Alpha interferon as an adenovirus-vectored vaccine adjuvant and antiviral in Venezuelan equine encephalitis virus infection

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There are no widely available vaccines or antiviral drugs capable of protecting against infection with Venezuelan equine encephalitis virus (VEEV), although an adenovirus vector expressing VEEV structural proteins protects mice from challenge with VEEV and is potentially a vaccine suitable for human use. This work examines whether alpha interferon (IFN-α) could act as an adjuvant for the adenovirus-based vaccine. IFN-α was either expressed by a plasmid linked to the adenovirus vaccine or encoded by a separate adenovirus vector administered as a mixture with the vaccine. In contrast to previous reports with other vaccines, the presence of IFN-α reduced the antibody response to VEEV. When IFN-α was encoded by adenovirus, the lack of a VEEV-specific response was accompanied by an increase in the immune response to the adenovirus vector. IFN-α also plays a direct role in defence against virus infection, inducing the expression of a large number of antiviral proteins. Adenovirus-delivered IFN-α protected mice from VEEV disease when administered 24 h prior to challenge, but not when administered 6 h post-challenge, suggesting that up to 24 h is required for the development of the IFN-mediated antiviral response.

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is a small, enveloped RNA virus belonging to the genus Alphavirus. It occurs naturally in South America and the southern United States, where the virus is maintained in a cycle between mosquitoes and small mammals (Weaver et al., 2004). Six serogroups of VEEV have been identified. Viruses in serogroup I are capable of infecting large mammals, and the IA/B and IC strains are associated with epizootic spread in equines. Infected equines can act as amplification hosts and virus may be transmitted to humans through mosquitoes, resulting in large outbreaks of febrile illness and neurological disease (Weaver et al., 2004). Viruses in other serogroups do not appear to be equine-virulent and persist in a stable enzootic cycle. Natural transmission of enzootic viruses to humans is rare, but may be associated with severe disease (Johnson & Martin, 1974).

An attenuated, live vaccine (TC-83), derived by repeated passage of a virulent IA/B strain in fetal guinea pig heart cells (Berge et al., 1961), is available for the immunization of equines. However, TC-83 is not generally available for use in humans, as it is poorly immunogenic (Pittman et al., 1996) and may cause serious side effects (Alevizatos et al., 1967; Casamassima et al., 1987; Rayfield et al., 1976). Several approaches have been taken to develop new candidate vaccines against VEEV, including the deliberate introduction of attenuating mutations (Pratt et al., 2003), the use of whole inactivated virus (Greenway et al., 1998) and the expression of virus structural proteins in vitro (Hodgson et al., 1999) and in vivo (Kinney et al., 1988; Phillpotts et al., 2000). Previously, we have utilized replication-defective adenovirus type 5 as a vector for expression of serogroup IA/B structural proteins (Phillpotts et al., 2005). After intranasal administration of the recombinant adenovirus/VEEV vaccine, good levels of protection (70–100 %) were achieved against challenge with serogroup I viruses, but there was less protection (50 %) against serogroup II and III viruses.

There is evidence to suggest that antibody reactivity to the two major glycoproteins on the surface of VEEV (E1 and E2) is responsible for immunity to infection (Mathews & Roehrig, 1982; Mathews et al., 1985) and that high levels of antibody are required for protection (Phillpotts, 1999). An increase in antibody levels generated by the adenovirus/VEEV vaccine may therefore result in an increased ability to protect against heterologous serogroups. Alpha interferon
(IFN-α) is a potent activator of B cells, capable of inducing a strong humoral immune response and isotype switching. IFN-α can act as an adjuvant and boost the immune response to various antigens (Bricci et al., 2005; de Avila Bottin et al., 2006; Le Bon et al., 2001; Proietti et al., 2002).

Here, we describe the effects of co-administering IFN-α (encoded by either a plasmid or an adenovirus vector) with the adeno virus/VEEV vaccine.

