

Chikungunya virus isolates with/without A226V mutation show different sensitivity to IFN- α , but similar replication kinetics in non human primate cells

Licia Bordi¹, Silvia Meschi¹, Marina Selleri¹, Eleonora Lalle¹, Concetta Castilletti¹, Fabrizio Carletti², Roberta Chiappini², Antonino Di Caro², Maria Rosaria Capobianchi¹

¹Laboratory of Virology, National Institute for Infectious Diseases "L. Spallanzani", Rome, Italy;

²Laboratory of Microbiology and Infectious Disease Biorepository, National Institute for Infectious Diseases "L. Spallanzani", Rome, Italy

SUMMARY

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus responsible for the first autochthonous Italian outbreak in 2007. A226V mutation in E1 has been associated with enhanced replication in *A. albopictus* vector. Possible involvement of this mutation in enhanced infection capability in primate cells and sensitivity to exogenous interferon (IFN)- α was investigated.

No significant differences were observed between the two isolates in terms of replication kinetic, virus yield and cytopathic effect (CPE). Interestingly, the A226V-carrying strain was more susceptible to the antiviral action of recombinant IFN- α . The interplay between A226V mutation and innate defence mechanisms needs further investigation.

KEY WORDS: Chikungunya, A226V mutation, Pathogenesis, Interferon α

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Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus belonging to Togaviridae family. CHIKV is geographically distributed in Africa, Asia, Indian Ocean Islands and India (Saluzzo *et al.*, 1983; Enserink *et al.*, 2006; *et al.*, 2006; AbuBakar *et al.*, 2007) and is also responsible for several imported cases in Southern Europe, giving rise, in 2007, to the first autochthonous European outbreak in Italy (Rezza *et al.*, 2007; Charrel *et al.*, 2008). At present, after a few years of relative dormancy in Réunion Island, CHIKV transmission has restarted, with one case imported in France (May 2010). This episode has refreshed concerns about the possibility of re-

newed autochthonous transmission in Mediterranean countries. Given the expanding geographic range of CHIKV and its potential to rapidly cause large scale epidemics, it has become important to understand the immune and pathogenic mechanisms active during CHIKV infections in order to guide the development of targeted and effective control and treatment strategies. Phylogenetic analysis of CHIKV strains circulating in *A. Albopictus*-humans transmission cycles, obtained during outbreaks, have identified the independent acquisition of a common mutation in E1 glycoprotein (E1gp), namely A226V, in strains isolated from different geographic regions (Schuffenecker *et al.*, 2006; de Lambellerie *et al.*, 2008). In a previous paper we analysed seven CHIKV isolates, five imported to Italy and two coming from the Italian outbreak, with respect to the presence of A226 mutation (Bordi *et al.*, 2008). All the imported and autochthonous strains showed the A226V mutation with the exception of the isolate imported from India in

Corresponding author

Maria Rosaria Capobianchi
Laboratory of Virology, Padiglione Baglivi
National Institute for Infectious Diseases
INMI "L. Spallanzani"
Via Portuense, 292
00149 Rome, Italy
E-mail: mrosaria.capobianchi@inmi.it

2006, suggesting that the acquisition and fixation of the A226V mutation may be a common pathway of CHIKV outbreak explosion, in a parallel interplay with the mosquito vector dynamics. Since this mutation has been associated with enhanced replication and fitness of CHIKV in *A. albopictus* vector and has also been shown to modulate cholesterol requirement for infection of insect cells (Tsetsarkin *et al.*, 2007), we investigated the possible involvement of A226V mutation in enhanced pathogenesis in non vector hosts, by testing the replication competence in primate cell cultures of two isolates, among those described (Bordi *et al.*, 2008), differing for the presence or absence of this mutation. In addition, since it has been clearly demonstrated that CHIKV replication is significantly influenced by type I and II IFNs (Sourisseau *et al.*, 2007; Courderc *et al.*, 2008; Schilte *et al.*, 2010), we considered the possibility that the A226V mutation could be associated with partial resistance to the inhibitory action of IFN- α in classical experiments of inhibition of virus replication.

