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Short Communication

Usutu virus growth in human cell lines: induction of and sensitivity to type I and III interferons

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The mechanisms of Usutu virus (USUV) pathogenesis are largely unknown. The aim of this study was to evaluate the sensitivity of USUV to interferon (IFN) and the capacity of USUV to stimulate IFN production. Initial experiments were conducted to characterize the susceptibility of human cell lines to USUV infection and to evaluate the single-growth cycle replication curve of USUV. Results indicate that USUV is able to infect a variety of human cell lines, completing the replication cycle in Hep-2 and Vero cells within 48 h. Pre-treatment of cells with types I and III IFNs significantly inhibited the replication of USUV. However, the inhibitory effects of IFNs were considerably less if IFN was added after viral infection had been initiated. Also, USUV weakly induced types I and III IFNs.

Usutu virus (USUV), an African mosquito-borne flavivirus of the Japanese encephalitis virus serocomplex, has been detected in dead birds and/or mosquitoes in several European countries since 2001 (Weissenböck *et al.*, 2002, 2003; Chvala *et al.*, 2007; Vazquez *et al.*, 2011). Moreover, human neuroinvasive USUV infections were reported in two immunocompromised patients in Italy recently (Cavrini *et al.*, 2009; Pecorari *et al.*, 2009) and USUV-specific IgG was detected in the serum of four subjects living in north-eastern Italy with no history of other flavivirus infections (Gaibani *et al.*, 2012). Although USUV has become a new emerging pathogen in Europe (Vazquez *et al.*, 2011), very little is known about its pathogenesis and about the activation of the innate immune response triggered by the virus infection.

In the light of lack of data on the sensitivity of USUV to interferon (IFN) and taking into consideration the implications for the underlying mechanism of USUV course of infection, we evaluated the susceptibility of different human cell lines to USUV infection, analysed the growth characteristics of USUV in various human cell lines and investigated whether USUV is sensitive to the antiviral activity of types I and III IFNs.

To allow careful examination of the effects of types I and III IFNs on USUV replication, initial experiments were conducted to evaluate the *in vitro* susceptibility of various human cell cultures to USUV infection. USUV *in vitro* infection has been studied in a variety of animal-derived cell lines including Vero (Bakonyi *et al.*, 2005), which was used as reference cell line in the current study. Different human cell lines [the human lung adenocarcinoma epithelial A549, human colon adenocarcinoma CaCo-2, human epitheloid cervix carcinoma HeLa, human hepatoblastoma Hep-G2, human epidermoid larynx carcinoma Hep-2, human colon adenocarcinoma SW480, human epidermoid oral carcinoma KB, human embryonic human lung MRC-5 and human colon adenocarcinoma grade II HT29 (1×10^6 cells per well in six-well plates)] were infected with USUV (Vienna 2001-blackbird strain, GenBank accession no. AY453411; Bakonyi *et al.*, 2005; Weissenböck *et al.*, 2002) at an m.o.i. of 0.1 $TCID_{50}$ per cell. After 48 h the culture supernatants were added to the

A supplementary table and figure are available with the online version of this paper.

