ORIGINAL ARTICLE

Substitutions of 169Lys and 173Thr in nonstructural protein 1 influence the infectivity and pathogenicity of XJ-160 virus

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Abstract An infectious clone (pBR-XJ160) was constructed using the full-length cDNA of the Sindbis-like XJ-160 virus. Two nucleotide mutations, causing amino acid changes at residue 169 from Lys to Arg and at residue 173 from Thr to Ile in the nonstructural protein (nsP) 1 coding region, strongly influenced the infectivity of in vitro-synthesized RNA. We used site-directed mutagenesis to obtain clones encoding a change to Arg at residue 169 of nsP1 (pBR-169), a change to Ile at residue 173 (pBR-173), or both changes (pBR-6973). Infectivity of RNA from pBR-169 was abolished, but viral forms BR-173 and BR-6973 were obtained from pBR-173 and pBR-6973, respectively. Further, BR-173 exhibited higher propagation than BR-XJ160 in cell culture and higher neurovirulence in a suckling mouse model. BR-6973 possessed an intermediate phenotype. BR-173 and BR-6973 showed increased sensitivity to 3-deaza-adenosine (3-DZA), which inhibits *S*-adenosylhomocysteine hydrolase. Thus, mutagenesis at residue 169 in the nsP1 region of XJ-160 is lethal, but mutation at residue 173 from Thr to Ile enhances viral infectivity and neurovirulence and suppresses the lethal effect of the mutation at residue 169. These mutations might be associated with the RNA methyltransferase (MTase) activity of nsP1.

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Introduction

Sindbis virus is the prototype member of the genus *Alphavirus* in the family *Togaviridae*. It is an enveloped virus with a single-stranded positive-sense RNA genome of about 12,000 nucleotides (nts) in length, which is capped and methylated at the 5′ terminus and polyadenylated at the 3′ terminus. The genomic RNA is infectious and serves as an mRNA for the four nonstructural proteins (nsPs), nsP1, nsP2, nsP3 and nsP4, named in order of their position in the 5′-terminal two-thirds of the 49S genomic RNA. By contrast, the structural proteins, capsid protein, and glycoproteins E3, E2, 6 K and El are translated from a 26S subgenomic RNA encoded by the 3′-terminal third of the genome [9, 15]. The precise roles of the nsPs have already been defined, and they are believed to be essential components of viral replication and transcription. The nsP1 region is thought to be involved in minus-strand synthesis [4, 16] and appears to be associated with methyltransferase activity and viral RNA capping [10–12, 17].

XJ-160 virus is a Sindbis-like virus isolated from a pooled sample of *Anopheles* mosquitoes collected in Xinjiang, China, in 1990 [8]. It has diverged significantly from the prototype Sindbis virus (S.A.AR86), with an 18% difference in nt sequence and an 8.6% difference in amino acid content. To elucidate the mechanism of propagation and pathogenesis of Sindbis virus, we have constructed the infectious full-length cDNA clone of XJ-160 [18, 19]. During this process, we obtained a rescued virus with a very low infectivity (almost no obvious CPE after three passages through BHK-21 cells). Sequence analysis indicates that there are 12 mutations in the full-length cDNA of XJ-160 virus used as the template for in vitro-synthesized RNA, including eight nonsense mutations, two sense mutations in the noncoding region and two mutations in the

nsP1 region, leading to a change at position 169 from Lys to Arg and at 173 from Thr to Ile. We then used a high-fidelity RT-PCR system to obtain the recombinant plasmid pBR-XJ-160, from which the rescued virus BR-XJ160, with similar characteristics to those of wild-type virus, could be produced. The sequencing results revealed that there were no mutations in the full-length cDNA of XJ-160 virus in pBR-XJ160. Therefore, we think that these two mutations greatly influenced the infectivity of XJ-160 virus. However, it is not clear which of the two mutations in nsP1 caused lethality of the initial cDNA construct or how the mutations influence the biological characteristics of XJ-160.

In this study, we constructed a panel of mutant viruses with single mutations at residues 169 or 173, or both, in the nsP1 coding region of XJ-160 and observed the effects of these mutations on infectivity and biological characteristics. Our results indicate that mutagenesis at residue 173 from Thr to Ile in nsP1 of XJ-160 not only enhances viral infectivity and neurovirulence but also compensates for the lethality to the virus caused by the mutation to residue 169. Moreover, these phenotypic changes to XJ-160 virus caused by the mutations might be associated with the viral RNA methyltransferase activity of nsP1.

Materials and methods

Virus strains, cell line, plasmid and antibody

XJ-160 virus is a Sindbis-like virus stored in our laboratory. Baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml each of penicillin and streptomycin. Recombinant plasmid pBR-XJ160 is an infectious full-length cDNA clone of XJ-160, from which rescued virus BR-XJ160 can be obtained by transcription in vitro followed by transfection. The BR-XJ160 virus produced in BHK-21 cells was indistinguishable from the XJ-160 virus in its biological properties, including its plaque morphology, growth kinetics and suckling mouse neurovirulence [18, 19]. The XJ-160-virus-specific antibody was prepared by our laboratory.