Two primary CHIKV isolates, one carrying the A226V mutation and one with wild type aminoacid were serially adapted on Vero E6 cells with identical passage history (6 passages) and culture conditions. Following those passages, the presence and absence of the mutation in the two isolates was confirmed by sequence analysis. The partial E1 sequences were identical to the original ones obtained from the primary isolates, deposited in GenBank (accession number: EU188924, named A226V and EU190884, named 226wt). The two virus preparations were used to infect Vero E6 cells, using either single replication cycle conditions (i.e. MOI 10, data not shown) or multiple replication (i.e. MOI 0.01, Figure 1A). After 1h of adsorption, cells were washed, treated with trypsin (0.05%)/EDTA (0.02%) for 2 min at 37°C and washed again, to eliminate residual inoculum and virus remained in the outer cell surface. At the indicated times post-infection, progeny virus was harvested by freezing/thawing the cultures three times. After supernatant clarification, virus yield was measured by both quantitative real time RT-PCR and viral infectivity assay. Quantitative real time RT-PCR targeting nsP1 gene was performed according to (Carletti *et al.*, 2007), and the results expressed as copies/ml. Virus titration was per-

formed on VeroE6 cells with limiting dilution assay, using three-fold serial dilutions. Infectivity is expressed as 50% tissue culture infectious dose (TCID₅₀). To establish the proportion of virus-infected cells, cells were seeded in chamber slides (Lab-Tek™, Thermo Fisher Scientific, Waltham, Massachusetts) infected at MOI 0.01 and stained with specific antibodies at different time points. Specifically, at each time point 5×10^5 cells were washed with phosphate-buffered saline, fixed and incubated with a human serum sample positive for Chikungunya IgG (IFA titer: 320) for 30 minutes at 37°C. Then, the slides were washed with PBS and incubated with fluorescein-labeled affinity-purified goat antibodies to human immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, Missouri). The enumeration of immunofluorescent cells, performed on five different fields by two investigators blinded to the experiment conditions, was highly reproducible for both isolates. For IFN- α experiments, Vero E6 cells were treated for 24h with increasing amounts of recombinant IFN- α (0-0.16-0.5-1.5-4.5-13.7-41-123-370-1,111-3,330-10,000 IU/ml), then infected with either of the two CHIKV isolates at MOI 0.01. After 24h, cells were fixed and stained with crystal violet (Fig.1B). In a separate series of experiments using different amounts of recombinant IFN- α (0-2-20-200-2,000-20,000 IU/ml), virus yield was measured by both quantitative real time RT-PCR and viral infectivity assay. Statistical analysis of the results was performed using unpaired t-test. No significant differences between the two isolates were observed in terms of replication kinetics on Vero E6 cells using either single replication cycle (MOI 10) and multiple replication cycle (MOI 0.01) conditions. Specifically, under single replication cycle conditions, the replication curve peaked at 24h post-infection and remained at plateau thereafter (data not shown); under multiple replication cycle conditions, replication kinetics peaked at 48h post-infection, with a subsequent plateau (Figure 1A).

Immunofluorescence staining of cultures infected at MOI 0.01 showed no significant difference between the two isolates. In both cases, the proportion of cells stained with virus-specific antibodies progressively rose from 1% at 6 hours to 70-80% at 24 hours, reaching a plateau of 100% thereafter (insert of Figure 1A). The shape and the size of the foci of fluorescent cells (insert of

Figure 1A) as well as the appearance of cytopathic effect (CPE) in infected cultures were indistinguishable between the two isolates. *In vitro* experiments of inhibition of virus replication by recombinant IFN- α on Vero E6 cells showed a dose-dependent reduction of CPE (Figure 1B) and of virus replication for both isolates, assessed by infectious virus yield (Figure 1D) and viral RNA titration (Figure 1C). However, the results from all standpoints indicated that the A226V carrying isolate is more sensitive to the antiviral effect of IFN- α : in fact, the limiting dilution at which IFN-induced protection vanished was 0.5-1.5 IU/ml for the A226V-carrying isolate, and 14 IU/ml for the wt strain (Figure 1B). A consistent 1 Log shift

of the inhibition curve for both RNA and TCID50 was observed for virus yield measurements (Figure 1C, 1D). Moreover, when measuring the ratio RNA/infectivity of the virus progeny obtained in the various experimental conditions, a significant increase was observed in the presence of IFN- α for the A226V-carrying isolate (1.44 ± 0.31 untreated vs 2.28 ± 0.06 , with 20,000 IU/ml IFN- α , $p=0.024$ in Student's t test), while no significant difference was observed for the wt strain (1.45 ± 0.62 untreated vs 1.30 ± 0.21 with 20,000 IU/ml IFN- α , $p=0.764$).