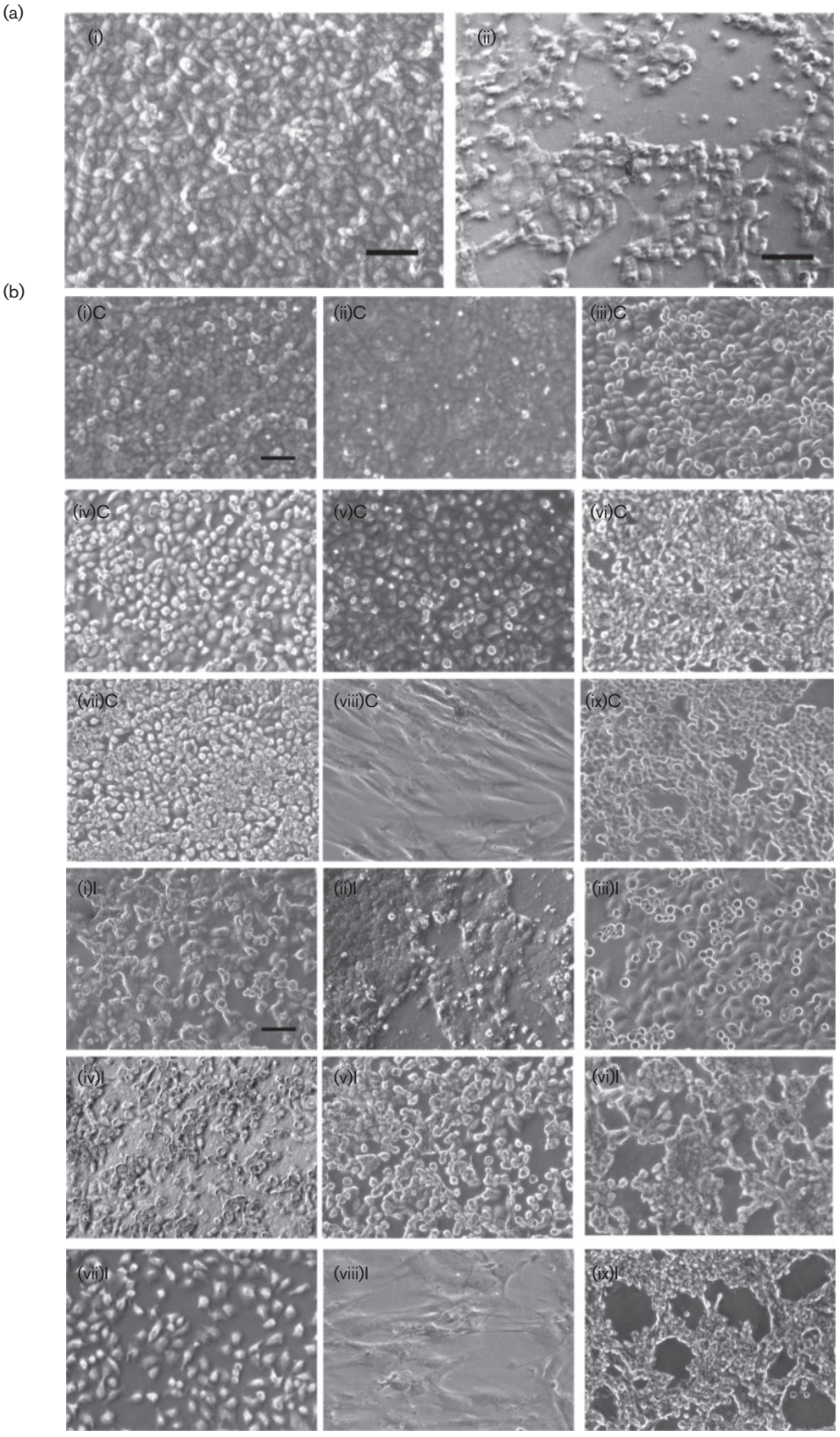
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Fig. 1. CPEs observed 2 days post-infection (p.i.) with USUV at an m.o.i. of 0.1 $TCID_{50}$ per cell. (a) Control (uninfected) Vero cells (reference cell line) (i) and corresponding infected cells (ii). (b) Control (C; uninfected) A549 (i), CaCo-2 (ii), HeLa (iii), Hep-G2 (iv), Hep-2 (v), SW480 (vi), KB (vii), MRC-5 (viii), HT29 (ix) cells and corresponding (i–ix) infected (I) cells. Bar, 1 μm.

cell lysate and titrated for USUV content by using either yield assay (Reed & Muench, 1938) or real-time RT-PCR (Cavrini *et al.*, 2011). The results showed that all cell lines tested were susceptible to USUV infection as evidenced, in most cells, by the appearance of characteristic cytopathic effects (CPEs), and resulted in USUV yield or viral RNA production of at least 4.5 log $TCID_{50}$ ml^{-1} and 8.1 log RNA copies ml^{-1}, respectively (Fig. 1a, b and Table S1, available in JGV Online). Three human cell lines, namely A549, Hep-2 and KB, developed a clear-cut CPE comparable to that produced in Vero cells with an USUV titre of at least 5.5 log $TCID_{50}$ ml^{-1} and 9.5 log RNA copies ml^{-1}, respectively.