Construction and identification of site-directed mutants

Nucleotides 565 and 577 (corresponding to residues 169 and 173, respectively) in nsP1 gene of XJ-160 virus play an important role in the infectivity of in vitro-synthesized RNA. In the order of the positions of mutagenic nts, oligonucleotides used to generate the mutant constructs are shown in Table 1. Using pBR-XJ160, recombinant clones with single or double mutations were constructed using the QuickChange II XL Site-directed Mutagenesis Kit. In brief, a mutated copy of the original plasmid was produced by a polymerase chain reaction (PCR)-like amplification using *Pfu* polymerase and primers containing the desired mutation. The parental template was then digested specifically by the restriction enzyme *Dpn*I, which cuts only DNA methylated by DNA adenine methylase. The nicked vector DNA incorporating the desired mutations was introduced into *Escherichia coli* by transformation. Reaction parameters were chosen according to Stratagene's recommendations. Finally, all mutant constructs were confirmed and identified by sequencing analysis (Shanghai SHENG-GONG Inc.).

Production and identification of mutant viruses

Three mutant cDNA clones, together with pBR-XJ160, were all linearized by *Xho*I, and then transcript RNAs were obtained by in vitro transcription using mMESSAGE mMACHINE™ transcription SP6 Kit (Ambion, UK) following the manufacturer's instructions. After purification, the in vitro-synthesized RNA were electroporated into BHK-21 cells in 0.2 cm Gene Pulser Cuvettes using the Gene Pulser Xcell apparatus (Bio-Rad) at 160 V, 25 µF and 200 Ω. After electroporation, the BHK-21 cells were

Table 1 Oligonucleotides used for site-directed mutagenesis in the nsP1 gene of XJ-160 virus

Primer	Sequence (5′ → 3′)	Position (nt)	Amino acid change
169 (+)	ggaactatttaccatcaagctatgaGaggcgtgcggacactttactgg	540–587	169Lys → Arg
169 (−)	ccagtaaagtgtccgcacgcctCtcatagcttgatggtaaatagttcc	587–540	
173 (+)	ccatcaagctatgaaaggcgtgcggaTactttactggattgggtttgacacc	552–602	173Thr → Ile
173 (−)	ggtgtcaaacccaatccagtaaagtAtccgcacgcctttcatagcttgatgg	602–552	
6973 (+)	ccatcaagctatgaGaggcgtgcggaTactttactggattggg	551–593	169Lys → Arg and 173Thr → Ile
6973 (−)	cccaatccagtaaagtAtccgcacgcctCtcatagcttgatgg	593–551	

The nucleotide position refers to the complete nucleotide sequence of XJ-160 virus (AF103728); Capital letters in the sequences of the primers indicate the directed mutations in nsP1, which caused the amino acid changes shown in the table

incubated on ice for 10 min, and then DMEM with 10% FBS was added. When the monolayers were confluent (after about 24 h), the culture medium was replaced with maintenance medium (DMEM supplemented with 2% FBS). The culture supernatants from the BHK-21 cells were collected to infect another monolayer of BHK-21 cells, and the cytopathic effect (CPE) was examined daily using light microscopy. The supernatants collected from BHK-21 cells were frozen at $-80°C$.

To confirm the nt sequence of the mutant viral RNA genome, cDNA fragments containing the point mutations were amplified from viral RNAs extracted from virus-infected supernatant by reverse transcription (RT)-PCR. Sequencing of the whole genome of each mutant virus was carried out commercially (Shanghai SHENGGONG Inc.).

Indirect immunofluorescence assay

To examine the expression of XJ-160-specific protein, immunofluorescence (IFA) tests were first performed 48 h after transfection with in vitro-synthesized RNA, then IFA tests were performed again after the first passage of culture supernatant to another monolayer of BHK-21 cells. In brief, the cells were seeded in 35-mm-diameter dishes with glass slides. When the cells were 80% confluent, they were transfected with either transcript RNAs or culture supernatants, and BHK-21 cells were harvested 48 h post-inoculation and fixed in cold acetone. After open-air drying and a second wash with cold phosphate-buffered saline (PBS), 1:100 diluted anti-XJ-160 virus antibodies were added and incubated at 37°C for 30 min in a moist chamber. After washing three times with PBS and air-drying, secondary antibodies diluted 1:100 with azovan blue (FITC-labeled sheep anti-mouse antibody) were added to each well and incubated at 37°C for 30 min. Finally, the slides were washed three times with PBS, mounted with 90% glycerin and observed under a fluorescence microscope.

Growth properties of the mutant viruses

Observation of plaque morphology and the determination of virus titers were performed by plaque testing in BHK-21 cells. Briefly, confluent monolayers were incubated and passaged on six-well plates at 37°C under 5% CO_2; 0.5-ml aliquots of the diluted samples were added to each well and incubated for 1 h. After the supernatant was aspirated, 2-ml aliquots of 1% low-melting-temperature agarose in $2 \times$ DMEM containing 4% fetal calf serum were added. Then, each plate was turned over and incubated under 5% CO_2 at 37°C in humidified air. When CPE was evident microscopically, another 2 ml of 1% agarose containing 10% neutral red was added, and the sizes and numbers of plaques were examined at 6 h postincubation.

