Type I interferons protect mice against enterovirus 71 infection

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In this study, the contribution of type I interferons (IFNs) to protection against infection with enterovirus 71 (EV71) was investigated using a murine model where the virus was administrated to neonatal Institute of Cancer Research (ICR) mice by either the intraperitoneal (i.p.) or the oral route. In i.p. inoculated mice, post-infection treatment of dexamethasone (5 mg kg\(^{-1}\)) at 2 or 3 days after infection) exacerbated clinical symptoms and increased the tissue viral titre. In contrast, polyribosinosine : polyribocytidylic acid [poly(I : C); 10 or 100 μg per mouse at 12 h before infection], a potent IFN inducer, improved the survival rate and decreased the tissue viral titres after EV71 challenge, which correlated with an increase in serum IFN-α concentration, the percentage of dendritic cells, their expression of major histocompatibility complex class II molecule and IFN-α in spleen. Treatment with a neutralizing antibody for type I IFNs (10\(^4\) neutralizing units per mouse, 6 h before and 12 h after infection) resulted in frequent deaths and higher tissue viral load in infected mice compared with control mice. In contrast, an early administration of recombinant mouse IFN-αA (10\(^4\) U per mouse for 3 days starting at 0, 1 or 3 days after infection) protected the mice against EV71 infection. In vitro analysis of virus-induced death in three human cell lines showed that human type I IFNs exerted a direct protective effect on EV71. It was concluded that type I IFNs play an important role in controlling EV71 infection and replication.

INTRODUCTION

A member of the genus Enterovirus of the family Picornaviridae, enterovirus 71 (EV71) is a neurotropic virus with a single-stranded, positive-polarity 7.5 kb RNA genome (McMinn, 2002). EV71 infections are generally mild, such as hand-foot-and-mouth disease and herpangina, but occasionally lead to severe diseases such as aseptic meningitis, poliomyelitis-like paralysis and possibly fatal encephalitis in neonates. It has caused significant morbidity and mortality worldwide, especially in the Asia-Pacific region since it was first described in 1969 in the USA (Ho et al., 1999; Lum et al., 1998; Komatsu et al., 1999). Brainstem encephalitis associated with pulmonary oedema and cardiac insufficiency are the primary manifestations in patients with neurological involvement (Chang et al., 2004; Huang et al., 1999; Lin et al., 2002a; Wang et al., 1999; Yan et al., 2000). At present, treatment for EV71 is supportive. Intravenous immunoglobulin has been used for therapy against EV71; however, its efficacy has not yet been demonstrated (Lin et al., 2002a; Nolan et al., 2003).

The induction of type I interferons (IFNs) is the earliest non-specific host response to viral infections (Goodbourn et al., 2000). The type I IFNs IFN-α and IFN-β are produced by leukocytes and fibroblasts, respectively, in response to infection, which leads to the induction of antiviral pathways within hours. Clinically, IFN-α/β has been used for the treatment of hepatitis C virus infection and certain tumours (Kirkwood, 2002). Sasaki et al. (1986) showed a therapeutic effect of murine IFN-α/β on coxsackievirus type A16 infection in newborn mice. In this study, we have demonstrated that type I IFNs represent an essential innate defence mechanism for controlling EV71 infection in mice.

METHODS

Cells and viruses. RD (rhabdomyosarcoma), Caco-2 (human colo-rectal carcinoma) and SK-N-SH (human neuroblastoma) cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% (for Caco-2 cells) or 10% (other cell lines) fetal bovine serum (FBS) with 2 mM L-glutamine ml\(^{-1}\), 100 IU penicillin ml\(^{-1}\) and 100 μg streptomycin ml\(^{-1}\). EV71/Taiwan/4643/98 stock virus (GenBank accession no. AF304458) (Yan et al., 2001) and EV71/M4, a mouse-adapted strain derived from the parental virus 4643 (Wang et al., 2004), were grown in RD cells. Both strains showed positive reactivity to anti-EV71 monoclonal antibody (mAb) (Chemicon) with an indirect immunofluorescence
staining of infected RD cell cultures. To prepare virus stocks, viruses were propagated for one further passage in RD cells. Working stocks contained $2 \times 10^6$ p.f.u. virus ml$^{-1}$.