IFN-α also plays a role in defense against virus infection. It is secreted by cells in response to virus infection and induces the expression of a large number of antiviral proteins, which generally act to inhibit virus genome translation (Zhang et al., 2007; and references therein). IFN-α is crucial for protection against VEEV infection, and mice that lack the IFN-α receptor are highly susceptible to VEEV disease (Grieder & Vogel, 1999; White et al., 2001).

However, the use of IFN-α for the prevention and treatment of virus infection is hampered by its short half-life. For example, daily injections of IFN-α could not prevent mice from developing VEEV disease (Lukaszewski & Brooks, 2000). In contrast, when IFN-α is conjugated with polyethylene glycol (to extend the half-life), mice are protected from VEEV infection, although daily injections are still required (Lukaszewski & Brooks, 2000). Delivery by an adenovirus vector offers an alternative strategy for improving the half-life and the therapeutic effect of IFN-α. A single injection of an adenovirus vector expressing IFN-α is effective against foot-and-mouth disease virus (FMDV; Chinsangaram et al., 2003) and another alphavirus, Western equine encephalitis virus (WEEV) (Wu et al., 2007). The ability of IFN-α to protect against VEEV disease was therefore tested in this study, using an adenovirus vector for delivery.

**METHODS**

**Cells, plasmids and viruses.** The L929 (murine fibroblast), A549 (human lung) and Vero (simian kidney) cell lines (European Collection of Animal Cell Cultures, UK) were propagated by standard methods using the recommended culture media. A plasmid encoding murine IFN-α subtype 5 (pIFN) was obtained from InvivoGen (catalogue reference porf-mifna). The plasmid encoding enhanced green fluorescent protein (pGFP) has been described previously (Perkins et al., 2005). The construction and properties of replication-defective adenovirus type 5 containing no VEEV sequence (RAd) or expressing serogroup IA/B structural proteins (RAd/VEEV #3) have been described previously (Phillpotts et al., 2005). Replication-defective adenovirus type 5 expressing murine IFN-α subtype 5 (Ad5-mIFNα) has been described previously (Wu et al., 2007). VEEV strain Trinidad donkey (TrD), from serogroup IA/B, was supplied by Dr R. E. Shope (University of Texas Medical Branch, Galveston, TX, USA).

A virulent virus stock was prepared and the titre determined as described by Phillpotts et al. (2005). All work with VEEV was carried out under UK Advisory Committee on Dangerous Pathogens Level 3 containment.

**Transfection of cells with plasmids.** A549 cells (1 x 10^6) were plated into each well of a 24-well plate and incubated overnight. The cells were then transfected with plasmid DNA by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s guidelines.

**Attaching plasmids to adenovirus.** The method used to link plasmids to adenoviruses has been described by Wei et al. (2000). Briefly, 2–20 μl adenovirus (3 x 10^10–3 x 10^11 p.f.u. ml^-1) was added to 14 μl poly-l-lysine (33 μg ml^-1; Sigma) and diluted in culture medium to a final volume of 267 μl. After an incubation of 30 min at room temperature, 0.7 μg plasmid DNA was added to the mixture. After a further 30 min incubation, 10 μl poly-l-lysine (33 μg ml^-1) was added and the mixture was incubated for an additional 10 min. Medium was then added to a total volume of 550 μl. This mixture was used either to immunize mice (diluted to 2 x 10^5 p.f.u. ml^-1 in PBS; see below) or to infect A549 cells (1 x 10^5 cells per well) that had been incubated overnight in 24-well plates. The culture medium was removed from the wells and replaced with 250 μl of the mixture. After 3 h incubation at 37°C, 750 μl medium was added to each well and the cells were incubated at 37°C until required for analysis.