The growth curves for the viruses were measured in 75-cm^2 flasks with BHK-21 cells at a multiplicity of infection (MOI) of approximately 0.01 plaque-forming units (PFU)/cell. After adsorption at 37°C for 1.5 h, 30 ml of maintenance DMEM with 2% FBS was added, and the cultures were incubated under 5% CO_2 in air at 37°C. Aliquots of culture medium were removed at 8-h intervals for about 3 days, and the titer of the virus released into the medium during each interval was determined by virus plaque assay. The results shown in this study are the means of three independent growth experiments.

Sensitivity of mutant viruses to 3-DZA

Several studies have indicated that nsP1 of Sindbis virus plays an important role in minus-strand synthesis [4, 16] and in viral RNA capping [10, 11], and these biological functions are all involved with the methyltransferase (MTase) activity of nsP1. In addition, 3-deazaadenosine (3-DZA) is an adenosine analog that inhibits S-adenosyl-homocysteine (AdoHcy) hydrolase [14]. Therefore, to investigate whether there was any association between the phenotypic alternation of XJ-160 virus and the site-directed mutagenesis in its nsP1 region, we examined the effect of each mutation on viral sensitivity to 3-DZA by observing plaque morphology on BHK-21 cells. Briefly, plaques were formed by BR-XJ160 virus, BR-173 virus and BR-6973 virus in the presence and absence of 3-DZA. In each case, the various virus yields were titrated by plaque assay on BHK-21 cells in the absence and presence of 3-DZA at 0.8, 1.0, 1.2 and 1.4 μg/ml, respectively. Each point represents the mean titer of three independent wells.

Animal experiments

To evaluate the effects of the mutated nsP1 proteins on the pathogenicity of XJ160, the neurovirulence of the mutant viruses in mice was assessed using a suckling mouse model. In brief, groups of 12 BALB/c strain suckling mice (3 days old) were inoculated intracerebrally (i.c.) with 30 μl of 10^8 PFU/ml virus sample, and an equal volume of DMEM with 1% fetal bovine serum was used as control. The numbers of dead mice and the time to death were recorded for 14 days.

Results

Viability of mutant viruses in BHK-21 cells

Based on the infectious clone of XJ160 virus, we obtained mutant cDNA clones using QuickChange II XL

Site-directed Mutagenesis Kits. The recombinant clones with the desired mutations were designated pBR-169, pBR-173 and pBR-6973, whose designation was based on the locations of the site-directed mutation within nsP1; thus, the mutant pBR-169 contains a mutation at residue 169. The compositions of all the recombinant clones were confirmed by sequencing.

The initial step of our characterization of the nsP1 site-directed mutants was to assess their competence for protein expression in BHK-21 cell culture. Because protein expression depends on both translation and replication of the viral genome, an inability to detect XJ-160 virus-specific protein in this experiment would suggest that one or both of these functions were impaired significantly by the mutation in nsP1. RNAs transcribed from the three mutated and the parental wild-type cDNA clones were introduced by transfection into BHK-21 cells, which were assayed for XJ-160-virus-specific protein expression 48 h postinfection. Wild-type XJ-160 virus was also included in the experiment. As can be seen in Fig. 1a, the wild-type virus and all of the mutants yielded positive immunofluorescence staining. In contrast, the control was negative. These results indicated that the mutant RNAs were competent for protein expression in BHK-21 cells. To investigate whether the released material contained infectious particles, each culture supernatant was transferred to new BHK-21 cells, and XJ-160-virus-specific protein expression was detected as before (Fig. 1b). Clearly, as with XJ-160 virus, all of the mutants except for the one carrying a single mutation at residue 169 in nsP1 (pBR-169) were transmitted to new BHK-21 cells. IFA tests were consecutively performed during three passages through BHK-21 cells, and XJ-160-virus-specific

DNA segment analysis by RT-PCR was simultaneously performed. All the results indicated that mutant RNA from pBR-169 never produced infectious progeny in BHK-21 cells.

When expression and particle formation were analyzed in more detail, as with the parental virus, typical 24-h-posttransfection CPEs were observed for BHK-21 cells with all of the transcripts except the one made from the mutant clone pBR-169. According to the location of the substitution within nsP1, the mutant viruses were designated BR-173 and BR-6973, respectively. The sequencing results of the entire genome cDNA in each rescued mutant virus confirmed the absence of any mutation except for the engineered mutations in these viral stocks (data not shown). Thus, the XJ-160 virus is capable of assembling infectious particles despite a single site-directed mutation at residue 173 or a double mutation at residues 169 and 173. In contrast, the mutation at residue 169 by itself was lethal.

