DNAzyme-mediated Inhibition of Japanese Encephalitis Virus Replication in Mouse Brain

Mohan Babu Appaiahgari[1] and Sudhanshu Vrati[1]

[1]*Virology Laboratory, National Institute of Immunology, New Delhi, India*

Japanese encephalitis virus (JEV) is an arthropod-borne flavivirus with a single-stranded RNA genome containing non-coding regions (NCRs) at its 5′ and 3′-ends. The NCRs have flavivirus-conserved sequences that are important for virus replication. Here we describe DNAzymes (Dzs) that cleave the RNA sequence of the 3′-NCR of JEV genome *in vitro*. The nuclease-resistant Dzs, containing phosphorothioate linkages, were efficiently taken up by mouse neuronal and glial cells, and addition of a continuous stretch of 10 guanosine residues (poly-$(G)_{10}$) to the 3′-end of a Dz led to its enhanced delivery to cells containing scavenger receptors (ScRs). These novel Dzs inhibited JEV replication in cultured mouse cells of neuronal and macrophage origin. JEV is a neurotropic virus that actively replicates in mouse brain. Here we show that intra-cerebral (i.c.) administration of a poly-$(G)_{10}$-tethered, phosphorothioated Dz in JEV-infected mice led to more than 99.99% inhibition of virus replication in brain, resulting in a dose-dependent extended lifespan or complete recovery of the infected animals. This is the first report of *in vivo* application of a Dz to control a virus infection in an animal model.

Received 20 February 2007; accepted 10 May 2007; published online 19 June 2007. doi:10.1038/sj.mt.6300231

INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus responsible for frequent epidemics of encephalitis, predominantly in children, in most parts of Southeast Asia. Up to 50,000 cases of Japanese encephalitis occur every year; of these cases, around 10,000 result in fatality and the remainder lead to serious neurological sequelae.[1] As a prophylactic measure, a mouse brain–derived Japanese encephalitis vaccine is available, but this has limitations in terms of availability, cost, and safety. No virus-specific chemotherapy is available for Japanese encephalitis.

The JEV genome is a single-stranded RNA of approximately 11 kilobases. The coding sequence of the genome is flanked by a 95-nucleotide (nt) 5′-non-coding region (NCR) and a 585-nt 3′-NCR.[2] The 3′-NCR is crucial for virus replication as it binds the RNA-dependent RNA polymerase and other proteins that initiate viral genomic RNA synthesis.[3,4] Although the size and the sequence of the NCRs are not well conserved among different flaviviruses, several conserved features and secondary structures have been elucidated.[5–7] Thus, among different flaviviruses, the last 80–90 bases at the extreme 3′-end of the 3′-NCR have been predicted to form a stable stem–loop structure that may make up part of the promoter region.[6,8,9] In addition, there are direct repeats consisting of 20–70 nts separated by non-repeated regions of different lengths.[2,9,10] These repeat sequences have been shown to be important for flavivirus replication.[11,12] Thus, in terms of interfering with JEV replication, these sequences within the 3′-NCR form an attractive target.

DNAzymes (Dzs) are single-stranded oligodeoxynucleotides (ODNs) with Mg^{2+}-dependent enzymatic activity capable of cleaving single-stranded RNA at specific sites under simulated physiological conditions.[13] The "10–23" Dz consists of a catalytic domain of 15 nts flanked by 7 nts on each side that form the hybridizing arms. The sequence of these arms determines the specificity of the Dz, which cleaves the target RNA between the paired pyrimidine base and a free purine base, with highest cleavage efficiency at AU and GU sites. In recent years Dzs have been used to inhibit messenger RNA expression or RNA virus replication in cultured cells.[14–22]

JEV is a neurotropic virus that replicates actively in human brain. In an experimental mouse model for the study of JEV infection, intra-cerebral (i.c.) administration of the virus leads to clinical symptoms of paralysis and death. Here we describe a poly-$(G)_{10}$-tethered Dz that cleaves the direct repeat sequences within the 3′-NCR of JEV RNA, leading to inhibition of virus replication *in vitro* in cultured cells and in mouse brain *in vivo*. We also show that reduction in JEV titer in mouse brain by the Dz led to an extended lifespan or recovery of the infected animal, depending on the dosage used. This is the first report of *in vivo* application of a Dz to control a virus infection in an animal model.

RESULTS

DNAzyme-mediated cleavage of JEV RNA

There are two direct repeat sequences of 27 bases located between nts 10,745 and 10,771 and between nts 10,823 and 10,849 within the 3′-NCR of the JEV genome (**Figure 1a**).[2,23] We designed a Dz 3Dz (**Figure 1b**) targeted to cleave this repeat sequence. To test the *in vitro* enzymatic activity of 3Dz, we used as the substrate a synthetic 25-nt RNA (**Figure 1b**) representing part of the repeat sequence. The Dz 3Dz cleaved the substrate RNA efficiently within 5 minutes in the presence of 2 mmol/l $MgCl_2$ (used to simulate

Correspondence: *Sudhanshu Vrati, Virology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, JNU Complex, New Delhi, India. E-mail: vrati@nii.res.in*

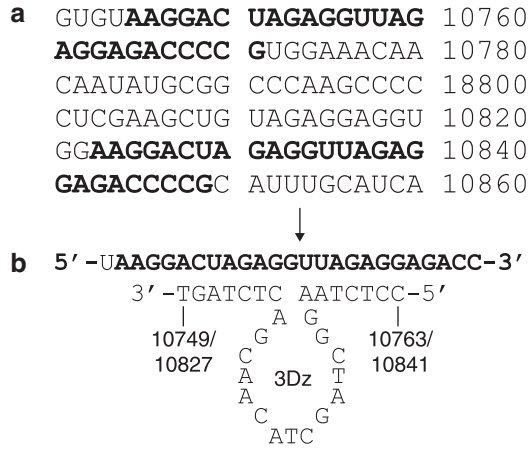

Figure 1 DNAzyme (Dz) 3Dz and its target sequence in the Japanese encephalitis virus (JEV) genome. (**a**) The JEV (JaOArS982) genome sequence between nucleotides (nts) 10,741 and 10,860 is shown. The 27-base repeat sequence between nts 10,745 and 10,771 and between nts 10,823 and 10,849 is shown in bold. **(b)** The sequence of 3Dz is shown, along with the synthetic RNA substrate sequence at the top. The sequence found in the 27-base repeat is shown in bold. The Dz 3Dz binds within these repeats between nts 10,749 and 10,763 and between nts 10,827 and 10,841. The nucleotide positions and cleavage site (arrow) are indicated.

the physiological concentration of magnesium; **Figure 2a**). The enzyme activity was magnesium dependent: no cleavage was observed in the absence of $MgCl_2$ (**Figure 2b**).