Since only three human cell lines (A549, Hep-2 and KB) developed a clearly visible CPE following USUV infection and considering that Hep-2 cells showed the highest IFN lambda-induced protection from vesicular stomatitis virus [VSV (Indiana strain) IFN-sensitive virus] infection (data not shown), this cell line was considered suitable for the evaluation of the sensitivity of USUV to the antiviral activities of types I and III IFNs. In order to validate the results on USUV sensitivity to type I IFN, the same experiments were conducted in the Vero cell line. Evaluation of antiviral activity of IFN lambda subtypes was not performed in Vero cells because this cell line is not sensitive to type III IFN preparations (data not shown). To enable careful examination of the effects of IFNs on USUV, initial experiments were conducted to characterize the growth of USUV in Vero and Hep-2 cell lines. The infection of both cell lines (1×10^6 cells per well in six-well plates) was carried out at an m.o.i. of 1 $TCID_{50}$ per cell. Aliquots of the cells and the cell culture fluid were collected at the time of virus adsorption [i.e. 90 min post-infection (p.i.)] and at 2, 4, 6, 8, 11, 24, 48, 60, 72, 96 and 120 h p.i., and were titrated in Vero cells by using Reed & Muench's (1938) method. The kinetics of USUV growth in Hep-2 and Vero cells are shown in Fig. 2(a, b). As shown, USUV titres reached $10^{4.25}$ and $10^{3.25}$ $TCID_{50}$ ml^{-1} in extracellular fluids 11 h after Hep-2 and Vero cell infections, respectively. The USUV yields peaked 48 h p.i. at approximately $10^{6.00}$ $TCID_{50}$ ml^{-1} in both cell lines. The titre decreased slowly after another 12 and 24 h in both cell lines, and successively declined over the next 3 days.

The cell-associated virus titre exhibited $10^{3.25}$ and $10^{3.75}$ $TCID_{50}$ ml^{-1} 6 h p.i. in Hep-2 and Vero cells, respectively, and rose rapidly until 24 h p.i. in both cell lines. Over the next 2 days, the titre declined, with no more cell-associated USUV production in the remaining 48 h.

After establishing that a single cycle of USUV replication in Hep-2 and Vero cells was completed in about 60 h and that most of the cycle was already complete after 48 h, the latter time was chosen to test the antiviral activities of human types I and III IFN preparations on USUV replication. The IFN preparations used were leucocyte IFN alpha (Alfa Wassermann SpA), IFN beta (Rebif Merck-Serono), IFN omega and IFN lambda 1–2 (PBL interferon source) and IFN lambda 3 (R&D systems). Briefly, Hep-2 and Vero cells (6×10^4 cells per well in 96-well plates) were infected at an m.o.i. of 1 $TCID_{50}$ per cell. For IFN pre-treatment experiments, cells were treated with half-log serial dilutions of IFN (from 10^6 to 0.6 pg ml^{-1}) for 24 h before USUV infection. In each test, 12 wells were not treated with IFN and filled with 0.1 ml of minimal essential medium (Euroclone) before USUV infection to serve as controls. Supernatants were then collected after 48 h p.i., and cells were subjected to three consecutive freeze (at −80 °C)–