Effects of site-directed mutations on the propagation characteristics of XJ-160

To evaluate the effects of the mutations on the plaque phenotype of XJ-160 virus, the plaque size and plaque-forming time of the mutant viruses, XJ-160 virus and its rescued virus BR-XJ160 were compared in BHK-21 cells. As shown in Fig. 2, both BR-173 and BR-XJ160 could form clear plaques that had similar morphological characteristics to those of the parental virus. However, unlike the plaques formed by BR-173, those formed by BR-6973 were smaller than those formed by the wild-type or rescued viruses. In contrast, no plaques could be formed on the BHK-21 cells
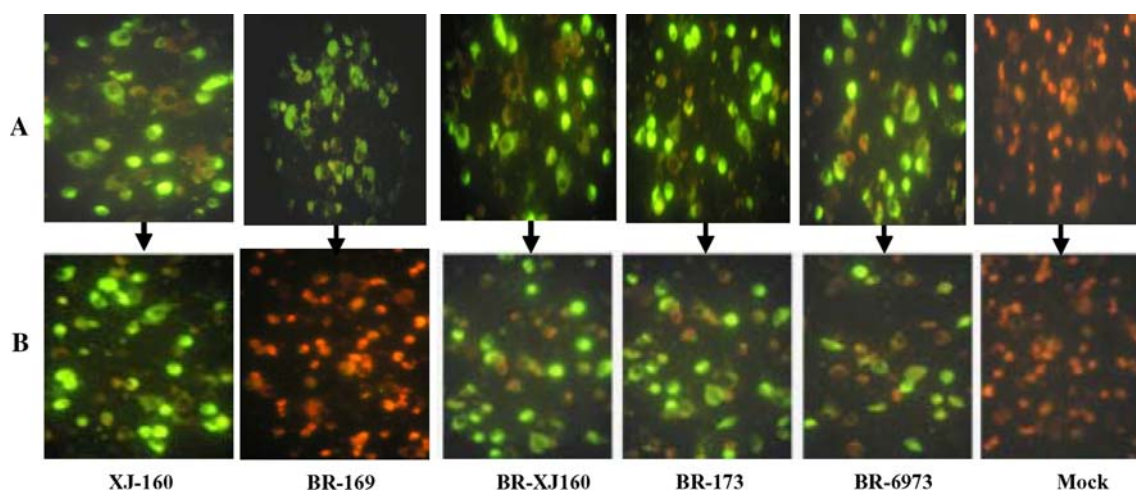

Fig. 1 IFA assay of expression of specific proteins of XJ-160 virus. a Protein expression of XJ-160 virus and transcript RNA from pBR-XJ160, pBR-169, pBR-173, and pBR-6973 into BHK-21 cells detected by IFA (48 h postinfection). b Expression of protein specific to XJ-160 virus determined by IFA after inoculation of new BHK-21 cells as symbolized by the *arrows*

Fig. 2 Plaque phenotypes of mutant viruses in BHK-21 cells. Plaque-forming assay of mutant viruses on BHK-21 cells. BHK-21 cells infected with XJ-160 virus were used as a positive control, and normal BHK-21 cells (mock) were used as a negative control in the experiment

XJ-160   BR-169   BR-XJ160

BR-173   BR-6973   Mock
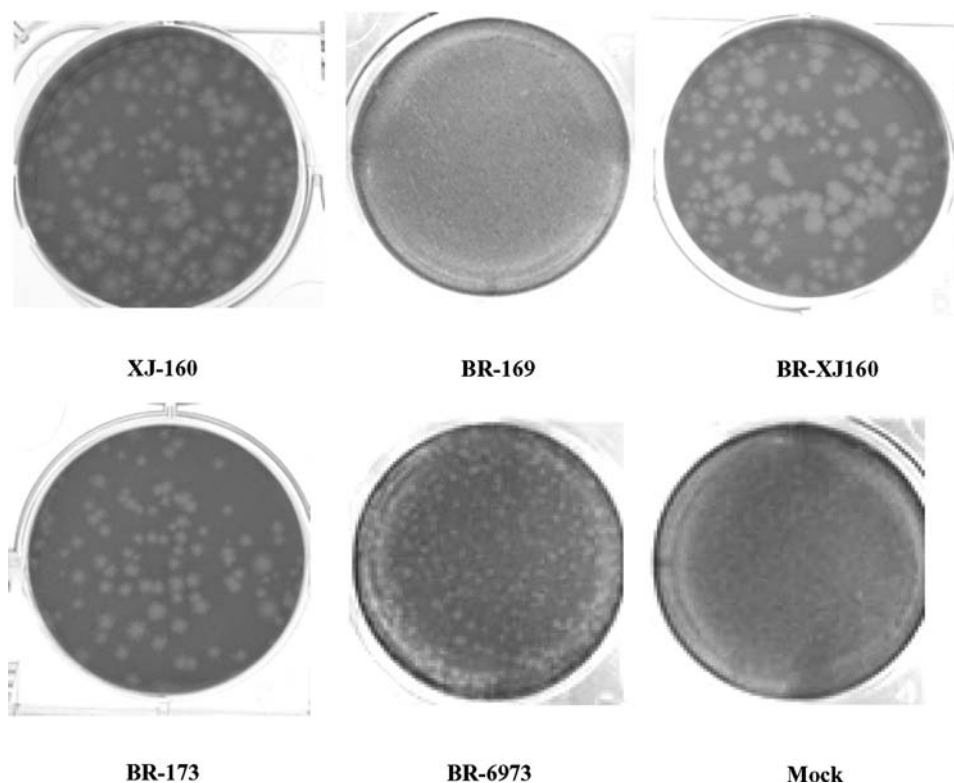
infected by transcript RNA from pBR-169, which was consistent with previous results. Thus, both BR-173 and BR-6973 were competent for the formation of plaques using BHK-21 cells, but the double mutation reduced plaque size and prolonged the plaque-forming time.

