the Golgi apparatus.

Effect of glycosylation on virulence in mice

The effect of glycosylation on pathogenicity was examined in a mouse model. Five-week-old female C57BL/6J mice were infected subcutaneously with Oshima-IC-pt or Δ Eg at 10^5 p.f.u. mouse⁻¹ and monitored for 28 days (Fig. 7). All mice infected with Oshima-IC-pt showed general signs of illness such as hunched posture, ruffled fur and general malaise; one mouse died. However, no mice infected with Oshima-IC- Δ Eg showed signs of illness or died.

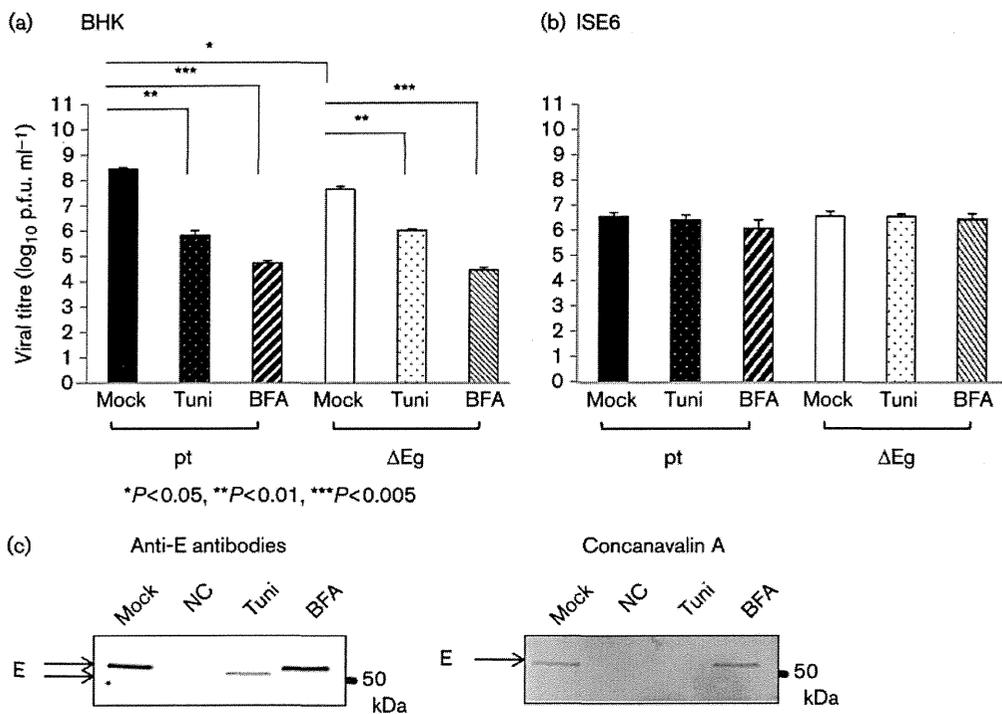


Fig. 6. The effect of inhibitors on the secretion of infectious virus. (a) BHK cells and (b) ISE6 cells were infected with Oshima-IC-pt or Oshima-IC-ΔEg at an m.o.i. of 0.01. At 24 h p.i. for BHK and 72 h for ISE6 cells, the medium was replaced with fresh medium containing 2 μg ml⁻¹ of tunicamycin (Tuni), 2 μg ml⁻¹ of brefeldin A (BFA), or DMSO (Mock). After 12 h, the medium was harvested and virus titres were determined using plaque assays in BHK cells. (c) After the various treatments, intracellular protein E in ISE6 cells infected with Oshima-IC-pt was immunoprecipitated using anti-E antibodies. Precipitated protein E was detected using anti-E antibodies (left panel) or concanavalin A (right panel). NC, Uninfected negative control cell.