**In vitro antiviral activity of Ad5-mIFNα.** This method was adapted from that described by Wu et al. (2007). Briefly, 5 x 10^5 L929 cells were plated into each well of a six-well plate. After overnight incubation, they were either mock-infected or infected with RAd or Ad5-mIFNα at an m.o.i. of 5. Twenty-four hours later, the cells were infected with VEEV strain TrD (m.o.i. of 0.1). Supernatants were harvested 24 and 48 h after VEEV infection and the yield of strain TrD was determined by plaque assay in Vero cells.

**Animals, immunization and challenge with virulent VEEV.** BALB/c mice, 7–9 weeks old (Charles River Laboratories), were immunized intranasally under halothane anaesthesia on days 0, 7 and 21 with 1 x 10^6 p.f.u. RAd or RAd/VEEV #3 in 50 μl PBS. Where appropriate, the virus was co-administered with an equal amount of Ad5-mIFNα (total dose, 2 x 10^6 p.f.u.). In addition, the ability of Ad5-mIFNα alone to protect against VEEV disease was tested. Mice were administered 1 x 10^6 p.f.u. Ad5-mIFNα intramuscularly in 50 μl PBS either 24 h before the challenge dose or 6 h after the challenge dose. A high (100 LD<sub>50</sub>, equivalent to approx. 30–50 p.f.u.) and a low (10 LD<sub>50</sub>, equivalent to approx. 3–5 p.f.u.) dose challenge were performed with VEEV strain TrD, administered by the subcutaneous route. After challenge, mice were observed twice daily by an independent observer for clinical signs of infection (Phillpotts et al., 2005). Animals were scored according to the severity of four clinical signs (piloerection, hunched posture, immobility and excitability), based on a truncated scale of 0 (absent), 1 (clearly observable) and 2 (pronounced). Any mice scoring 2 for two or more clinical signs were humanely culled. In addition, any mice exhibiting signs of paralysis were also humanely culled. These experiments therefore record the occurrence of severe disease rather than mortality (Wright & Phillpotts, 1998). Even though it is rare for animals infected with virulent VEEV and showing signs of severe illness to survive, our use of humane end points should be considered when interpreting any virus doses expressed here as 50% lethal doses (LD<sub>50</sub>).

**Immunofluorescence.** The expression of VEEV structural proteins in the presence of IFN-α was detected by immunofluorescence. L929 cells were infected with RAd/VEEV #3 alone (m.o.i. of 1000), Ad5-mIFNα alone (m.o.i. of 200) or with RAd/VEEV #3 (m.o.i. of 1000) plus Ad5-mIFNα (m.o.i. of 200) and incubated for 48 h. The supernatants were harvested and the cells fixed in acetone. Fixed cells were incubated with 10 μg 1A4A1 ml^-1 (a VEEV E2-specific monoclonal antibody; supplied by Dr J. T. Roehrig, Division of Vector-borne Infectious Diseases, CDC, Fort Collins, CO, USA), followed by a 1:400 dilution of anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Sigma) before being examined under UV illumination.

**Enzyme immunoassay.** Murine IFN-α was assayed by using a commercially available ELISA kit (R&D Systems, catalogue no. 42100-1), following the manufacturer’s instructions. The concentration of
IFN-α in mouse sera was determined by using samples that had been obtained from the marginal tail vein.

Mouse sera, harvested from the marginal tail vein or by cardiac puncture, were assayed for VEEV-specific antibodies by using sucrose density gradient-purified, β-propiolactone-inactivated antigen from strain TC-83 (Phillpotts et al., 2000, 2005) or sucrose density gradient-purified antigen from strain TrD (Phillpotts, 2006). Immunoglobulin concentrations were estimated by comparison of the A₄₅₀ values generated by diluted serum samples (three replicates) with a standard curve prepared from dilutions of mouse IgG (Sigma).

Pooled mouse sera, obtained from the marginal tail vein, were assayed for adenovirus-specific antibodies by using antigen prepared from cells infected with RAd (Phillpotts et al., 2005). Immunoglobulin concentrations were estimated as described above.