**Mouse experiments.** Specific-pathogen-free, 3-day-old Institute of Cancer Research (ICR) mice (Laboratory Animal Center, National Cheng Kung University, College of Medicine, Tainan, Taiwan) were inoculated intraperitoneally (i.p.) with 100 p.f.u. of a pre-determined dose of EV71/Taiwan/4643/98 that would result in a 20 or 80% mortality rate (Yu et al., 2000). Inocula were diluted by plaque assay immediately after the challenge. Mice were treated with dexamethasone, polyribosinic-polyribotydilic acid [poly(I:C)] (both from Sigma-Aldrich), murine IFN-α/β neutralizing antibody (murine L-cell antiseraum; NIAID Reference Reagent Repository) or recombinant mouse IFN-α (R&D Systems). Control mice were given control sera (NIAID Reference Reagent Repository) or PBS. Mice were observed twice daily for clinical illness and death until 21 days of age. Clinical disease was graded as follows: 0, healthy; 1, ruffled fur and hunched-back appearance; 2, wasting; 3, limb weakness; 4, limb paralysis; and 5, moribund and death. For oral infection experiments, 7-day-old mice were force-fed with 200 μl EV71/MP4 using a 24-gauge feeding tube after fasting for 8 h (Wang et al., 2004). The Institutional Animal Care and Use Committee approved all animal protocols.

**Tissue viral titres.** After euthanasia, blood samples were collected following axilla dissection. Blood and serum samples were stored at −70°C. After perfusion with isotonic saline containing EDTA, tissue samples were aseptically removed, weighed and stored at −70°C. Tissue samples were homogenized in 1 ml DMEM containing 2% FBS, disrupted by three freeze–thaw cycles and centrifuged. Clarified supernatants and blood samples were inoculated onto monolayers of RD cells, which were inspected daily for a minimum of 14 days for cytopathic effect (CPE) (Hsiung, 1994). Viral titres were expressed as log p.f.u. (mg tissue)$^{-1}$ or (ml blood)$^{-1}$. The lower limit of virus detection was 10 p.f.u.

**IFN-α ELISA.** Concentrations of serum IFN-α were measured by a sandwich ELISA technique according to the manufacturer’s instruction (Mouse IFN-α ELISA kit; R&D Systems). The detection limit was 7.5 pg ml$^{-1}$.

**Flow cytometry for CD11c$^+$ dendritic cells and IFN-α expression.** The spleen was removed from mice after euthanasia and mechanically disrupted. Red blood cells were removed into lysing solution containing 20 mM NH$_4$Cl, 10 mM KHCO$_3$ and 0.1% EDTA. After washing, a single-cell suspension ($10^6$ cells in 1 ml Hank’s balanced salt solution containing 2% fetal calf serum and 0.1% sodium azide) was incubated with anti-CD16/CD32 mAb (Fc blocker, 2.4G2; BD PharMingen), followed by fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (H129.19; BD PharMingen) and phycoerythrin (PE)-conjugated anti-CD8α (53-6.7; BD PharMingen) and PE-Cy5-conjugated anti-major histocompatibility complex (MHC) class II (M5/114.15.2; ebioscience) or PE–Cy7-conjugated anti-B20 (RA3-6B2; ebioscience) mAbs for 30 min on ice. After washing, stained cells were quantified using flow cytometry (BD Immunocytometry Systems). Isotype-matched mAb-stained cells were used as a background control in all experiments. For intracellular cytokine staining, single-cell suspensions of splenocytes were stimulated with 10 μg phorbol myristate acetate ml$^{-1}$ plus 1 μM ionomycin (Sigma-Aldrich) in the presence of monensin (ebioscience) for 5 h in six-well plates. Cells ($10^6$ ml$^{-1}$) were first stained with PE-conjugated anti-CD11c (N418; ebioscience), followed by anti-mouse IFN-α mAb (R&D Systems) and secondary antibody after fixation and permeabilization. Cells were then analysed using flow cytometry as described above.

**In vitro CPE protection assay.** RD, Caco-2 or SK-N-SH cells ($5 \times 10^5$ cells per well) were plated on 24-well plates, grown overnight to obtain >80% confluence and then primed with human leukocyte IFN-α (R&D Systems) for 6 h or left untreated. After washing, the monolayers were infected with EV71/Taiwan/4643/98 at an m.o.i. of 0.001. When 90% of the cells in the non-treated wells had CPE, the wells were stained with 0.5% crystal violet. In other experiments, RD or SK-N-SH cells ($2 \times 10^5$ cells per well) were plated on 96-well plates and cultured for 18 h before treatment with increasing doses of IFN-α (final concentrations ranging from 0 to 2500 U ml$^{-1}$) for 6 h. After washing, the monolayers were infected with EV71/Taiwan/4643/98 (dilutions of 10$^{-1}$ to 10$^{-7}$). CPE was examined after incubation for 72 h and TCID$_{50}$ values were determined by the method of Hsiung (1994) using the Reed & Muench formula.