To examine the correlation between disease development and viral replication in organs, viral loads in the blood, spleen and brain were compared between mice inoculated with 10⁵ p.f.u. of Oshima-IC-pt and 10⁵ p.f.u. of -ΔEg (Fig. 8). The levels of transient viraemia and multiplication in the spleen were lower in mice infected with Oshima-IC-ΔEg than in those infected with -pt. In the brain, the virus was detected from 6 days p.i. in mice infected with Oshima-IC-pt, while with -ΔEg a low titre of virus was detected in only one mouse at 12 days p.i. These data indicate that the Oshima-IC-ΔEg virus cannot multiply efficiently in organs, leading to a loss of virulence in mice.

Similar high titres of neutralizing antibodies (>320) were observed in mice infected with Oshima-IC-pt or with -ΔEg at 12 days p.i. (data not shown), suggesting that lack of the N-linked glycan on protein E did not affect the induction of neutralizing antibodies.

DISCUSSION

N-linked glycans on viral glycoproteins play important roles in viral multiplication, immunogenicity and pathogenicity (Vigerust & Shepherd, 2007). In this study, we

used an infectious TBEV cDNA clone to generate infectious virus with or without protein E N-linked glycan and investigated specific phenotypic changes in mammalian and tick cells.

The defect in protein E glycosylation reduced the secretion of infectious virions in mammalian cells. In studies of West Nile virus and dengue virus, a defect in glycosylation caused similar reductions in the release of infectious virions (Hanna *et al.*, 2005; Lee *et al.*, 2010; Li *et al.*, 2006). Although the total level of secreted protein E remained constant, the conformational structure of protein E was affected by the lack of glycosylation, resulting in reduced virion infectivity. However, cleavage of the N-linked glycan after secretion did not affect virion infectivity in mammalian cells. These results indicate that glycosylation is important in retaining the conformational structure of protein E, which is necessary for virion infectivity during the intracellular secretory process in mammalian cells. In the endoplasmic reticulum, two homologous resident lectins (calnexin and calreticulin) bind N-linked core glycans and promote proper folding of glycoproteins (Ellgaard *et al.*, 1999). It is known that the loss of glycosylation alters West Nile virus virion stability at mildly acidic pH (Beasley *et al.*, 2005). Defects in protein E glycosylation may affect the proper folding and/or stability of virions, reducing the infectivity of TBEV in mammalian cells.