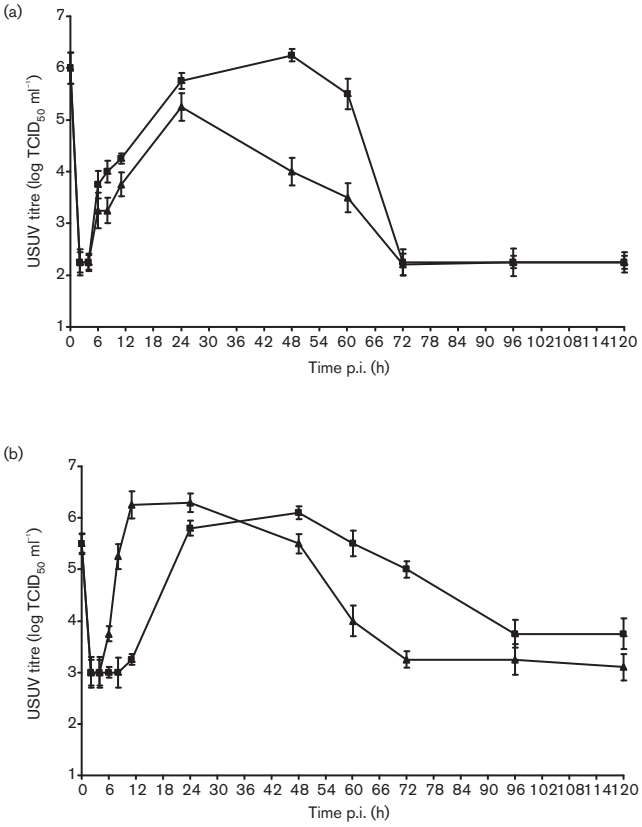

Fig. 2. USUV replication cycle. Hep-2 (a) and Vero cells (b) were infected with USUV at an m.o.i. of 1 $TCID_{50}$ per cell. At the time of virus absorption and at 2, 4, 6, 8, 11, 24, 48, 60, 72, 96 and 120 h p.i., pelleted cells (▲) and supernatants (■) were collected separately and titrated for virus content by using the yield assay. Each dot represents the means ± SD of three separate experiments run in duplicate.

thaw cycles. The culture supernatant was then added to the cell lysate and titrated for USUV yield in Vero cells (Reed & Muench's method). For IFN post-treatment experiments, Hep-2 and Vero cells were infected with USUV, and then stimulated with IFN at two time points after viral replication had been initiated (1.5 and 12 h p.i.). Evaluation of USUV yield was performed 48 h p.i. as described above. The concentration of IFN that inhibited 50 % of USUV yield (i.e. the IC_{50}) was calculated from a dose–response curve obtained by plotting the percentage of viral yield reduction obtained in IFN-treated cells, with respect to the viral yield from USUV-infected control cells, versus the IFN dose. IC_{50} values were calculated for each IFN triplicate independently, and mean values were compared using ANOVA followed by a Student's *t*-test with Bonferroni correction; *P*-values <0.05 were considered significant (SPSS version 13.0 for Windows). In order to better characterize the Hep-2 and Vero cell lines from the IFN sensitivity point of view, the same experiments were performed with VSV (Scagnolari *et al.*, 2004, 2011). The results are shown in Fig. 3(a, b), which demonstrate that the pre-treatment of cells with types I and III IFN potently inhibits USUV replication. In particular it can be seen that the IC_{50} values of leucocyte IFN alpha, IFN beta, IFN omega and IFN lambda 1–3 preparations observed in pre-treated Hep-2 or Vero cells were lower or similar to those observed for VSV. In addition, the effect of IFN pre-treatment was indirectly related to virus inoculum, and the inhibition was significantly greater with infection by a low dose of USUV (Fig. S1).

However, results showed that treatment of cells with IFNs as little as 1.5 h after exposure to USUV revealed a marked loss in the inhibitory effect of IFN on virus production. As shown in Fig. 3(a, b), the IC_{50} values observed in Hep-2 or Vero cells after addition of IFN immediately after the USUV-cell absorption were significantly higher than those recovered when cells were treated prior to USUV infection ($P<0.05$). At 12 h p.i, the highest concentration used for each IFN preparation to treat cells was not able to inhibit the replication of USUV (IC_{50} >1 μg ml^{-1}). Furthermore, as expected (Faul *et al.*, 2009), the effects of post-IFN treatment on VSV replication were also completely lost at 1.5 and 12 h p.i. In particular, the concentrations of all IFN preparations required to inhibit 50 % of VSV replication were always >1 μg ml^{-1} in both cell lines, except for the IC_{50} values recorded in Hep-2 cells for type I IFN preparations (IC_{50} $\leqslant 3000$ pg ml^{-1}), which were also similar or lower compared with those of USUV ($P<0.05$) (Fig. 3a, b).