To examine the roles of the site-directed mutations in the propagation of rescued viruses, growth curves for the mutant and BR-XJ160 viruses were constructed using BHK-21 cells. As shown in Fig. 3, BR-6973 with a double mutation released fewer infectious particles than did the rescued BR-XJ160 virus produced from a wild-type clone in BHK-21 cells, whereas BR-173 released more infectious particles than did BR-XJ160. However, the growth tendencies of the mutant viruses were indistinguishable from those of the rescued virus without any mutation, which exhibited rapid propagation from 8 to 32 h after inoculation and peaked at 48 h after infection.
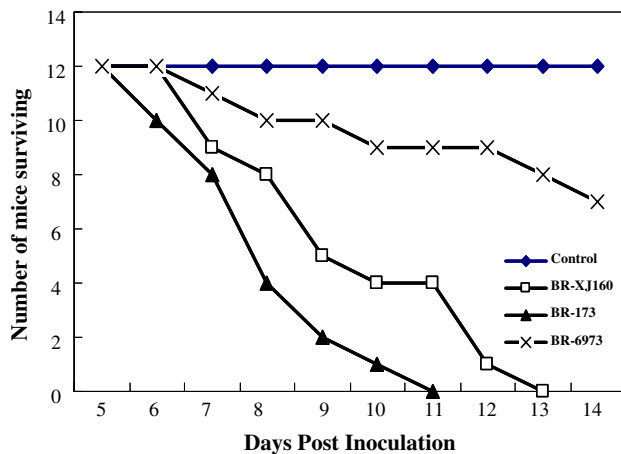
Suckling mouse neurovirulence of mutant viruses

The inoculation of mice with Sindbis-group viruses provides an excellent model for studying virally induced neurologic disease. The outcome of Sindbis virus infection in the mouse model correlates with the age and strain of the animal, virus dose, route of inoculation and virus strain. Because of the neurovirulence properties of XJ-160 virus, we inoculated 3-day-old mice with mutant viruses to
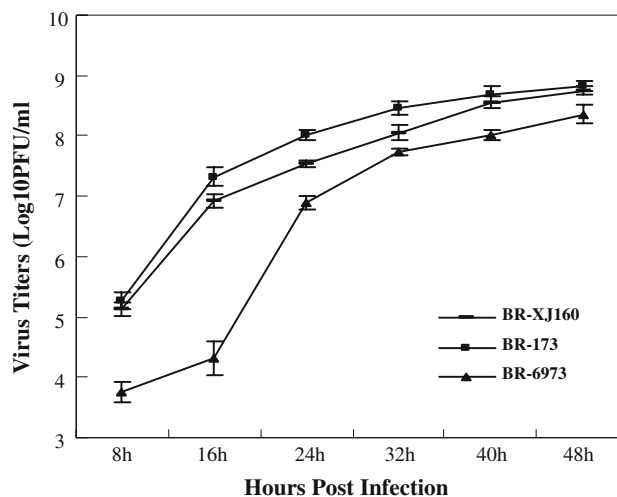
Fig. 3 Growth properties of mutant viruses in BHK-21 cells. The mutant BR-173 (173Thr → Ile) virus grows to higher levels than BR-XJ160 derived from pBR-XJ160 in in vitro growth curves, while the mutant BR-6973 (169Lys → Arg and 173Thr → Ile) releases the lowest number of virus particles in BHK-21 cells. BHK-21 cells were infected with BR-XJ160, BR-173 or BR-6973 at an MOI of 0.01. Samples of supernatant were removed at the indicated time points and evaluated for virus yield by plaque assay. The data shown are from one of three representative experiments. Each point represents the average titer of three independent wells with the standard deviation

observe the effects of the substitution on the virulence properties of XJ-160. The numbers of mice killed and the time to death are shown in Fig. 4. Both BR-173 and the

Fig. 4 Neurovirulence of mutant viruses for suckling mice. Twelve suckling mice (3 days old) as a group were inoculated intracerebrally (i.c.) with 10^8 PFU/ml parental viruses or recombinant viruses in 30 μL, and an equal volume of Eagle's medium with 1% fetal bovine serum was used as control

parental virus were fatal for suckling mice; however, the time until death varied somewhat with the different viruses. Thus, the time to death of suckling mice inoculated by BR-173 was from days 6 to 11 postinoculation, while that of BR-XJ160 from the wild-type clone was between days 7 and 13, and the mean time to death of the mice killed by BR-173 virus was 8.1 ± 2.5 days, which was apparently shorter than that of the mice killed by BR-XJ160 virus (10.3 ± 2.4 days). On the other hand, BR-6973 appeared less neurovirulent, because only 4 of 12 mice inoculated with the virus died during 14 days postinoculation, and the others in this group recuperated. In addition, none of the rescued viruses produced fatality in adult mice, which

agrees with the reported biological characteristics of XJ-160. These results indicate that the mutation at residue 173 in nsP1 enhanced the neurovirulence of XJ-160 in this suckling mouse model. In contrast, BR-6973 with double mutations within nsP1 became less pathogenic.