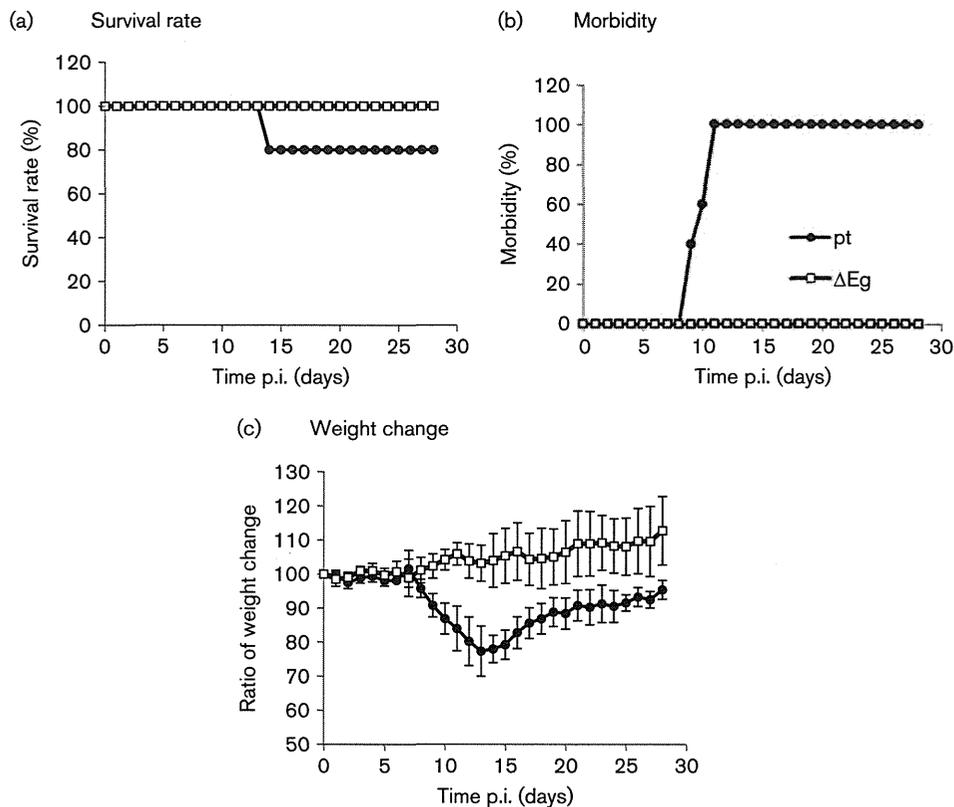


Fig. 7. (a) Survival rate, (b) morbidity and (c) weight change following infection with TBEV. B6 mice were subcutaneously infected with 10^5 p.f.u. of Oshima-IC-pt (filled circles) or Oshima-IC- Δ Eg (open squares) and monitored for 28 days. Mouse morbidity was estimated based on >10% weight loss. The mean daily weight change was calculated based on the ratio of the daily weight to the weight at day 0. Error bars represent standard deviations.

In the mouse model, protein E glycosylation affected TBEV pathogenicity. TBEV without protein E glycosylation did not multiply efficiently in peripheral organs, and eventually the virus could not enter the brain or cause disease in mice. Similarly, reduced neuroinvasiveness due to a defect in glycosylation was reported in West Nile virus studies (Beasley *et al.*, 2005; Shirato *et al.*, 2004). The mechanism of neuroinvasiveness of TBEV is unclear, but it has been reported that efficient viral multiplication in peripheral organs is required for TBEV entry into the brain (Mandl, 2005). Reduced infectivity of secreted virions owing to a defect in the glycosylation of protein E, as observed in cultured cells, is thought to reduce viral multiplication in peripheral organs and to reduce neuroinvasiveness.

TBEV with non-glycosylated protein E could efficiently induce neutralizing antibodies against TBEV without any clinical symptoms. Also, no revertant or compensatory mutation occurred during passaging. These data suggest that deletion of the protein E glycosylation site could attenuate TBEV.

The lack of protein E glycosylation did not affect the TBEV secretory process in tick cells, unlike in mammalian cells. Furthermore, the inhibition of transport from the

endoplasmic reticulum to the Golgi apparatus did not affect TBEV multiplication in tick cells. In a previous report, nascent TBEV particles were observed inside vacuoles, and free nucleocapsids were seen in the cytosol or attached to the membrane of virus particle-containing vacuoles in tick cells, whereas viral particles appeared in the endoplasmic reticulum, Golgi apparatus and secretory pathway in mammalian cells (Šenigl *et al.*, 2006). Taken together, our data suggest that TBEV secretion in tick cells occurs through an unidentified mechanism different from the traditional secretory pathway through the Golgi apparatus.

Glycosylation-independent virus secretion was observed only in TBEV-infected tick cells. However, studies of mosquito-borne flavivirus have shown that glycosylation of protein E is important in both mammalian and mosquito cells (Hanna *et al.*, 2005; Lee *et al.*, 2010). The difference in virus maturation between arthropod vectors may be associated with the different ecology of tick-borne and mosquito-borne flaviviruses in their arthropod vectors. Unlike mosquito-borne flaviviruses, tick-borne flaviviruses establish and maintain a persistent infection across the various life-stages of the tick vector, through transstadial