The addition of a continuous stretch of 10 deoxyguanosine residues [poly-$(G)_{10}$] at the 3′-end of a Dz was shown to enable efficient delivery of the Dz to cells bearing scavenger receptors (ScRs) without affecting its enzymatic activity. The poly-$(G)_{10}$-bearing Dz (3DzG) was, however, found to be 25–30% slower than the unmodified Dz.[24] We also found that although 3DzG cleaved the synthetic RNA substrate efficiently, it worked more slowly than 3Dz (**Figure 2c**). In addition, control Dzs (**Table 1**) in which the nt sequence of the hybridizing arms (3DzG-AR) or the catalytic domain of the 3Dz (3DzG-CR) had been randomized failed to cleave the synthetic RNA (**Figure 2d**), demonstrating Dz activity–specific cleavage of JEV RNA.

Phosphorothioated Dzs were shown to have remarkable stability in human serum ($t_{1/2}$ > 90 hours), but they were up to 100-fold less efficient than their phosphodiestered counterparts.[25] Consistent with this, 3Dz (**Table 1**) containing the phosphorothioate-linked nts cleaved the synthetic RNA substrate much less efficiently and much more slowly than 3Dz (**Figure 2e**). Here too, poly-$(G)_{10}$-bearing 3DzG worked more slowly than 3Dz.

The Dz 3Dz also efficiently cleaved the 597-nt *in vitro*–transcribed RNA containing at its 3′-end the 582-nt 3′-NCR sequence of JEV (**Figure 2f**). The target for 3Dz was present twice within this sequence (**Figure 1a**), and the Dz was able to cleave efficiently at both positions. 3DzG containing the poly-$(G)_{10}$ sequence at its 3′-end also cleaved the *in vitro*–transcribed JEV 3′-NCR RNA efficiently.

Uptake of poly-G-tethered DNAzyme by cultured cells

JEV is transmitted to human hosts by infected mosquitoes. The virus initially replicates locally in the skin and is then transported to the regional lymph nodes. This is followed by a brief viremia that allows the virus to move to other sites within the body and enter the central nervous system after breaching the blood–brain barrier. The virus then replicates in brain, leading to encephalitis. In brain, JEV replicates to different extents in neurons, microglia, astrocytes, and macrophages.[26–29] ScRs are known to be present on microglia, astrocytes, and macrophages.[30–33] Microglia are known to take up the fragmented DNA via different ScRs.[31] Neuronal cells take up ODNs in a very rapid and potent manner, but the uptake mechanism is unknown.[34,35] It has been shown that G-rich ODNs are involved in the formation of G-tetrads that can be recognized by ScRs. Thus ODNs containing the poly-$(G)_{10}$ sequence at their 3′-ends are taken up rapidly by murine[36] and human macrophage cell lines.[24] We studied the transport of 3Dz in cultured murine macrophage cells, J774E; murine neuroblastoma cells, Neuro 2a; and murine microglia cells, EOC 2. **Figure 2a** shows that J774E cells take up 3Dz very slowly and only in small amounts. In contrast, 3DzG with the poly-$(G)_{10}$ sequence at its 3′-end was taken up efficiently by J774E cells; the uptake of 3DzG was approximately tenfold higher than that of 3Dz. EOC 2 cells took up Dz efficiently; the uptake of 3Dz was approximately fivefold higher than in J774E cells (**Figure 2b**). This is consistent with the earlier finding that microglial cells take up fragmented DNA efficiently through multiple ScR types.[31] The addition of the poly-$(G)_{10}$ sequence to 3Dz enhanced its uptake by EOC 2 cells marginally but consistently at all time points studied (**Figure 2b**). This increased uptake of 3DzG may be related to the involvement of the poly-$(G)_{10}$-specific ScRs. Consistent with earlier findings,[37–40] the phosphorothioated Dzs 3Dz and 3DzG were taken up more efficiently; their uptake was approximately twofold higher than that of their phosphodiestered counterparts. In Neuro 2a cells, phosphorothioated Dzs were taken up approximately sixfold more efficiently than phosphodiestered Dzs; after 8 hours, the uptake was approximately 25%, and this continued to increase with time (**Figure 3c**). In these cells, addition of the poly-$(G)_{10}$ sequence did not result in enhanced uptake of the Dz. The mechanism behind the efficient ODN uptake by neurons is not clear; it may be noted that neurons are not known to express ScRs.

Inhibition of JEV replication in cultured cells

We studied the biological activity of the Dzs in cultured cells. Thus, if Dzs cleave the JEV RNA *in vivo*, this should result in inhibition of JEV replication in cultured cells, reflected in a reduction in extracellular virus titers. **Figure 4a** shows that in J774E cells, 3Dz used at 1 μmol/l concentration had no more effect on JEV titers than the "No Dz" control, whereas titers were reduced approximately fivefold when 3Dz was used at 5 μmol/l concentration ($P = 0.001$). Addition of the poly-$(G)_{10}$ sequence to 3Dz significantly enhanced its efficacy. Thus JEV titers were 9-fold ($P = 0.0008$) and 19-fold ($P = 0.0006$) lower in the presence of 1 and 5 μmol/l 3DzG, respectively. Phosphorothioated Dz was most effective in inhibiting JEV replication. Thus, 3DzG reduced JEV titers by 28-fold ($P = 0.0006$) and 108-fold ($P = 0.0005$) when used at 1 and 5 μmol/l concentrations, respectively. This pronounced reduction in JEV titers in the presence of 3DzG may be due to the enhanced uptake of the phosphorothioated Dz and to its known resistance to nuclease degradation. The inhibition

of virus replication, reflected in the lowering of virus titers seen here, was due to the Dz activity of the ODNs and not to the antisense effect of the RNA hybridizing arms of the Dz: 3DzG-CR, where the catalytic domain of 3DzG had been randomized, and 3DzG-AR, where the antisense arms' sequence had been randomized (**Table 1**), failed to show inhibition of JEV replication. Compared with the results in J774E, JEV replication was slower in Neuro 2a cells (**Figure 4b**). In these cells, too, at 1 μmol/l concentration 3Dz had little effect on JEV titers ($P = 0.087$), whereas at 5 μmol/l concentration JEV titers were approximately fivefold lower than those in the "No Dz" control ($P = 0.0005$). Addition of the poly-$(G)_{10}$ sequence to 3Dz did not add to its ability to inhibit JEV replication. This was consistent with our finding that addition of the poly-$(G)_{10}$ sequence to the Dz did not enhance its uptake in Neuro 2a cells. Phosphorothioated Dz was more efficient than the phosphodiestered Dz in inhibiting JEV replication in Neuro 2a cells ($P = 0.002$). Thus, approximately 30-fold lower titers were seen in the presence of 5 μmol/l 3DzG than for the "No Dz" control. There was no significant effect on JEV titers in Neuro 2a cells in the presence of the control Dzs 3Dz-AR ($P = 0.21$) and 3Dz-CR ($P = 0.084$).