Changed sensitivity of mutant viruses to 3-DZA

S-adenosylmethionine (AdoMet) as substrate and adenosylhomocysteine (AdoHcy) as a product and competitive inhibitor of all transmethylation reactions are potential regulators of methylation-dependent cellular processes. In mammalian cells, the only metabolic route of AdoHcy elimination is hydrolysis to adenosine and homocysteine by S-adenosylhomocysteine hydrolase (AdoHcyase). 3-deazaadenosine (3-DZA) is an adenosine analog that inhibits AdoHcyase, which leads to an accumulation of AdoHcy in host cells. Thus, 3-DZA is potentially an inhibitor of methyltransferase (MTase) activity. In addition, previous investigations proved that there was a close correlation between retention of MTase activity in nsP1 and infectivity of the Sindbis RNA transcripts [10–12, 16]. Therefore, 3-DZA was used to investigate whether there was association between the phenotypic alternation of XJ-160 virus and the site-directed mutagenesis in the nsP1 region.

First, we observed plaque morphology of mutants on BHK-21 cells preincubated with or without 3-DZA. As can be seen in Fig. 5, the sizes of plaques formed on BHK-21 cells by BR-XJ160 virus, BR-173 virus and BR-6973 virus were not obviously affected by 3-DZA. However, virus yields in the presence of 3-DZA varied markedly among the three viruses (Table 2). 3-DZA markedly reduced the

Fig. 5 Sensitivity of mutant viruses to 3-DZA. Plaques were formed by BR-XJ160 virus, BR-173 virus and BR-6973 virus in the absence and presence of 3-DZA (1.0 μg/ml)
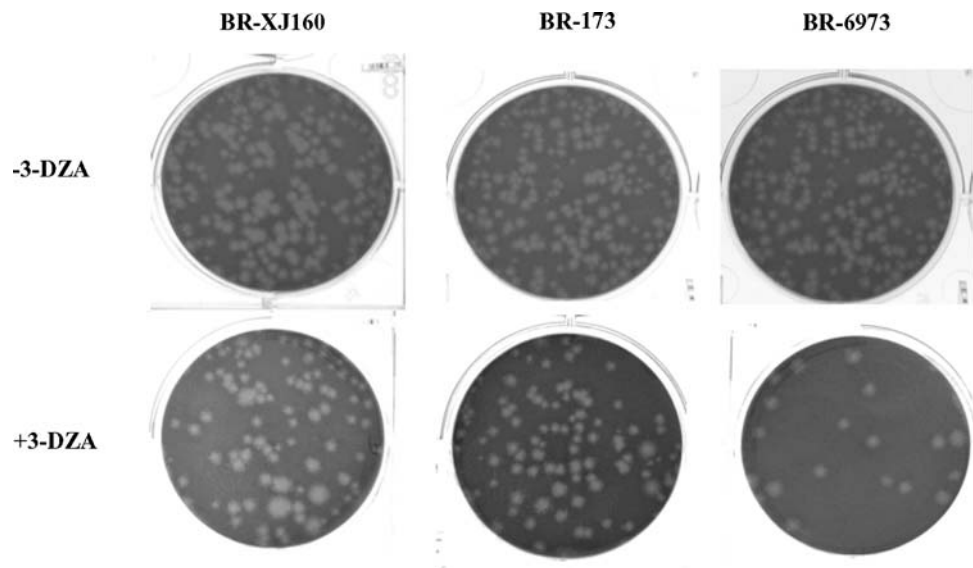
Table 2 Sensitivity of mutant viruses to 3-DZA

3-DZA (μg/ml)	Virus yield (PFU/ml)					
	BR-XJ160[a]	BR-XJ160[b]	BR-173[a]	BR-173[b]	BR-6973[a]	BR-6973[b]
0.8	5.0×10^8	8.0×10^7	7.1×10^8	5.1×10^8	8.6×10^7	5.0×10^6
1.0	5.0×10^8	4.1×10^6	7.1×10^8	8.0×10^7	8.6×10^7	4.5×10^4
1.2	5.0×10^8	5.7×10^5	7.1×10^8	5.4×10^7	8.6×10^7	6.3×10^3
1.4	5.0×10^8	6.3×10^4	7.1×10^8	9.3×10^5	8.6×10^7	5.1×10^2

[a] and [b] The various virus stocks were titrated by plaque assay on BHK-21 cells in the absence and presence of 3-DZA. Each point represents the mean titer of three independent wells

yield of BR-6973 virus, almost 10^5-fold when the concentration of 3-DZA was 1.4 μg/ml. In contrast, 3-DZA did reduce the numbers of plaques formed by both BR-173 virus and BR-XJ160 virus, but the effect of 3-DZA on BR-173 virus was less than that on BR-XJ160 virus. 1.4 μg/ml 3-DZA reduced the yield of BR-XJ160 virus and BR-173 virus almost 10^4 and 10^3-fold. Therefore, the mutation at nsP1 residue 173 decreased the sensitivity to 3-DZA of XJ-160 virus, while BR-6973 virus with double mutations has an increased sensitivity to 3-DZA.