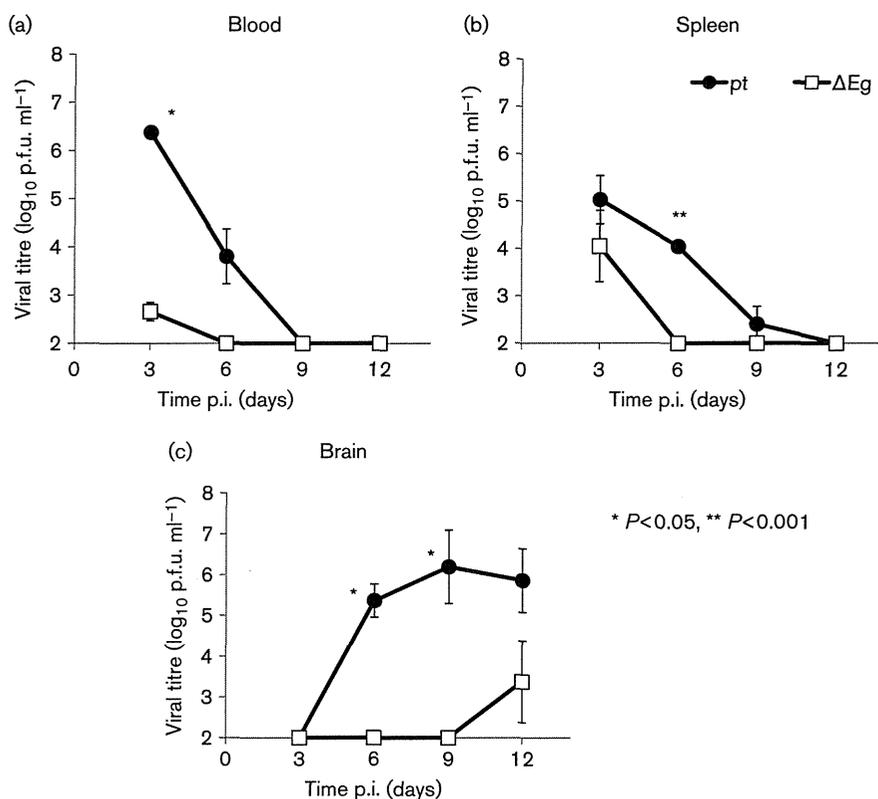


Fig. 8. Virus replication in organs. Mice were subcutaneously infected with 10^5 p.f.u. of Oshima-IC-pt (filled circles) or Oshima-IC-ΔEg (open squares). At the indicated days after infection, virus titres in (a) blood, (b) spleen and (c) brain were determined using plaque assays. Error bars represent standard deviations ($n=3$).

and transovarial transmission in nature (Nuttall & Labuda, 2003). In persistent tick infections, viruses are thought to multiply regardless of the glycosylation of the E proteins. It is possible that because TBEV with glycosylated protein E has more effective transmission from tick vectors to mammals, it had a selective advantage during viral evolution.

In summary, we generated recombinant TBEV with or without glycosylated protein E. Deletion of the glycosylation site affected the maturation of TBEV infectious virions in mammalian cells and reduced TBEV virulence in mice. Our results suggest that TBEV is secreted in a glycosylation-independent manner in tick cells. Overall, these results increase our understanding of the molecular mechanism of TBEV maturation and can be applied to attenuate TBEV infection.

METHODS

Cells. BHK cells were grown at 37 °C in Eagle's MEM supplemented with 8% fetal bovine serum (FBS) and L-glutamine. The ISE6 cell line from *Ixodes scapularis* was grown at 34 °C in L-15B medium with 10% FBS and 5% tryptose phosphate broth.

Virus. TBEV Oshima-IC was prepared from infectious cDNA clones of Oshima 5-10 strain (accession no. AB062003) (Hayasaka *et al.*,

2004), isolated in Hokkaido, Japan in 1995 (Takashima *et al.*, 1997). Standard PCR mutagenesis techniques were used to construct the Oshima-IC-ΔEg virus, in which nucleotides for the glycosylation site in protein E were mutated as shown in Fig. 1(a).

RNA was transcribed from the Oshima-IC plasmid using a mMESAGE mMACHINE SP6 kit (Life Technology) and was transfected into BHK cells using TransIT-mRNA (Mirus Bio) as described previously (Yoshii *et al.*, 2004, 2011).

Reagents. N-glycosidase F (Roche) was used to cleave protein E N-linked glycan in infectious virions. A total of 100 TBEV p.f.u. were treated with serially diluted N-glycosidase F ($10 \mu\text{U ml}^{-1}$ to 1U ml^{-1}) for 1 h at 37 °C and the virus was titrated.