As shown in previous studies with other flaviviruses (Diamond *et al.*, 2000; Anderson & Rahal 2002; Samuel & Diamond 2005), cells respond to IFN by efficiently inhibiting USUV replication when the IFN treatment happened prior to but not after virus infection. This phenomenon is particularly evident for type I IFN preparations. Several research groups demonstrated that flaviviruses use several targeted strategies to hinder the antiviral effects of IFN (Hoenen *et al.*, 2007; Daffis *et al.*, 2009; Tu *et al.*, 2012; Morrison *et al.*, 2012). Furthermore, the ability of West Nile virus (WNV) to decrease the expression of eIF-3 (Pastorino *et al.*, 2009) together with the observations that other host cell physiological processes may be altered during flavivirus infections (Westaway *et al.*, 1997; Su *et al.*, 2002; Yu *et al.*, 2006; Sangiambut *et al.*, 2008; Pastorino *et al.*, 2009; Colpitts *et al.*, 2011) might suggest that flaviviruses indirectly affect the response to IFN through a profound modulation of the cell metabolism. Therefore, in consideration of the above evidence, it is possible to speculate that USUV infection might inhibit IFN antiviral action through an uncharacterized mechanism that allows USUV to at least partially overcome the IFN response in order to establish a productive infection. An alternate, non-mutually exclusive explanation, in part sustained by the ability of some IFN-stimulated genes to inhibit the early replication of flaviviruses (Brass *et al.*, 2009), is that IFN protects against *de novo* viral infection but cannot restrain USUV replication in cells in which viral infection has been previously established because it interferes with an early step of USUV replication but has little or no effect on later steps. In line with this consideration, we observed that the IFN-induced inhibition of USUV replication was essentially lost after the viral infection had been established.

Looking at differences in antiviral activity between different IFNs, we observed that, in general, although there was some variation depending on the cell type, the antiviral effects of type I IFNs against USUV were comparable independently of the time of IFN treatment (Fig. 3a, b). By contrast as reported for influenza A/H1N1, WNV and hepatitis C virus (Kelly *et al.*, 2011; Marcello *et al.*, 2006; Meager *et al.*, 2005), the potential of IFN lambda 1–3 to inhibit USUV replication was lower than that of type I IFNs ($P<0.05$) (Fig. 3a, b).

In order to gain insights into the mechanisms of the USUV natural history of infection we determined the time-course and amount of IFN produced following USUV infection of Hep-2 cells. Briefly Hep-2 cells (1×10^6 cells per well in six-well plates) were infected with USUV or VSV at an m.o.i. of 0.1 $TCID_{50}$ per cell. After 3, 6, 10, 24 and 48 h p.i., supernatants were collected. The experiment was run in triplicate. IFN activity was determined evaluating the reduction of CPE on A549 cells by encephalomyocarditis virus (Scagnolari *et al.*, 2008). The analyses of the production of IFN subtypes were performed using ELISA tests [IFN alpha multi-subtype ELISA kit, IFN beta ELISA kit, IFN omega ELISA kit, Verikine-DIY IFN lambda 1–3 (PBL interferon source)], following the manufacturer's instructions. Mean values were compared using ANOVA followed by a Student's *t*-test with Bonferroni correction; *P*-values <0.05 were considered significant (SPSS version 13.0 for Windows).