DNAzyme-mediated inhibition of JEV replication in mouse brain

Ogawa *et al.* (1995) showed that ODNs diffuse very quickly in mouse brain after i.c. injection and are taken up by many cells around the injection site as early as 15 minutes after administration.[35] They showed that although concentrations of phosphodiestered ODNs were greatly reduced by 4 hours after injection, the phosphorothioated ODNs were stable beyond at least 8 hours after injection. They also showed that most of the ODNs were localized in the neuronal cells and to some extent in the astrocytic cells after i.c. injection. Similar experiment in rats showed ODN localization in neurons, astrocytes, and microglia.[34] Thus Dzs can be delivered to various cells in the mouse brain by direct i.c. injection. To examine whether Dzs could be used to block JEV infection *in vivo*, we injected JEV (1,000 plaque-forming units, PFU) into mouse brain that simultaneously received 500 pmol of different ODNs encoding Dzs or their rearranged sequences. Brain tissues were harvested 72 hours post-infection (p.i.) and assayed for JEV titer. **Figure 5a** shows that 3Dz had no effect on JEV titers: mice that received 3Dz had JEV titers similar to those in the "No Dz" control mice ($P = 0.96$). However, virus titers were approximately tenfold lower in mice that received the phosphorothioated form of the Dz, 3Dz ($P = 0.01$). Virus titers were reduced very significantly in the presence of the phosphorothioated Dz with the poly-$(G)_{10}$ sequence, 3DzG. Thus, compared with the control, JEV titers were 873-fold lower in mice

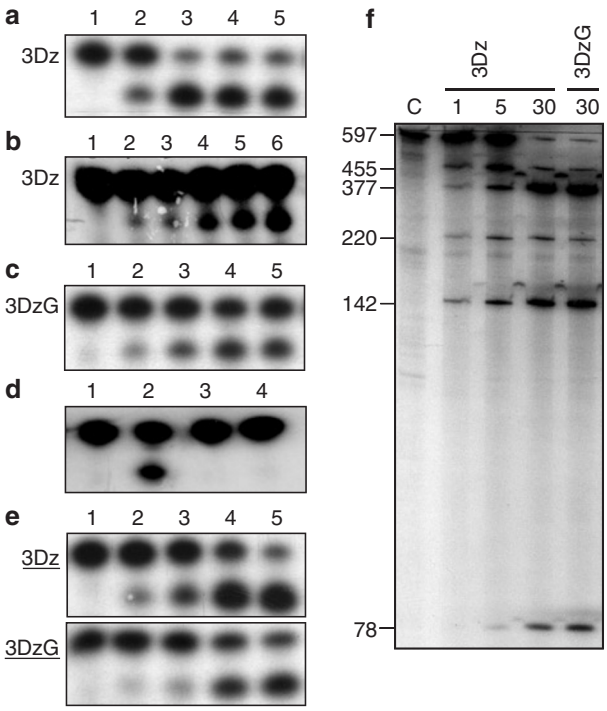

Figure 2 DNAzyme (Dz)-mediated RNA cleavage. (**a**) Cleavage of synthetic RNA substrate by 3Dz. ^{32}P-labelled synthetic RNA substrate (100 pmol) was incubated with 1 pmol of 3Dz in a reaction mix containing 50 mmol/l Tris–HCl, pH 7.5, and 2 mmol/l $MgCl_2$ for various times at 37 °C. The reaction was quenched with formamide and products were separated on a 15% denaturing polyacrylamide gel and autoradiographed. Lane 1, control reaction for 60 minutes where no Dz was added; lanes 2–5, reaction products after incubation for 5, 10, 15, and 30 minutes, respectively. (**b**) Magnesium-dependent Dz activity. ^{32}P-labelled synthetic RNA substrate (100 pmol) was incubated with 1 pmol of 3Dz in a reaction mix containing 50 mmol/l Tris–HCl, pH 7.5, and different amounts of $MgCl_2$ for 5 minutes at 37 °C. The reaction was quenched with formamide and products were separated on a 15% denaturing polyacrylamide gel and autoradiographed. Lanes 1–6, reaction products in the presence of 0, 0.1, 0.5, 1, 2, and 5 mmol/l $MgCl_2$, respectively. (**c**) Cleavage of synthetic RNA substrate by 3DzG. ^{32}P-labelled synthetic RNA substrate (100 pmol) was incubated with 1 pmol of 3DzG in a reaction mix containing 50 mmol/l Tris–HCl, pH 7.5, and 2 mmol/l $MgCl_2$ for various times at 37 °C. The reaction was quenched with formamide and products were separated on a 15% denaturing polyacrylamide gel and autoradiographed. Lane 1, control reaction for 60 minutes where no Dz was added; lanes 2–5, reaction products after incubation for 5, 10, 15, and 30 minutes, respectively. (**d**) Specificity of Dz-mediated cleavage of RNA. ^{32}P-labelled synthetic RNA substrate (100 pmol) was incubated with 1 pmol of Dz or sequence-modified versions in a reaction mix containing 50 mmol/l Tris–HCl, pH 7.5, and 2 mmol/l $MgCl_2$ for 30 minutes at 37 °C. The reaction was quenched with formamide and products were separated on a 15% denaturing polyacrylamide gel and autoradiographed. Lane 1, control reaction product where no Dz was added; lane 2, 3DzG product; lane 3, 3DzG-CR product; lane 4, 3DzG-AR product. **(e)** Cleavage of synthetic RNA substrate by phosphorothioated Dzs. ^{32}P-labelled synthetic RNA substrate (100 pmol) was incubated with 1 pmol of 3Dz or 3DzG in a reaction mix containing 50 mmol/l Tris–HCl, pH 7.5, and 2 mmol/l $MgCl_2$ for various times at 37 °C. The reaction was quenched with formamide and products were separated on a 15% denaturing polyacrylamide gel and autoradiographed. Lane 1, control reaction for 60 minutes where no Dz was added; lanes 2–5, reaction products after incubation for 1, 2, 3, and 4 hours, respectively. (**f**) Cleavage of Japanese encephalitis virus (JEV) 3′-non-coding region (NCR) RNA by Dzs. ^{32}P-labelled *in vitro*–transcribed 597-nucleotide (nt) RNA substrate (100 pmol) containing the 582-nt JEV 3′-NCR sequence at its 3′-end was incubated with 1 pmol of Dz (indicated at the top of the panel) for various times (indicated at the top of the panel in minutes) at 37 °C. C is the control reaction for 30 minutes where no Dz was added. The reaction was quenched with formamide and products were separated on a 6% denaturing polyacrylamide gel and autoradiographed. Two cleavage sites for 3Dz exist in JEV 3′-NCR RNA. At the completion of the reaction, cleaved RNA products of 377, 142, and 78 nts are expected. In addition, small amounts of the partial cleavage products of 455 and 220 nts are seen. The product size (in nts) is indicated at the left.