To examine the expression of VEEV structural proteins in the presence of IFN-α, L929 cells were infected with RAd/VEEV#3 alone (m.o.i. of 1000) or with RAd/VEEV#3 (m.o.i. of 1000) plus Ads-mIFNα (m.o.i. of 200) and incubated for 48 h. Antigen was then prepared from cells as described by Phillpotts et al. (2005) and VEEV proteins were detected by using 10 μg 1A4A1 ml⁻¹.

**Titration of virus in the brain.** The amount of VEEV strain TrD present in mouse brains was determined by titration in Vero cells. Brains were removed and homogenized in 2 ml PBS by passing them through a 70 μm nylon cell strainer (BD Falcon). Cell suspension (200 μl) was added to each well of the first column of a 96-well plate and the homogenate was then diluted serially (1:10) in cell-culture medium across the plate. Diluted homogenate (100 μl) from each well was added to the corresponding well of a 96-well plate containing confluent monolayers of Vero cells. The cells were incubated for 72 h, after which time the monolayers were fixed by the addition of 10 % (v/v) formal saline and stained with 0.1 % (w/v) crystal violet. The concentration of VEEV, expressed as tissue culture infectious dose 50 % (TCID₅₀), was calculated by Reed–Muench analysis of virus-positive wells (Butcher & Ulaeto, 2005). This figure was converted to p.f.u. by multiplying the TCID₅₀ value by 0.69 (Dubois et al., 2004).

**Statistical methods.** Statistical analysis was performed by using Minitab (http://www.minitab.com) or GraphPad Prism (http://www.graphpad.com) software.

**RESULTS**

**Transport of pIFN into cells using adenovirus**

Initially, the plasmid encoding murine IFN-α was transfected into A549 cells by using a standard transfection reagent (Lipofectamine 2000), and IFN-α production was measured by ELISA. The mean concentration of IFN-α was 7.9, 30.3 and 56.8 μg ml⁻¹ at 24, 48 and 72 h post-transfection, respectively. The ability of RAd/VEEV#3 to transport plasmids into cells was then examined. The plasmids encoding IFN-α and GFP were linked to adenoviruses with poly-L-lysine and the mixtures were used to infect A549 cells. IFN-α was not detected in the supernatant of those infected with RAd/VEEV#3 carrying pGFP. However, IFN-α was detected in the supernatant of cells infected with virus carrying pIFN (a mean of 253.5 pg ml⁻¹ at 24 h and 270.1 pg ml⁻¹ at 48 h). Therefore, plasmids may be transported into cells via adenovirus infection, although the concentration of IFN-α expressed was considerably lower than that produced by transfection of the cells with plasmid alone. IFN-α was not assayed at 72 h, as by this time the cytopathic effects were extensive, due to the extremely high m.o.i. used (approx. 6000).

**In vivo effects of attaching pIFN to adenovirus with poly-L-lysine**

We have shown previously that replication-defective adenovirus type 5 expressing serogroup IA/B structural proteins (RAd/VEEV#3) induces a protective immune response against VEEV (Phillpotts et al., 2005). However, protection against viruses in serogroups II and III was not complete, suggesting that an increase in immunogenicity would be beneficial. In order to ascertain whether IFN-α augmented the immune response to VEEV antigens, the plasmid encoding IFN-α was attached to RAd/VEEV#3 and this mixture was administered to mice. To control for the protective effects of IFN alone, pIFN was also attached to an empty replication-defective adenovirus type 5 (RAd). Mice were immunized on days 0, 7 and 21 with RAd, RAd-PLL-IFN, RAd/VEEV#3 or RAd/VEEV#3-PLL-IFN.

The concentration of VEEV-specific antibody in sera was determined after two and three doses (on days 20 and 26, respectively). Mice administered vaccine with pIFN attached had statistically significantly lower levels of
VEEV-specific antibodies ($P<0.001$, general linear model; Fig. 1). The mean levels of VEEV-specific antibody in the group receiving RAd/VEEV#3 were 0.81 and 4.11 µg ml$^{-1}$ after two and three doses, respectively, compared with 0.12 and 1.56 µg ml$^{-1}$, respectively, in the group receiving RAd/VEEV#3-PLL-IFN.