Fig. 3(c) shows the IFN yield induced after infection with USUV. It can be seen that USUV induces a lower yield of IFN activity than VSV at 24 and 48 h p.i. ($P<0.05$). No

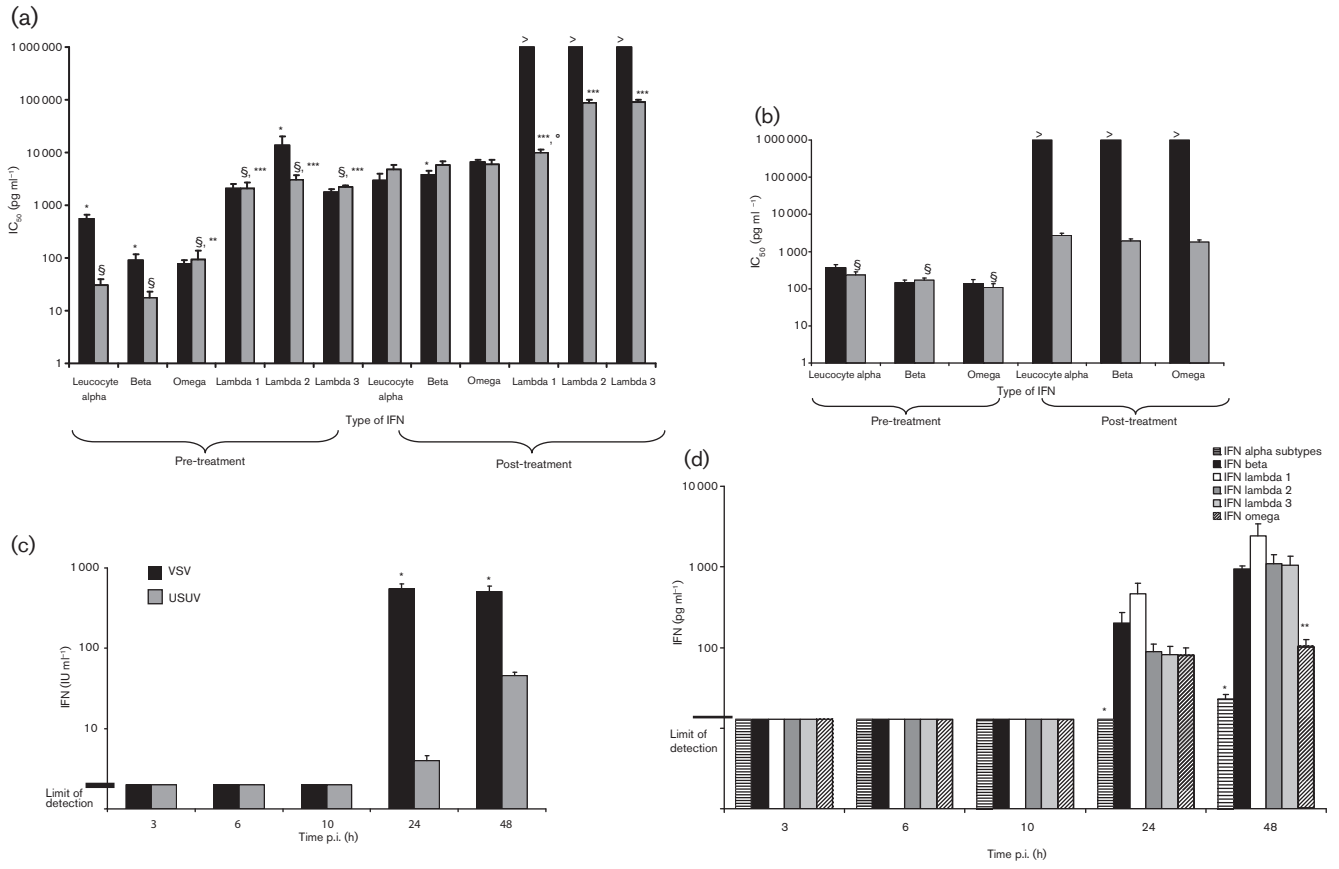

Fig. 3. Evaluation of *in vitro* sensitivity of USUV to IFN and its capacity to induce IFN. (a, b) Antiviral activity of human IFN preparations were evaluated against USUV (grey bars) in Hep-2 (a) and Vero cells (b) before (pre-treatment) and at 1.5 h post-USUV infection (post-treatment) at an m.o.i. of 1 $TCID_{50}$ per cell. The same experiments were performed with VSV (black bars). >, The highest IFN concentration used was not able to inhibit the replication of VSV. (c, d) Induction of IFN after infection of Hep-2 with USUV or VSV at an m.o.i. of 0.1 $TCID_{50}$ per cell. After 3, 6, 10, 24 and 48 h p.i., supernatants were collected and IFNs were detected by biological assay (c) and different ELISA tests (d). The limit of detection of the biological assay and ELISA tests were: 5 international units (IU) ml^{-1} (biological assay), 13 pg ml^{-1} (IFN alpha subtypes), 50 pg ml^{-1} (IFN beta, IFN lambda 1–3), 10 pg ml^{-1} (IFN omega). Symbols for statistical comparison: (a) §, USUV (pre-treatment) vs USUV (post-treatment), $P<0.05$ for all IFNs; *, USUV vs VSV, $P<0.05$ for leucocyte IFN alpha (pre-treatment), IFN beta (pre-treatment and post-treatment), IFN lambda 2 (pre-treatment); **, (USUV) IFN omega vs leucocyte IFN alpha, IFN beta (pre-treatment), $P<0.05$; ***, (USUV) IFN lambda 1–3 vs type I IFN preparations (pre-treatment and post-treatment), $P<0.05$; °, IFN lambda 1 vs IFN lambda 2–3 (post-treatment), $P<0.05$. (b) §, USUV (pre-treatment) vs USUV (post-treatment), $P<0.05$ for all IFNs. (c) *, USUV vs VSV, $P<0.05$. (d) *, IFN alpha subtypes vs IFN beta, IFN omega, IFN lambda 1–3, $P<0.05$; **, IFN omega vs IFN beta, IFN lambda 1–3 at 48 h p.i., $P<0.05$. For the statistical comparison of protein levels of different IFNs induced at 24 h p.i., the levels of IFN alpha subtypes were considered to be 13 pg ml^{-1}. Data shown are means ± SD for three independent experiments.