Table 1 Oligonucleotides used in the study

Oligonucleotide	Nucleotide sequence	Definition
3Dz	CCT CTA AGG CTA GCT ACA ACG ACT CTA GT	Phosphodiestered DNAzyme targeted against 3′-NCR
3DzG	CCT CTA AGG CTA GCT ACA ACG ACT CTA GTG GGG GGG GGG	3Dz with phosphodiestered poly-$(G)_{10}$
3DzG-AR	ACC TTA CGG CTA GCT ACA ACG ATC TGA TCG GGG GGG GGG	3DzG with sequence of the antisense arms randomized
3DzG-CR	CCT CTA AGA GAC CAG TAG TCA CCT CTA GTG GGG GGG GGG	3DzG with sequence of the catalytic site randomized
3Dz	CCT CTA AGG CTA GCT ACA ACG ACT CTA GT	Phosphorothioated 3Dz
3DzG	CCT CTA AGG CTA GCT ACA ACG ACT CTA GTG GGG GGG GGG	3Dz with phosphorothioated poly-(G)10
3DzG	CCT CTA AGG CTA GCT ACA ACG ACT CTA GTG GGG GGG GGG	3Dz with phosphodiestered poly-(G)10
3DzG-AR	ACC TTA CGG CTA GCT ACA ACG ATC TGA TCG GGG GGG GGG	3DzG with sequence of the antisense arms randomized
3DzG-CR	CCT CTA AGA GAC CAG TAG TCA CCT CTA GTG GGG GGG GGG	3DzG with sequence of the catalytic site randomized
3DzC	CCT CTA AGG CTA GCT ACA ACG ACT CTA GTC CCC CCC CCC	3Dz with phosphorothioated poly-(C)10
R29G	GAT ACC TGA GAC CAG TAG TCA CAC CTT ACG GGG GGG GGG	Phosphorothioated random 29 nucleotides with poly-$(G)_{10}$

Abbreviations: NCR, non-coding region.

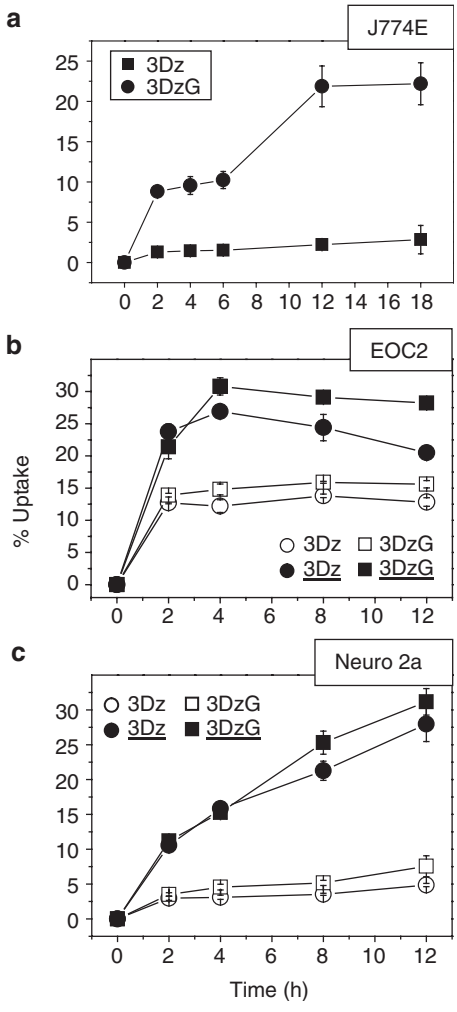

Figure 3 Uptake of DNAzymes (Dzs) by cultured cells. Monolayers of (**a**) J774E, (**b**) EOC 2, and (**c**) Neuro 2a cells were incubated at 37 °C with ^{32}P-labelled Dz (40 pmol/l) in 200 µl culture medium. Cells and culture supernatants were harvested at various time points and counted for cell-free and cell-associated radioactivity. The counts were used to calculate the percentage uptake of the Dz shown in the figure.

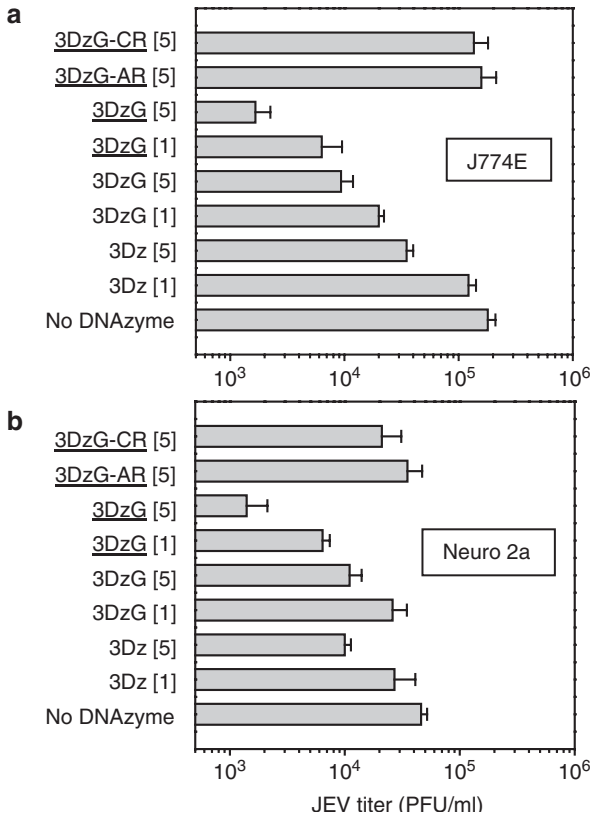

Figure 4 DNAzyme (Dz)-mediated inhibition of Japanese encephalitis virus (JEV) replication in cultured cells. (**a**) J774E or (**b**) Neuro 2a cells were infected with JEV at a multiplicity of infection of 0.1 and incubated at 37 °C in culture medium containing different Dzs at concentrations of 1 or 5 µmol/l (shown in brackets next to the oligodeoxynucleotide (ODN)). Samples were removed 24 hours after infection and assayed for JEV titers by plaque formation on porcine kidney cell monolayers. Shown above are mean titers and SDs. None of the ODNs at the concentrations used in the experiment showed any cytotoxic effects. PFU, plaque-forming units.