Discussion

During the process of constructing an infectious full-length cDNA clone of the XJ-160 virus, the nts at 565A and at 577C in nsP1 were found to be highly associated with the infectivity of in vitro transcript RNAs. Further analysis revealed that the 565A nt is highly conserved in all viruses in the genus *Alphavirus*, whereas the 577C nt is conserved in several Sindbis viruses (Fig. 6a). Whereas the corresponding amino acids 169Lys and 173Thr are also highly conserved, 169Lys is conserved in all of the viruses in the genus *Alphavirus* in the family *Togaviridae* (Fig. 6b). In addition, Ahlquist et al. [1] observed that there are significant sequence similarities between the Sindbis nsP1, nsP3 and nsP4 and nsPs encoded by three plant viruses, alfalfa mosaic virus (AMV), brome mosaic virus (BMV) and tobacco mosaic virus (TMV). Presumably, the proteins that show these sequence similarities carry out similar functions with respect to viral replication. It is of considerable interest in this respect that in the AMV and TMV proteins, which are homologous to Sindbis virus nsP1, the amino acid at the position that corresponds to residue 169 of XJ-160 is also a lysine and that of BMV is a histidine, which has similar characteristics to lysine. For example, both of them are small, hydrophilic, charged and basic amino acids. However, the residue in two of the three plant viruses at the position corresponding to residue 173 of XJ-160 is not threonine. We postulate that the 169Lys in nsP1 possibly plays a more important role than 173Thr does in

the replication of XJ-160. In this study, we found that while mutation of nsP1 169Lys to Arg led to a loss of the infectivity of RNA transcripts, the substitution of Thr173 to Ile in nsP1 could increase propagation in BHK-21 cells and intensified the neurovirulence of XJ-160 in the suckling mouse model. It also compensated for the impaired viral replication ability and neurovirulence caused by the mutation at residue 169. These results are all identical to the results obtained in the above analysis.

One motivation for the site-directed mutational analysis was that alphavirus nsP1 is structurally related to methyltransferase, and several of the nsP1 mutant proteins have previously been proved to depress the nsP1 methyltransferase activity [2, 6, 10–12, 17]. Meanwhile, we found that relative to the wild-type virus, both BR-173 virus and BR-6973 virus exhibited an altered sensitivity to 3-DZA, and BR-6973 virus exhibited a much higher sensitivity to 3-DZA than did BR-173 virus. In addition, 3-DZA is potentially an inhibitor of *S*-adenosylhomocysteine hydrolase. Thus, BR-6973 virus with an increased sensitivity to 3-DZA reflected a decreased level of viral RNA MTase activity of nsP1. In contrast, the mutation at nsP1-173 had no substantial effect on RNA MTase activity of XJ-160 virus, and RNA MTase activity of nsP1 in BR-173 virus increased somewhat. Therefore, we suggest that the characteristic biological changes of XJ-160 virus caused by these forms of site-directed mutagenesis might be associated with the viral RNA methyltransferase activity of nsP1.

Nonstructural protein 1 may bind to replication-associated enzymes to form replication enzyme complexes, which participate in viral replication. This protein plays a key role in the initiation of RNA negative strand synthesis [5, 7]. In addition, nsP1 shows both MTase and guanylate transferase activity and participates in viral RNA capping. His39, Arg91, Asp94 and Tyr249 in nsP1 of Sindbis virus are highly conserved, and alteration of each leads to a loss of both MTase activity and viral infectivity, which can also be caused by deletions of amino acid residues 442–492 [17]. With Sindbis virus, other single mutations at sites A120, A176, G176 or U188 are all lethal [3, 13]. Our data indicate that mutagenesis at residue 169 from Lys to Arg in nsP1 is