detectable IFN antiviral activity was observed at the other time points analysed for both viruses. Next, we characterized the antiviral activity in the supernatant of Hep-2 cultures infected with USUV confirming that types I and III IFN protein productions could be recorded only after 24 and 48 h p.i. (Fig. 3d). In addition we observed that comparable IFN beta and IFN lambda 1–3 protein levels were induced at 24 and 48 h p.i. In contrast, IFN omega levels were lower compared with IFN beta and IFN lambda 1–3 only at 48 h p.i ($P<0.05$). Interestingly, results also showed that USUV induces lower production of IFN alpha subtypes compared with IFN beta, IFN omega and IFN lambda subtypes both at 24 and 48 h p.i. ($P<0.05$). In the light of these observations it is tempting to speculate that USUV has evolved by developing countermeasures to evade or attenuate the IFN antiviral response. There are several examples in the literature. Flaviviruses are able to overcome host innate immunity and productively infect the host by evading the interaction of pathogen-associated molecular patterns (PAMPs) with the cellular pattern recognition receptors and by the expression of antagonist molecules which directly block the intracellular pathways that lead to type I IFN production and signalling (Morrison *et al.*, 2012; Daffis *et al.*, 2009). Interestingly,

regardless of these considerations, our results confirmed that IFN lambda 1–3, like type I IFNs, are produced after viral infection (Ank *et al.*, 2006) and showed that USUV differentially regulates the induction of IFNs.

In summary, the present study demonstrates for the first time that USUV replicates efficiently in human cell lines derived from different tissues and organs, and that the virus induces a weak antiviral response. Furthermore we show that USUV is highly sensitive to the antiviral actions of types I and III IFNs only when cells were treated prior to but not after viral infection, suggesting that an established USUV infection is able to overcome the antiviral effect of IFNs.

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