that received 3DzG ($P = 0.005$). Compared with the 3Dz-treated mice, JEV titers in 3DzG-treated mice were approximately 100-fold lower ($P = 0.013$). The addition of poly-$(G)_{10}$ sequence to the Dz was required for this massive reduction in JEV titers: 3DzC,

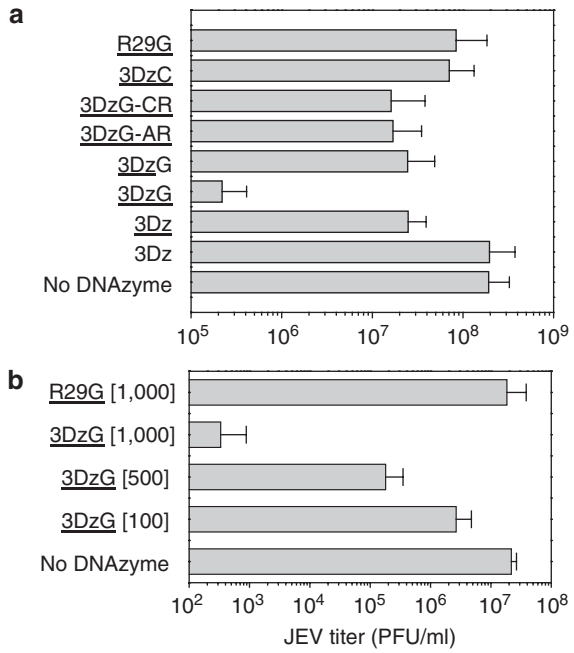

Figure 5 DNAzyme (Dz)-mediated inhibition of Japanese encephalitis virus (JEV) replication in mouse brain. Groups of 1-week-old mice ($n = 6$) were injected intra-cerebrally with 1,000 plaque-forming units (PFU) of JEV with or without a Dz. Brain tissues were harvested 72 hours later and assayed for JEV titers by plaque formation on porcine kidney cells. The "No Dz" control mice received phosphate-buffered saline in place of the Dz. (**a**) Mice received 500 pmol of the oligodeoxynucleotides (ODNs) each; (**b**) mice received 100–1,000 pmol of the ODNs shown in parentheses.

a Dz containing poly-$(C)_{10}$ residues at the 3′-end, lowered JEV titers only threefold. Furthermore, 3DzG, containing a phosphodiestered poly-$(G)_{10}$ sequence, inhibited JEV replication approximately 100-fold less efficiently than 3DzG ($P = 0.03$), suggesting an important role for the nuclease-resistant poly-$(G)_{10}$ sequence in 3DzG-mediated inhibition of JEV replication in mouse brain. It may be noted that in the presence of 3DzG-CR, where the nt sequence of the catalytic domain had been randomized, or 3DzG-AR, where the hybridizing arms' sequence had been randomized, only about tenfold lower JEV titers were recorded in comparison with the controls. In addition R29G, a 39-nt phosphorothioated ODN containing a random sequence of 29 bases with 10 G residues at its 3′-end, did not inhibit JEV replication in mouse brain. Thus, inhibition of JEV replication by 3DzG was largely due to its Dz activity and not simply an interferon-mediated response. The lowering of JEV titers was not due to the *in vitro* inactivation of JEV by the Dzs during the plaque assays, as the brain lysates from mice injected with 3DzG when mixed with JEV did not cause inhibition of JEV plaque formation. We have subsequently found that 3DzG-mediated inhibition of JEV replication in mouse brain is dose dependent (**Figure 5b**). Thus, a very high level of JEV inhibition was seen when 3DzG was used at a dose of 1,000 pmol per mouse. Compared with the "No Dz" controls (titer 2.18×10^7 PFU/ml), the mean JEV titer was 65,000-fold lower in 3DzG-treated animals (3.3×10^2 PFU/ml), resulting in 99.998% inhibition of virus replication. In fact, no virus was detectable in plaque assays from four out of six mice used in the experiment. The sensitivity of the plaque assay used here was 50 PFU/ml. Similarly, near-complete inhibition of JEV replication was achieved in 3-day-old mice using 500 pmol of 3DzG. The effectiveness of this smaller Dz dose in younger animals may simply be related to the smaller mass of the brain tissue in younger animals; whereas the brain mass is approximately 400 mg in 1-week-old animals, it is only approximately 250 mg in 3-day-old mice. The 3DzG-mediated inhibition of JEV replication in mouse brain was observed reproducibly with different lots of ODNs using different batches of mice, although it varied between 100-fold and 1,000-fold at a dose of 500 pmol Dz per mouse at 1 week of age.

DNAzyme-mediated protection of mice against lethal JEV infection

JEV replication in mouse brain leads to clinical symptoms of paralysis, followed by death. Thus, Dz-mediated reduction of JEV load in mouse brain could extend the lifespan of the infected animal. To test this, 1-week-old mice infected by i.c. injection of 1,000 PFU of JEV were given 500 pmol of Dzs. **Figure 6a** shows that all JEV-infected untreated mice died by the sixth day p.i., with an average survival time (AST) of 4.5 days. JEV-infected mice that received a single dose of 500 pmol of 3DzG at the time of the virus inoculation showed an extended lifespan, with an AST of 7.7 days; all mice in this group died by day 10 p.i. JEV-infected mice that received two doses of 500 pmol each of 3DzG at 0 and 2 days p.i. showed a further extended lifespan, with an AST of 8.6 days. It is important to note that 2 out of 12 mice completely overcame the infection and were alive on day 21 p.i. when the experiment was terminated. In contrast, two injections of R29G given on days 0 and 2 p.i. did not extend the lifespan of JEV-infected mice (AST = 4.9 days), and all mice were dead by day 7 p.i. The Dz-mediated protection of mice was dose dependent, as 100% of mice survived JEV infection when a single dose of 1,000 pmol of 3DzG was given at the time of virus inoculation (**Figure 6b**). Different experiments showed that a single dose of 500 pmol of 3DzG given on day 0 just extended the AST of JEV-infected mice, whereas two doses given on days 0 and 2 p.i. led to the survival of 15–60% of infected animals. However, 100% of JEV-infected mice that were given a single dose of 1,000 pmol of 3DzG on day 0 invariably survived. These experiments were repeated several times; none of the mice given a single dose of 1,000 pmol of 3DzG on day 0 died in eight independent experiments involving 8–12 mice each using two different commercial preparations of the Dz. These results demonstrate that Dz-mediated inhibition of JEV replication by 3DzG in mouse brain is sequence specific and dose dependent.