**Co-administration of VEEV vaccine with an adenovirus expressing murine IFN-α**

An alternative strategy for testing the effects of IFN-α on the anti-VEEV immune response generated by RAd/VEEV#3 is to co-administer the vaccine with Ad5-mIFNα. Mice were immunized on days 0, 7 and 21 with RAd/VEEV#3, either alone or mixed with an equal amount of Ad5-mIFNα. Serum samples were taken after two and three doses (on days 16 and 23, respectively) and the concentration of VEEV-specific antibody was determined by ELISA. The ability of RAd/VEEV#3 to generate an anti-VEEV immune response was inhibited completely by the inclusion of Ad5-mIFNα. Mice immunized with the vaccine alone had a mean concentration of 0.33 and 1.6 µg VEEV-specific IgG ml$^{-1}$ after two and three doses, respectively (95% confidence intervals, 0.39 and 1.3 µg ml$^{-1}$, respectively; $n=10$), whereas in mice immunized with the mixture of RAd/VEEV#3 and Ad5-mIFNα, there was no detectable VEEV-specific antibody.

IFN-α expressed by Ad5-mIFNα may have induced an antiviral response, thereby inhibiting expression of VEEV structural proteins by RAd/VEEV#3-infected cells and preventing the development of an immune response. To test this, immunofluorescence and ELISA techniques were used to measure VEEV antigen expression in L929 cells infected with RAd/VEEV#3 and Ad5-mIFNα (Fig. 2; the expression of IFN-α was confirmed in the appropriate samples by ELISA, results not shown). At the high m.o.i. used in this experiment, the expression of VEEV proteins was not affected by IFN-α. Although it is problematic to extrapolate the results of any in vitro experiment to the intact animal (the cellular and cytokine environments are different and the m.o.i. is likely to be lower), the data suggest that, in vivo, the antiviral activity of IFN-α is not responsible for the lack of VEEV-specific antibody. When serum samples from mice that had been immunized with RAd/VEEV#3, in the presence or absence of Ad5-mIFNα, were tested for adenovirus-specific antibody, it was found that mice administered vaccine plus Ad5-mIFNα had noticeably increased levels of antibody to the adenovirus vector (after two and three immunizations, antibody levels increased from 14.51 to 900.00 ng ml$^{-1}$ and from 17.13 to 3523.91 ng ml$^{-1}$, respectively, in the presence of Ad5-mIFNα).

**Antiviral activity of Ad5-mIFNα**

IFN-α induces the production of numerous antiviral proteins that are responsible for limiting virus infection. An experiment demonstrating the ability of Ad5-mIFNα to inhibit the replication of VEEV in cultured cells, described by Wu et al. (2007), was repeated, using VEEV strain TrD rather than strain TC-83 because it is more relevant to the in vivo challenge experiment described below. Ad5-mIFNα inhibited the replication of VEEV in cultured cells (Fig. 3). Infection of L929 cells with Ad5-mIFNα, prior to infection with VEEV strain TrD, reduced VEEV titres at both time points, compared with those in cells pre-infected with RAd ($P<0.001$, Bonferroni’s post-hoc test). The yields of TrD virus from cells pre-infected with Ad5-mIFNα were 3.37
Fig. 3. Pre-infecting cells with recombinant adenovirus expressing murine IFN-α inhibits VEEV replication. L929 cells (5×10⁴ per well) were either mock-infected (empty bars) or infected with RAd (filled bars) or Ad5-mIFNα (shaded bars) (m.o.i. of 5). The cells were infected with VEEV strain TrD (m.o.i. of 0.1) 24 h later. Supernatants were harvested 24 and 48 h after VEEV infection and the yield of virus was determined. n=3; 95% confidence intervals are shown; *P<0.001.

and 2.81 logs lower than those from RAd-infected cells at 24 and 48 h after VEEV infection, respectively. The titre of VEEV in supernatants harvested from cells pre-infected with Ad5-mIFNα was greater than the original infectious dose, indicating that the murine IFN-α did not prevent replication of VEEV completely, but did slow the rate of replication dramatically.