**Statistical analysis.** The frequencies of survival and mortality in treated mice versus control mice were assessed using Fisher’s exact two-tailed test. Differences in the mean survival time, tissue viral titres, serum IFN-α concentrations and TCID$_{50}$ were determined using a two-tailed Mann–Whitney U test or Kruskal–Wallis test. Values were considered significant for values of $P<0.05$.

**RESULTS**

**Dexamethasone exacerbates EV71 infection in mice**

To examine the extent of the inflammatory response contribution to EV71 infection, we tested whether dexamethasone, a potent anti-inflammatory steroid, affected the outcome of EV71 infection in a murine experimental infection model (Yu et al., 2000). As expected, infection of 3-day-old mice with an i.p. dose of 4x10$^6$ p.f.u. EV71 resulted in 80–100% mortality by 10 days post-infection (p.i.) (Fig. 1). Viable viruses were detected in the brain and limb muscles [3 and 5x10$^6$ p.f.u. (mg tissue)$^{-1}$, respectively] of infected mice before death (7 days p.i.) (Fig. 1). The limb muscles seemed to be a major site for virus replication since the tissue contained a high titre. However, whether its damage led to fatality was not known. Post-infection treatment of dexamethasone (i.p., 5 mg kg$^{-1}$, either 2 and 3 days p.i. or 3 and 4 days p.i.) did not improve the survival rate of EV71-infected mice. All the animals died by 10 days p.i. with or without treatment. Furthermore, tissue viral loads in both the brain and limb muscles of dexamethasone-treated mice were significantly higher than those of untreated mice (Fig. 1).

**Poly(I:C) ameliorates EV71 infection in mice and is associated with an upregulation of dendritic cells and type I IFNs**

In contrast to treatment with dexamethasone, a pre-infection administration of poly(I:C) (10 or 100 μg per mouse, 12 h before inoculation, i.p.) improved the survival rate of mice infected with EV71 (4x10$^6$ p.f.u., i.p.) (Fig. 1). The mortality of poly(I:C)-treated mice was 50% (10 μg) and 37.5% (100 μg) compared with 100% in untreated mice infected in parallel. The improvement in survival rate correlated with a decrease in viral titre in the brain tissue [control vs 10 μg, 5x10$^2$ vs 0 p.f.u. (mg tissue)$^{-1}$], indicating that EV71-induced brain damage might be
responsible for the death of the animals (Fig. 1). Flow cytometry revealed that poly(I:C) treatment (10 μg) increased the percentage of splenic CD4+ T cells [control vs poly(I:C), 5.3 ± 0.5% vs 9.0 ± 0.8%, n = 3–6 mice, $P<0.05$] and dendritic cells (B220+CD11c+) (3.5 ± 0.6% vs 7.3 ± 1.9%, $P<0.05$) but not CD8+ T cells (1.2 ± 0.2% vs 1.4 ± 0.4%). Poly(I:C) also upregulated the expression of MHC class II molecules (2.0 ± 0.4% vs 4.4 ± 0.9%) and intracellular IFN-α of the CD11c+ cells (0.9 ± 0.1% vs 1.8 ± 0.2%, both $P<0.05$) (Fig. 2). An ELISA revealed that poly(I:C), but not EV71, also augmented IFN-α production in mice 12 h after administration. The increased production was sustained for at least 24 h (Fig. 3).

**Anti-IFN-α/β antibody exacerbes and type I IFNs protect against EV71-induced disease**

Next, we directly tested the effect of type I IFNs in the EV71 infection model. Mice were infected with a sublethal dose of EV71 (1 × 10^6 or 2 × 10^6 p.f.u. per mouse) and treated with a neutralizing antibody to IFN-α/β [10^4 neutralizing units (NU) per mouse] 6 h before and 12 h after infection. Fig. 4 shows that mice treated with the neutralizing antibody developed a more severe disease with a higher clinical score and a lower survival rate than untreated infected control mice. Specifically, the survival rates of the control and treated mice were 100 and 75%, respectively, in the low-dose challenge groups (1 × 10^6 p.f.u.). Upon high-dose challenge (2 × 10^6 p.f.u.), the control and treated mice had an 83.3 and 16.7% survival rate, respectively. Concomitantly, the neutralizing antibody not only increased the tissue viral titre by more than 1 log [control vs anti-IFN-α/β, 3.2 vs 2.8 × 10^6 p.f.u. (mg tissue)^-1], but also decreased the serum IFN-α concentration of EV71-infected mice 12 h after infection compared with untreated mice (control vs anti-IFN-α/β, 220 ± 7 pg ml^-1 vs 170 ± 6 pg ml^-1, $P<0.05$). In another series of experiments, 3- and 7-day-old mice were first infected with an 80–100% lethal dose of EV71 by the i.p. route (EV71/Tainan/4643/98, 4 × 10^6 p.f.u. per mouse) and oral route (EV71/MP4, 5 × 10^6 p.f.u. per mouse), respectively. Recombinant murine IFN-α (10^6 U per mouse per day) was then i.p. administered once daily for 3 days, starting at 0, 1 or 3 days p.i. The mice in the control groups showed paralysis, and survival rate was 16.7% in the i.p. infection experiment and 18.5% in the oral infection experiment (Fig. 5). The administration of recombinant IFN-αA immediately after infection reduced clinical symptoms and prevented death (Fig. 5). Thirty-three and 0% of the mice survived if the treatment started at 1 and 3 days p.i., respectively, in the i.p. infection experiment, and 100 and 40% of the mice survived when initial treatment was given at 0 and 3 days p.i., respectively, in the oral infection experiment.