DISCUSSION

The ability of Dzs to cleave RNA specifically with high efficiency under simulated physiological conditions makes them potential agents to block gene expression. These molecules have the advantage of being cost-effective and more stable than other RNA-cleaving nucleic acid molecules such as ribozymes and siRNAs. Thus, a number of groups have used Dzs to down-regulate gene expression in cultured cells by specific cleavage of the messenger RNA. A few experiments have demonstrated the use of Dzs for targeting specific messenger RNAs in animal models.[16–18] In recent years Dzs have been designed to inhibit the replication of RNA viruses such

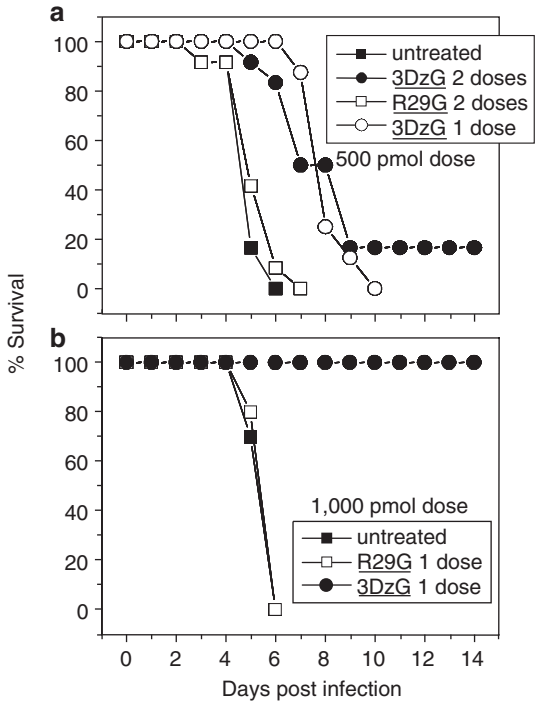

Figure 6 Survival of the Japanese encephalitis virus (JEV)–infected mice following intra-cerebral (i.c.) injection of DNAzyme. **(a)** Groups of 1-week-old BALB/c mice ($n = 12$) were injected i.c. with 1,000 plaque-forming units (PFU) of JEV with or without 500 pmol of 3DzG or R29G. The 3DzG- and R29G-treated mice were given another injection of 500 pmol of 3DzG and R29G, respectively, 2 days later. **(b)** Groups of 1-week-old BALB/c mice ($n = 8$) received 1,000 PFU JEV with or without 1,000 pmol of 3DzG or R29G. These mice were followed for clinical symptoms of sickness and death for the next 3 weeks. Shown above are the percentage survival rates of mice in different groups on various days after infection.

as human immunodeficiency, hepatitis B, hepatitis C, and influenza A viruses.[19–22] In all these cases the inhibition of virus replication was shown in an *in vitro* system using cultured cells. We have reported here a novel Dz that efficiently cleaved *in vitro* a 27-nt sequence repeated twice within the 3′-NCR of the JEV genome. This sequence is conserved among different flaviviruses and is predicted to be important for virus replication. Thus, cleavage of this sequence within the genome should inhibit JEV replication. Indeed, JEV titers were around 100-fold lower in J774E cells in the presence of 3DzG. We found that the extent of reduction in JEV titer by 3DzG was dose dependent, was related to the catalytic activity of the molecule, and was not simply due to the antisense effect. 3DzG reduced JEV replication in mouse brain in a very significant manner. Thus, in the presence of 3DzG, JEV titers in mouse brain were 65,000-fold lower, resulting in 99.998% inhibition of virus replication. Again, the extent of reduction in JEV titer in mouse brain by 3DzG was dose dependent and was related to the catalytic activity of the molecule and not simply its antisense effect. This is the first report of use of a novel Dz to inhibit virus replication *in vivo* using the mouse model.

In the mouse model of JEV infection, i.c. injected virus replicates to high titer in brain, leading to death of the animal. Neurons, which form an important site for JEV replication, are known to take up i.c. administered phosphorothioated ODNs in a very rapid and potent manner.[34] The Dz 3Dz, which would have been taken up by the brain cells in a non-specific manner, caused only a small reduction in JEV titers in mouse brain, and the role of interferon in virus inhibition by 3Dz cannot be ruled out. In addition to neurons, other major cell types found in the brain that support JEV replication are of glial or macrophage origin, such as astrocytes. Mouse and human macrophage cells have been shown to take up poly-G-containing ODNs efficiently via their ScRs. In our experiments, microglial EOC 2 cells showed improved uptake of poly-G-bearing Dzs. Glial and astrocytic cells constitute 50–90% of brain cells. We have shown that JEV replicates more efficiently in mouse macrophage J774E cells than in neuroblastoma Neuro 2a cells. Thus 3DzG, which was taken up efficiently by microglia and astrocyte cells as well as neurons, was most potent in inhibiting JEV replication in mouse brain.

In the mouse model of JEV infection, virus replication in the brain leads to microglial activation and subsequent release of various pro-inflammatory mediators, inducing neuronal death.[41] These events culminate in encephalitis and the death of the animal. Dz-mediated inhibition of JEV replication led to a significant reduction in virus load in mouse brain. Depending on the dose of the Dz 3DzG used, this resulted in an extended lifespan for the JEV-infected animals or their complete recovery. Most significant, 100% of mice injected with the lethal dose of JEV survived when they received a single dose of 1,000 pmol 3DzG. This is the first report of the application of a Dz to overcome a virus infection in an animal model. This work thus opens up the possibility of using such molecules with suitable delivery methods for treating patients with Japanese encephalitis by inhibiting virus replication in the brain.