Fig. 6 Analysis of conserved nucleotides and amino acids at positions 565 and 577 in XJ-160 virus. **a** Conservation of nucleotides 565A and 577C among several alphaviruses, **b** conservation of amino acids 169Lys and 173Thr among several alphaviruses. *YN87448* Sindbis-like virus (AF103734), *S. A. AR86* Sindbis standard train (U38305), *AR339* wild Sindbis virus (V01404), *WEE* Western equine encephalomyelitis virus (AY348559), *EEE* Eastern equine encephalitis virus (EU257810), *SFV* Semliki Forest virus (NC 003215), *RR* Ross river virus (NC 001544), *Mayaro* NAY virus (DQ138320), *O'nyong-nyong* (ONN) virus (AF079456), *VEE* Venezuelan equine encephalomyelitis virus (DQ228210), *Aura* (AURA) virus (AY348562)
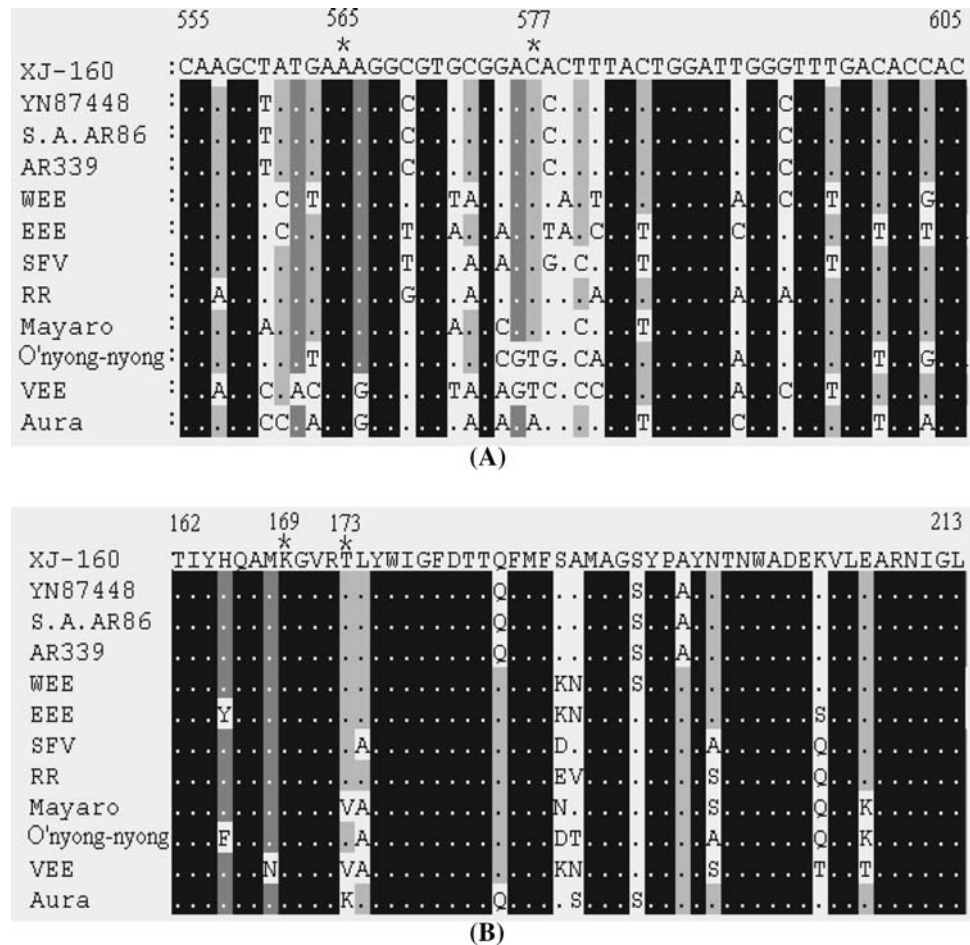
also lethal, but high levels of proteins specific to XJ-160 virus were detected by IFA after BHK-21 cells were electroporated with mutant RNA from pBR-169. Concerning transcript RNAs of alphaviruses, subgenomic RNAs coding structure proteins (capsid and E3E26KE1) can be efficiently produced, so high levels of proteins specific to XJ-160 virus could be detected. However, mutant RNA from pBR-169 never produced infectious particles in BHK-21 cells. Since changes in the viral nonstructural region are likely to affect viral RNA replication, we investigated the replication of XJ-160 viral genomic RNA by RT-PCR in the electroporated BHK-21 cells as well as in BHK-21 cells after 2–3 passages. The results indicated that XJ-160 viral RNA could be replicated only in the transfected cells and could not be transferred to the next BHK-21 cells (data not shown). This implies that the mutation at nsP1 residue 169 did not completely prevent the replication of the viral RNA genome. On the other hand, nsP1-169Lys is absolutely conserved in all of the viruses in the genus *Alphavirus* in the family *Togaviridae*, which indicates that an important function is associated with that particular residue. The important function harmed by the mutation 169Lys → Arg in the N-terminal part of nsP1 maybe associated with

XJ-160 viral RNA methyltransferase. The reasons for this are as follows: (1) Sindbis virus nsP1 is associated with a MTase activity that is presumably responsible for methylating the 5′ caps of positive-strand genomic and subgenomic RNAs, (2) the domains of nsP1 involved with MTase activity are mainly located in the N-terminal half of the protein, and (3) there was a close correlation between retention of MTase activity in nsP1 and infectivity of the viral RNA transcripts [10–12, 17].

The functions of nsPs of SINV have already been well investigated. They play an essential role in viral RNA synthesis and presumably affect viral replication in some manner. In contrast, the roles of SINV nsP1 in viral pathogenesis remain relatively unclear. Therefore, we assessed the effect of mutations at residues 169 or 173 within nsP1 on viral neurovirulence in a suckling mouse model. Our results show that the mutation of nsP1 Thr173 → Ile increased viral neurovirulence, while the BR-6973 virus with double mutations exhibited an attenuated phenotype. The simplest explanation for these phenotypes of the mutant viruses is that nsP1 mutations of XJ-160 virus affected viral propagation capacity and thus caused a quantitative increase or decrease of viral particles in the brain of the inoculated

mice. Consistent with this view is that BR-173 virus released more infectious particles than did BR-XJ160 from wild-type clones in BHK-21 cells, whereas the BR-6973 virus released fewer infectious particles (Fig. 3). However, whether these changes to viral neurovirulence are all caused by a simple growth accentuation or a defect in vitro or within the brains of infected animals remains unknown, and the role of nsP1 in the pathogenesis of alphaviruses needs to be investigated further.

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