Next, the ability of Ad5-mIFNα to protect mice against VEEV disease was determined (Fig. 4). Mice given either a high (100 LD₅₀) or a low (10 LD₅₀) dose of strain TrD were treated with Ad5-mIFNα, either 24 h before or 6 h after challenge. Mice that were challenged with VEEV but received no treatment succumbed to disease (no survivors out of eight mice in the 100 LD₅₀ challenge group, one survivor out of eight in the 10 LD₅₀ challenge group). When administered 24 h before challenge, Ad5-mIFNα was able to protect mice (eight survivors out of eight in the 10 LD₅₀ challenge group, six survivors out of eight in the 100 LD₅₀ challenge group; P=0.0014 and 0.007, respectively; Fisher’s exact test). In contrast, Ad5-mIFNα did not protect mice when administered 6 h after challenge, although it was able to confer a slight delay in the time to death (the median time to death for mice challenged with 10 LD₅₀ was 7 days, which increased to 8 days when mice were treated with Ad5-mIFNα; the median time to death for mice challenged with 100 LD₅₀ was 6 days, which increased to 8 days when mice were treated with Ad5-mIFNα).

Two days after challenge, serum samples were harvested from individual mice and the concentration of IFN-α in pooled samples was determined (Fig. 5). Mice that had not been treated with Ad5-mIFNα had the highest levels of IFN-α (10.6 and 9.3 ng ml⁻¹ in the 10 and 100 LD₅₀ challenge groups, respectively), whereas mice that had been treated with Ad5-mIFNα had lower levels of IFN-α. The relative contribution of the recombinant adenovirus and VEEV infection to the amount of IFN-α detected in mice treated with Ad5-mIFNα cannot be determined. It has been shown that the sera of mice administered Ad5-mIFNα alone contain 1.18 and 2.26 ng IFN-α ml⁻¹ at 48 and 72 h after administration, respectively (Wu et al., 2007; J. Q. H. Wu & D. Huang, unpublished results). The 48 and 72 h time points correspond to serum samples taken from mice treated with Ad5-mIFNα either 6 h after challenge or 24 h before challenge, respectively. However, it is difficult to compare the two experiments as it appears that VEEV infection inhibits the production of IFN-α by the recombinant adenovirus.

Surviving mice, given Ad5-mIFNα 24 h prior to challenge with 10 or 100 LD₅₀ VEEV, had mean serum anti-VEEV antibody concentrations of 0.47 and 4.01 µg ml⁻¹, respectively (95% confidence intervals, 0.43 µg ml⁻¹, n=8, and 3.03 µg ml⁻¹, n=6, respectively). However, mice that became ill and were culled during the experiment had higher levels of VEEV-specific antibody (Table 1): mean serum concentration of VEEV-specific IgG, 21.23 µg ml⁻¹; 95% confidence interval, 5.82 µg ml⁻¹, n=8).

VEEV was not detectable in brain tissue from any of the surviving mice (at day 34). In contrast, virus was detected in the brains of mice that were culled during the course of the experiment (Table 1). Virus titres were similar and were indicative of severe VEEV infection.

**DISCUSSION**

Although a VEEV vaccine suitable for human use is the ideal preventative measure, in its absence, the availability of effective antiviral therapies is extremely desirable. This work has investigated two ways by which IFN-α may be used to combat disease caused by VEEV: as an adjuvant for an adenovirus-based VEEV vaccine and as an antiviral therapy delivered by an adenovirus vector.