MATERIALS AND METHODS

Virus and cells. The JaOArS982 strain of JEV was used in these studies. Virus was grown in neonatal mouse brain and titrated by plaque formation on porcine kidney cells (National Centre for Cell Science, Pune, India) as described before.[42] The murine macrophage cell line, J774E, was kindly provided by Dr. P. Stahl, Washington University, St. Louis, MO. The murine neuronal cell line, Neuro 2a, was obtained from the National Centre for Cell Science (Pune, India), and the murine glial cell line, EOC 2, was obtained from the American Type Culture Collection (Marassas, VA).

DNAzyme synthesis. Dzs were synthesized commercially and high-performance liquid chromatography purified (Biobasic, Canada, and Sigma-Genosys, UK). Their nt sequences are shown in **Table 1**. An underlined Dz indicates an ODN with phosphorothioate linkages.

DNAzyme uptake assay. Dz ODNs were radiolabeled with γ^{32}P-ATP using T4 polynucleotide kinase and purified on G-25 columns (Amersham Biosciences, UK). Cells were cultured (10^5 per well) in a 24-well tissue culture plate. On the following day, radiolabeled Dzs (40 pmol/l) were added to cells in 200 μl culture medium; they were then incubated at 37 °C. At different intervals, cells and the culture supernatants were harvested. The cells were washed twice with phosphate-buffered saline and counted for cell-free and intracellular radioactivity using a gamma counter. Percentage uptake of the Dz was calculated from these values.

RNA synthesis. A 25-mer oligoribonucleotide (U*AAGGACUAGAGG UUAGAGGAGACC*) was commercially synthesized (Integrated DNA Technologies, Coraville, IA) and used as substrate for the *in vitro* enzyme assays using Dzs. This contained (shown in italics) part of the 27-base repeat sequence located between nts 10,745 and 10,771 and between nts

10,823 and 10,849 in the 3′-NCR of JEV.[2,23] The synthetic RNA was radiolabeled using γ^{32}P-ATP and T4 polynucleotide kinase and purified on a G-25 column (Amersham Biosciences, UK). A 597-nt ^{32}P-labelled RNA was transcribed from *Xba*I-digested plasmid pJE3NCR as described before.[3] This RNA contained at its 3′-end a stretch of 582 bases corresponding to nts 10,395–10,976 of JEV RNA.[2]

RNA cleavage assay. ^{32}P-labelled RNA substrate (100 pmol) was incubated with 1 pmol of DNAzyme in a reaction mix containing 50 mmol/l Tris–HCl, pH 7.5, and 2 mmol/l $MgCl_2$ at 37 °C for various times. The reaction was quenched by transferring aliquots to tubes containing formamide dye. Samples were separated by electrophoresis on a denaturing polyacrylamide gel containing 7 mol/l urea and autoradiographed.

Mice experiments. Groups of 1-week-old BALB/c mice (n = 12) were injected i.c. with 1,000 PFU of JEV along with the indicated amounts of ODNs in 30-µl volumes into the left frontal lobe using a 26G needle. Mice were killed 72 hours later. Their brain tissues were removed and homogenized in minimal essential medium (Sigma-Aldrich, UK) to prepare a 10% suspension that was centrifuged to remove debris. The supernatant containing the virus was stored at −70 °C. The virus titers were assayed by plaque formation on porcine kidney cells. In the survival experiments, mice were observed for 21 days (or until death). All the animal experiments were approved by the National Institute of Immunology's animal ethics committee.

ACKNOWLEDGMENTS

This work was supported by the core grant provided by the Department of Biotechnology, Government of India, to the National Institute of Immunology and by a National Bioscience Career Development Award to S.V. We thank Rajendra Prasad Roy and Satyajit Rath (National Institute of Immunology, New Delhi, India) for helpful discussions and for reading the manuscript.