**Type I IFNs protect human cell lines from EV71-induced cytopathology in vitro**

To characterize further the protective effect of type I IFNs, we examined whether RD, Caco-2, and SK-N-SH cell lines were protected from EV71-induced cell death in vitro. The cells were pre-treated with human IFN-α (0–10^10 U) for 6 h before infection with EV71. EV71 infection resulted in an obvious CPE in all three cell lines, while it decreased following pre-treatment with IFN-α/α (Fig. 6a), indicating that type I IFNs exert a direct anti-EV71 effect. Furthermore, the IFN-treated cells were more resistant to EV71 infection than those without treatment, as reflected by a high TCID^50 (Fig. 6b).

**DISCUSSION**

An inflammatory response with induction of toxic inflammatory cytokines has been considered as one possible pathogenesis mechanism of EV71 infection (Lin et al., 2002b; 2003; Wang et al., 2003). Thus, we sought to test whether the host inflammatory response might contribute...
to the disease process of EV71. Dexamethasone, which has been shown to exert a beneficial effect for bacterial meningitis (Chaudhuri, 2004) and non-purulent meningoencephalitis (Somer et al., 2000), did not ameliorate EV71 infection as shown by the findings that its treatment resulted in uncontrolled virus replication and disease in EV71-infected mice. These data indicated that direct viral lysis rather than an effect of the immune system contributes to the pathology of EV71 infection and that early antiviral activity is crucial for controlling the infection.

The host factors that influence whether infection with EV71 culminates in a terminal disease or a mild infection state are not known (Chang et al., 1999). Alteration of cellular immunity of the hosts has been suggested to be related to disease severity (Yang et al., 2001). Immunodeficient mice including nude, B-cell deficient, SCID, CD28 knockout and tumour necrosis factor-α receptor knockout mice have been used to delineate the role of the immune system in EV71 infection. However, none of these mouse strains was more susceptible to infection than the wild type (Wu et al., 2002).

On the other hand, we observed that nitric oxide might represent an important innate immunity for EV71 infection in our murine infection model (P. W. Kuo & J. R. Wang, unpublished observation). Since the type I IFN system is the most important innate defence against viral infection, we next attempted to define the role of type I IFNs in EV71 infection. Poly(I : C) is a potent IFN inducer and poly(I : C) administration resulted in a rapid accumulation of high concentrations of IFN at the local site of virus replication, which acted in an autocrine manner (Poast et al., 2002; Pyo et al., 1993). Therefore, we first tested the prophylactic effect of poly(I : C). We demonstrated that poly(I : C) augmented IFN-α production and activated splenic IFN-α-producing dendritic cells in mice. Furthermore, poly(I : C) not only improved the survival rate but also decreased the viral titre in the brain tissue of EV71-infected mice. Similarly, Padalko et al. (2004) showed that poly(I : C) could markedly protect mice from coxsackie B3 virus-induced myocarditis. Recent studies have shown that poly(I : C) can activate natural killer

Fig. 2. Poly(I : C) increases the percentage of CD11c+ dendritic cells and their expression of MHC class II molecules and IFN-α in spleen. Three-day-old ICR mice were inoculated i.p. with poly(I : C) (10 μg per mouse) for 12 h. Spleen cells were stained with anti-CD16/CD32 mAb (Fc blocker, 2.4G2) followed by different fluorochrome-conjugated mAbs (FITC–CD4, FITC–CD11c, PE–CD8a, PE–Cy7–B220 and PE–Cy5–MHC class II) or rat anti-mouse IFN-α mAb followed by FITC-conjugated secondary antibody. The numbers represent the mean ± SEM of three to six mice per group. Spleen cells from an adult mouse were assayed in parallel.