IFN-α, whether secreted by a plasmid or by an adenovirus vector, failed to enhance the antibody response to an intranasally delivered adenovirus-vectored VEEV vaccine. The inclusion of IFN-α reduced the anti-VEEV response, and when IFN-α was produced from a separate adenovirus vector the antibody response was eradicated. The lack of VEEV-specific antibody appeared to be caused by an IFN-α-generated bias in the immune response towards the vector rather than the transgene. These results are in direct contrast to other published work (Bracci et al., 2005; Le Bon et al., 2001; Proietti et al., 2002) and in particular that of de Avila Botton et al. (2006), in which co-administration of an adenovirus-based vaccine for FMDV and an adenovirus vector expressing porcine IFN-α increased...
FMDV-specific antibody levels in swine. These adenoviruses were administered to swine by the intramuscular route, but it seems unlikely that this is the cause of the discrepancy; the reason for the conflicting results is unclear. Our findings suggest that the adjuvant effects of IFN-α for adenovirus-vectored immunogens cannot be assumed for all constructs and must be evaluated for each case.

Adenovirus-encoded IFN-α was also tested for its ability to induce anti-VEEV activity. Binding of IFN-α to type I IFN receptors on the cell surface triggers the JAK/STAT signalling cascade, which leads to the induction of IFN regulatory factor-7 and an array of IFN-stimulated genes that mediate the antiviral response (Zhang et al., 2007; and references therein). Here, the ability of IFN-α to protect mice from disease when administered pre- and post-challenge was investigated. There is a need for an antiviral that is able to prevent or at least modify disease after infection, because VEEV is generally diagnosed only once neurological symptoms become apparent. Additionally, VEEV is a potential bioterrorism agent (Hilleman, 2002) and it is likely that there would be a delay between a malicious release of the virus and either the acquisition of intelligence indicating that a release has taken place or the detection of virus in the environment. Although a single dose of adenovirus-encoded IFN-α provided good protection when administered 24 h prior to challenge, it was not effective 6 h post-challenge and, at best, elicited a delay in time to death. This lack of activity indicates that adenovirus-encoded IFN-α would not be useful in the treatment of VEEV infection after exposure. However, IFN-α may still have utility as a post-exposure treatment for other alphaviruses, as adenovirus-encoded IFN-α was shown to be more effective when administered 6 h after challenge with WEEV (five survivors out of eight mice; Wu et al., 2007).

Upon infection, alphaviruses induce transcriptional shut-off, thereby inhibiting the cellular antiviral response (Garmashova et al., 2007). IFN-α would therefore not be expected to have any effect on already infected cells, but should still induce an antiviral state in surrounding, non-infected cells. For WEEV, this appears to be sufficient to prevent disease developing when IFN-α is administered post-challenge (Wu et al., 2007), but it is not effective against VEEV.
Mice that were challenged with VEEV but received no treatment developed a strong immune response to the virus, which was manifested in high levels of circulating IFN-α and anti-VEEV antibody. Nevertheless, immunity was generated too late to overcome the infection and to prevent death from encephalitis. A lesser immune response was observed in mice administered adenovirus-encoded IFN-α and it seems likely that IFN-α prevented VEEV from establishing an infection of the central nervous system in those mice that remained well throughout the course of the challenge experiment. In these and many other experiments, the antiviral mechanisms induced by IFN-α appear to be particularly effective when generated prior to infection.

A number of approaches have previously been taken to inhibit VEEV, both in vitro and in vivo, such as IFN-α conjugated to polyethylene glycol (Łukaszewski & Brooks, 2000), monoclonal antibodies (Hunt et al., 2006; Phillpotts et al., 2002), short interfering RNA (O’Brien, 2007) and phosphorodiamidate morpholino oligomers (Paessler et al., 2008). The antiviral activity of IFN-α is mediated via activation of the host’s antiviral mechanisms, whereas monoclonal antibodies, short interfering RNA and phosphorodiamidate morpholino oligomers all act directly on the virus, whether by neutralizing infectivity and promoting phagocytosis (monoclonal antibodies) or by inhibiting virus genome replication and protein production (short interfering RNA and phosphorodiamidate morpholino oligomers). Unfortunately, treatments shown to be effective in mice (Hunt et al., 2006; Łukaszewski & Brooks, 2000; Paessler et al., 2008; Phillpotts et al., 2002) have still to be successfully transitioned into humans.