REFERENCES

1. World Health Organization (1998). Japanese encephalitis vaccines. *Wkly Epidemiol Rec* **73**: 337–344.
2. Vrati, S, Giri, RK, Razdan, A and Malik, P (1999). Complete nucleotide sequence of an Indian strain of Japanese encephalitis virus: sequence comparison with other strains and phylogenetic analysis. *Am J Trop Med Hyg* **61**: 677–680.
3. Ta, M and Vrati, S (2000). Mov34 protein from mouse brain interacts with the 3′ noncoding region of Japanese encephalitis virus. *J Virol* **74**: 5108–5115.
4. Chambers, TJ, Hahn, CS, Galler, R and Rice, CM (1990). Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* **44**: 649–688.
5. Brinton, MA, Fernandez, AV and Dispoto, JH (1986). The 3′-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* **153**: 113–121.
6. Proutski, V, Gould, EA and Holmes, EC (1997). Secondary structure of the 3′ untranslated region of flaviviruses: similarities and differences. *Nucleic Acids Res* **25**: 1194–1202.
7. Takegami, T, Washizu, M and Yasui, K (1986). Nucleotide sequence at the 3′ end of Japanese encephalitis virus genomic RNA. *Virology* **152**: 483–486.
8. Shi, PY, Brinton, MA, Veal, JM, Zhong, YY and Wilson, WD (1996). Evidence for the existence of a pseudoknot structure at the 3′ terminus of the flavivirus genomic RNA. *Biochemistry* **35**: 4222–4230.
9. Gritsun, TS and Gould, EA (2006). Direct repeats in the 3′ untranslated regions of mosquito-borne flaviviruses: possible implications for virus transmission. *J Gen Virol* **87**: 3297–3305.
10. Hahn, CS, Hahn, YS, Rice, CM, Lee, E, Dalgarno, L, Strauss, EG *et al.* (1987). Conserved elements in the 3′ untranslated region of flavivirus RNAs and potential cyclization sequences. *J Mol Biol* **198**: 33–41.
11. Men, R, Bray, M, Clark, D, Chanock, RM and Lai, CJ (1996). Dengue type 4 virus mutants containing deletions in the 3′ noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. *J Virol* **70**: 3930–3937.
12. Mandl, CW, Holzmann, H, Meixner, T, Rauscher, S, Stadler, PF, Allison, SL *et al.* (1998). Spontaneous and engineered deletions in the 3′ noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus. *J Virol* **72**: 2132–2140.
13. Santoro, SW and Joyce, GF (1997). A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci USA* **94**: 4262–4266.
14. Achenbach, JC, Chiuman, W, Cruz, RP and Li, Y (2004). DNAzymes: from creation *in vitro* to application *in vivo*. *Curr Pharm Biotechnol* **5**: 321–336.
15. Stearns, ME, Kim, G, Garcia, F and Wang, M (2004). Interleukin-10 induced activating transcription factor 3 transcriptional suppression of matrix metalloproteinase-2 gene expression in human prostate CPTX-1532 Cells. *Mol Cancer Res* **2**: 403–416.
16. Isaka, Y, Nakamura, H, Mizui, M, Takabatake, Y, Horio, M, Kawachi, H *et al.* (2004). DNAzyme for TGF-beta suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* **66**: 586–590.
17. Mitchell, A, Dass, CR, Sun, LQ and Khachigian, LM (2004). Inhibition of human breast carcinoma proliferation, migration, chemoinvasion and solid tumour growth by DNAzymes targeting the zinc finger transcription factor EGR-1. *Nucleic Acids Res* **32**: 3065–3069.
18. Santiago, FS, Lowe, HC, Kavurma, MM, Chesterman, CN, Baker, A, Atkins, DG *et al.* (1999). New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury. *Nat Med* **5**: 1264–1269.
19. Oketani, M, Asahina, Y, Wu, CH and Wu, GY (1999). Inhibition of hepatitis C virus-directed gene expression by a DNA ribonuclease. *J Hepatol* **31**: 628–634.
20. Goila, R and Banerjea, AC (2001). Inhibition of hepatitis B virus X gene expression by novel DNA enzymes. *Biochem J* **353**: 701–708.
21. Toyoda, T, Imamura, Y, Takaku, H, Kashiwagi, T, Hara, K, Iwahashi, J *et al.* (2000). Inhibition of influenza virus replication in cultured cells by RNA-cleaving DNA enzyme. *FEBS Lett* **481**: 113–116.
22. Takahashi, H, Hamazaki, H, Habu, Y, Hayashi, M, Abe, T, Miyano-Kurosaki, N *et al.* (2004). A new modified DNA enzyme that targets influenza virus A mRNA inhibits viral infection in cultured cells. *FEBS Lett* **560**: 69–74.
23. Sumiyoshi, H, Mori, C, Fuke, I, Morita, K, Kuhara, S, Kondou, J *et al.* (1987). Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* **161**: 497–510.
24. Unwalla, H and Banerjea, AC (2001). Inhibition of HIV-1 gene expression by novel macrophage-tropic DNA enzymes targeted to cleave HIV-1 TAT/Rev RNA. *Biochem J* **357**: 147–155.
25. Sioud, M and Leirdal, M (2000). Design of nuclease resistant protein kinase calpha DNA enzymes with potential therapeutic application. *J Mol Biol* **296**: 937–947.
26. Chen, CJ, Liao, SL, Kuo, MD and Wang, YM (2000). Astrocytic alteration induced by Japanese encephalitis virus infection. *Neuroreport* **11**: 1933–1937.
27. Suri, NK and Banerjee, K (1995). Growth and cytopathic effect of Japanese encephalitis virus in astrocyte-enriched cell cultures from neonatal mouse brains. *Acta Virol* **39**: 143–148.
28. Iwasaki, Y, Zhao, JX, Yamamoto, T and Konno, H (1986). Immunohistochemical demonstration of viral antigens in Japanese encephalitis. *Acta Neuropathol (Berl)* **70**: 79–81.
29. Wang, JJ, Liao, CL, Chiou, YW, Chiou, CT, Huang, YL and Chen, LK (1997). Ultrastructure and localization of E proteins in cultured neuron cells infected with Japanese encephalitis virus. *Virology* **238**: 30–39.
30. Husemann, J, Loike, JD, Anankov, R, Febbraio, M and Silverstein, SC (2002). Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. *Glia* **40**: 195–205.
31. Li, Y, Liu, L, Liu, D, Woodward, S, Barger, SW, Mrak, RE *et al.* (2004). Microglial activation by uptake of fDNA via a scavenger receptor. *J Neuroimmunol* **147**: 50–55.
32. Christie, RH, Freeman, M and Hyman, BT (1996). Expression of the macrophage scavenger receptor, a multifunctional lipoprotein receptor, in microglia associated with senile plaques in Alzheimer's disease. *Am J Pathol* **148**: 399–403.
33. Bell, MD, Lopez-Gonzalez, R, Lawson, L, Hughes, D, Fraser, I, Gordon, S *et al.* (1994). Upregulation of the macrophage scavenger receptor in response to different forms of injury in the CNS. *J Neurocytol* **23**: 605–613.
34. Sommer, W, Cui, X, Erdmann, B, Wiklund, L, Bricca, G, Heilig, M *et al.* (1998). The spread and uptake pattern of intracerebrally administered oligonucleotides in nerve and glial cell populations of the rat brain. *Antisense Nucleic Acid Drug Dev* **8**: 75–85.
35. Ogawa, S, Brown, HE, Okano, HJ and Pfaff, DW (1995). Cellular uptake of intracerebrally administered oligodeoxynucleotides in mouse brain. *Regul Pept* **59**: 143–149.
36. Prasad, V, Hashim, S, Mukhopadhyay, A, Basu, SK and Roy, RP (1999). Oligonucleotides tethered to a short polyguanylic acid stretch are targeted to macrophages: enhanced antiviral activity of a vesicular stomatitis virus-specific antisense oligonucleotide. *Antimicrob Agents Chemother* **43**: 2689–2696.
37. Acosta, R, Montanez, C, Gomez, P and Cisneros, B (2002). Delivery of antisense oligonucleotides to PC12 cells. *Neurosci Res* **43**: 81–86.
38. Takakura, Y, Oka, Y and Hashida, M (1998). Cellular uptake properties of oligonucleotides in LLC-PK1 renal epithelial cells. *Antisense Nucleic Acid Drug Dev* **8**: 67–73.
39. Zhao, Q, Matson, S, Herrera, CJ, Fisher, E, Yu, H and Krieg, AM (1993). Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res Dev* **3**: 53–66.
40. Beck, GF, Irwin, WJ, Nicklin, PL and Akhtar, S (1996). Interactions of phosphodiester and phosphorothioate oligonucleotides with intestinal epithelial Caco-2 cells. *Pharm Res* **13**: 1028–1037.
41. Ghoshal, A, Das, S, Ghosh, S, Mishra, MK, Sharma, V, Koli, P *et al.* (2007). Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. *Glia* **55**: 483–496.
42. Vrati, S, Agarwal, V, Malik, P, Wani, SA and Saini, M (1999). Molecular characterization of an Indian isolate of Japanese encephalitis virus that shows an extended lag phase during growth. *J Gen Virol* **80**: 1665–1671.