Fig. 3. Poly(I : C), not EV71, induces type I IFN production in mice. Three-day-old ICR mice (n = 6–12) were inoculated i.p. with either poly(I : C) (10 μg per mouse) or EV71/Tainan/4643/98 (4 × 10⁶ p.f.u. per mouse). Concentrations of serum IFN-α were determined by ELISA. Bars represent the mean ± SEM of three experiments. *, P < 0·05 when compared with naïve mice. **, P < 0·05 when compared with poly(I : C)-treated mice.
Neutralizing antibody to type I IFN increases clinical symptoms and mortality of EV71-infected mice. Three-day-old ICR mice \((n=6-12)\) were inoculated i.p. with EV71/Tainan/4643/98 \((1 \times 10^6 \text{ or } 2 \times 10^6 \text{ p.f.u. per mouse})\) with or without an administration i.p. of a neutralizing antibody to type I IFN \((10^4 \text{ NU per mouse})\) 6 h before and 12 h after infection. The clinical symptoms and survival rates were monitored daily after infection. The results shown are representative of three independent experiments.

Recombinant murine type I IFNs protect mice from EV71 challenge. Three- (upper panel) or 7-day-old (lower panel) ICR mice \((n=3-6)\) were inoculated i.p. with EV71/Tainan/4643/98 \((4 \times 10^6 \text{ p.f.u. per mouse})\) or orally with EV71/MP4 \((5 \times 10^5 \text{ p.f.u. per mouse})\), with or without administration i.p. of recombinant murine IFN-αA \((10^4 \text{ U per mouse per day})\) for 3 days starting at 0, 1 or 3 days p.i. Survival rates were monitored daily after infection. The results shown are representative of three independent experiments.

Human leukocyte IFNs protect human cell lines from EV71 infection. (a) RD, SK-N-SH and Caco-2 cells \((5 \times 10^5 \text{ cells per well})\) were treated with human IFN-α2b (h-IFN) for 6 h and then infected with EV71/Tainan/4643/98 at an m.o.i. of 0.001. When 90% of the cells without treatment had CPE \((44 \text{ h for RD cells, } 48 \text{ h for SK-N-SH cells and } 60 \text{ h for Caco-2 cells})\), cells were stained with 0.5% crystal violet staining solution. (b) RD and SK-N-SH cells were treated as described in Methods and TCID_{50} values were determined at 72 h p.i. *, \(P<0.05\) when compared with the no-treatment group. The results shown are representative of three independent experiments.
(NK) cells (Schmidt et al., 2004) and γδT cells via type I IFNs derived from toll-like receptor-3-expressing CD11c+ dendritic cells (Kunzmann et al., 2004). Thus, the observed protective effect of poly(I:C) might be attributed to the activation of CD11c+ dendritic cells, NK cells and γδT cells, and the production of IFN-α. Poly(I:C) has been given intravenously to patients with viral drug, but the low titre and short duration of IFN induced [8–500 U ml⁻¹ in response to doses of poly(I:C) of 0–1-1·0 mg kg⁻¹] might limit its clinical usefulness as an antiviral drug, as stated by Guggenheim & Baron (1977). In contrast to poly(I:C), EV71 inoculation was unable to elicit the production of type I IFNs. More studies are needed to clarify whether EV71 exerts any inhibitory effect on the production of type I IFNs.

Although EV71 inoculation did not elicit an increase in serum type I IFNs, two series of in vivo experiments confirmed the protective role of type I IFNs on EV71 infection. First, pre-treatment with a neutralizing antibody to IFN-α/β dramatically increased the susceptibility of mice to EV71. Secondly, the therapeutic effect of recombinant murine IFN-α/α was clearly demonstrated in mice infected by either the i.p. or the oral route. An early treatment of recombinant IFN-α was essential for the outcome of the infection, as no antiviral activity was observed once neuroinvasion was evident at 3 days p.i. EV71-infected mice developed clinical symptoms around 4 or 5 days p.i. IFN treatment at this time point may offer no benefit but may worsen the infection, which may explain why the survival rate of IFN-treated mice (treated at 3, 4 and 5 days p.i.) was lower in comparison with infected non-treated control mice. Furthermore, in vitro experiments demonstrated that human IFN-α/α could protect against EV71 infection of the three major target cell types of EV71, namely RD, Caco-2 and SK-N-SH cells.

Collectively, our data indicate that type I IFNs play an important role in the host defence of EV71 and that an earlier detection of viral involvement of the central nervous system followed by immediate type I IFN therapy should be considered an appropriate treatment regimen for EV71.

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REFERENCES