The pathogenesis of VEEV disease in mice and humans has common features. In mice, virus enters the central nervous system 2 or 3 days after peripheral inoculation (Bennett et al., 2000). After airborne infection, there is the additional possibility that virus may multiply in the olfactory neuroepithelium and thereby gain direct access to the brain via the olfactory nerve. This mechanism may also occur in humans. When monoclonal antibodies were tested as post-exposure antiviral therapies for VEEV in mice, they were effective only when administered 24 h after infection (Hunt et al., 2006; Phillpotts et al., 2002) and not after 48 h (Hunt et al., 2006) or 72 h (Phillpotts et al., 2002). The

Table 1. Concentration of VEEV-specific IgG in sera and titres of VEEV in the brains of mice culled before termination of the challenge experiment (day 34)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Challenge dose (LD₅₀)</th>
<th>Day culled</th>
<th>VEEV-specific IgG (μg ml⁻¹)</th>
<th>Virus titre in brain (p.f.u.)</th>
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<tbody>
<tr>
<td>Ad5-mlIFNα (-24 h)</td>
<td>100</td>
<td>16</td>
<td>32.66</td>
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<td>10.52</td>
<td>1.096 × 10⁷</td>
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<tr>
<td>Ad5-mlIFNα (+6 h)</td>
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<td>7</td>
<td>19.04</td>
<td>0.776 × 10⁷</td>
</tr>
<tr>
<td>Ad5-mlIFNα (+6 h)</td>
<td>100</td>
<td>7</td>
<td>32.49</td>
<td>3.698 × 10⁷</td>
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<tr>
<td>Ad5-mlIFNα (+6 h)</td>
<td>100</td>
<td>7</td>
<td>16.73</td>
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</tbody>
</table>
window of opportunity available for prevention of disease once infection with VEEV has taken place may therefore be extremely narrow in both mice and humans. Generally, to avoid deaths from encephalitis, VEEV must be prevented from accessing the brain or virus replication in the brain must be inhibited [exceptions have been noted in mice vaccinated with a chimeric Sindbis virus/VEEV vaccine (Paessler et al., 2006) and in γδ knockout mice (Paessler et al., 2007)]. If antivirals cannot be administered quickly enough to achieve the former, then they must be designed to cross the blood–brain barrier to achieve the latter.

In our experiments, IFN-α did indeed have an adjuvant effect when administered with a recombinant adenovirus/VEEV vaccine. However, rather than increasing the immune response to the transgene, it inappropriately potentiated the response to the adenovirus vector. Such a response could prevent boosting and may lead to a pathological immune response to further doses of adenovirus-vectored vaccine. Whilst adenovirus-delivered IFN-α did have antiviral activity against VEEV in vivo, it was only effective when administered before exposure. This indicates that a period of time is required for the development of the antiviral response within the host. Although IFN-α may be able to act as an antiviral therapy if administered immediately after VEEV infection, it seems unlikely that it could be administered quickly enough after exposure to be effective.

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REFERENCES


Phillpotts, R. J. (1999). Immunity to airborne challenge with Venezuelan equine encephalitis virus develops rapidly after immunisation with the attenuated vaccine strain TC-83. Vaccine 17, 2429–2435.


Phillpotts, R. J., Jones, L. D. & Howard, S. C. (2002). Monoclonal antibody protects against infection and disease when given either before or up to 24 h after airborne challenge with virulent Venezuelan equine encephalitis virus. Vaccine 20, 1497–1504.