The effects of tunicamycin (Sigma-Aldrich) and brefeldin A (Wako) on the secretion of viral particles were examined. At the indicated times p.i., virus-infected cells were treated with $2 \mu\text{g ml}^{-1}$ of tunicamycin, or $2 \mu\text{g ml}^{-1}$ of brefeldin A and the secreted virus was titrated after 12 h.

Immunoprecipitation, SDS-PAGE, immunoblotting and lectin-blotting. BHK cells were infected with Oshima-IC-parent (pt) or -ΔEg. At 48 h p.i., the cells were lysed with 1% Triton X-100 in 10 mM TBS, incubated on ice for 20 min and centrifuged ($16\,000 \text{g}$, 20 min). The supernatant (excluding the nuclear fraction) was precleared on protein G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C. The precleared lysates were precipitated with protein G-Sepharose beads with mouse monoclonal anti-E antibody

IH4 (Komoro *et al.*, 2000) for 2 h at 4 °C. Immune complexes were collected by centrifugation (10 000 g, 10 s) and washed four times with 1 % Triton X-100 in 10 mM TBS. Protein samples were electrophoresed through 12 % (w/v) polyacrylamide–SDS gels. Protein bands were transferred onto PVDF membranes and incubated with 1 % (w/v) gelatin in 25 mM TBS containing 0.01 % (v/v) Tween 20. After a wash with 25 mM TBS containing 0.01 % (v/v) Tween 20, the membranes were reacted with rabbit polyclonal anti-E antibodies (Yoshii *et al.*, 2004) or biotinylated lectin concanavalin A (J-Oil Mills), followed by alkaline phosphatase-conjugated secondary antibody or streptavidin (Jackson ImmunoResearch), respectively. Protein bands were visualized using an alkaline phosphatase detection kit (Merck) according to the manufacturer's protocol.

ELISA. The TBEV protein E was detected by sandwich-ELISA using a set of anti-E polyclonal antibodies or monoclonal antibodies recognizing conformational epitopes of protein E. Briefly, to prepare samples, virus-infected cells were lysed with 1 % (v/v) Triton X-100 in 10 mM TBS and the supernatants were treated with 1 % Triton X-100.

For ELISA using a set of anti-E polyclonal antibodies, Triton X-100-solubilized samples were added to 96-well microtitre plates coated with rabbit polyclonal anti-E antibodies. After blocking with 3 % (w/v) BSA, protein E was detected by incubation with TBEV-infected mouse serum and horseradish peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch).

For ELISA using monoclonal antibodies recognizing conformational epitopes of protein E, samples were added to wells coated with mouse monoclonal anti-E antibody 1H4, previously blocked with 3 % (w/v) BSA. Protein E was detected by incubation with biotinylated monoclonal antibody (mAb) 4H8 and peroxidase-conjugated streptavidin (Sigma). Peroxidase activity was detected by adding *o*-phenylenediamine dihydrochloride (Sigma) in the presence of 0.03 % (v/v) H₂O₂ and the absorbance was measured at 450 nm.

Virulence in mouse. Viruses were inoculated subcutaneously into 5-week-old female C57BL/6J mice (Charles River Laboratories). Morbidity was defined as >10 % weight loss. The mice were monitored for 28 days p.i. to determine the survival curve and mortality rate. To analyse the virus distribution in tissues, the serum, brains and spleens were collected from mice 3, 6, 9 and 12 days p.i. The organs were weighed individually, homogenized and prepared as 10 % suspensions in PBS (w/v) containing 10 % FBS. The suspensions were clarified by centrifugation (1200 g for 5 min, 4 °C) and the supernatants were titrated using plaque assays in BHK cells.

Titration and neutralization test. For titration, cell monolayers prepared in 12-well plates were incubated with serial dilutions of the virus for 1 h, overlaid with minimal medium containing 2 % FBS and 1.5 % carboxymethyl cellulose, and incubated for 5 days. After incubation, the cells were fixed and stained with 0.25 % crystal violet in 10 % buffered formalin. Plaques were counted and expressed as p.f.u. ml⁻¹. For the neutralization test, serum samples that induced a 50 % reduction in Oshima-IC-pt plaque formation were examined.

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