In conclusion, a complex interplay of factors such as virus genetic variations as well as environmental factors may influence the emergence of

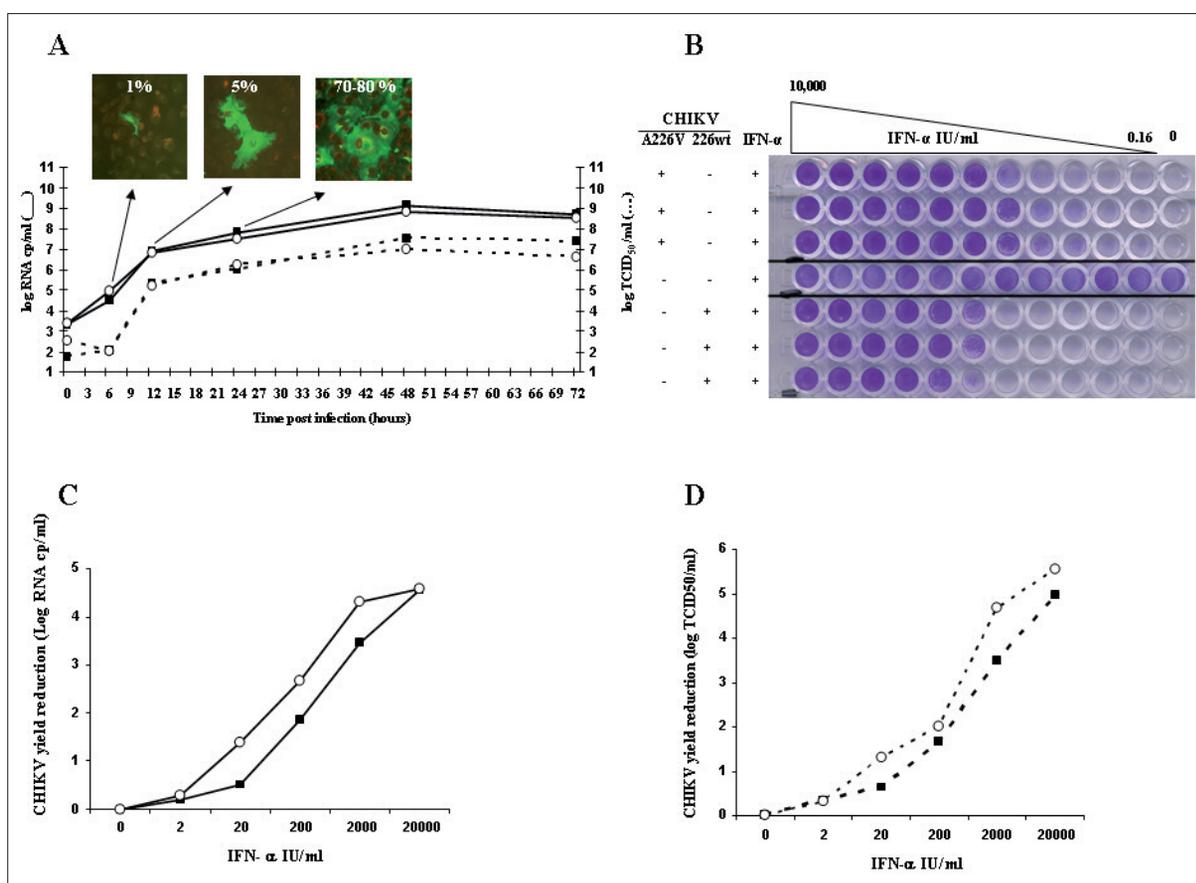


FIGURE 1 - Time course of the replication of 226wt and A226V CHIKV isolates on primate Vero E6 cells (Panel A), and the inhibition of virus replication by recombinant IFN- α (Panels B-D). **Panel A:** Cells were infected at MOI 0.01 and virus replication was monitored as viral RNA and infectivity yield; at the time points marked by arrows, the shape of infected cell foci is shown in the insert, and the percentage of cells stained by virus-specific antiserum is indicated. **Panel B:** Dose-dependent reduction of viral CPE by recombinant IFN- α . **Panels C and D:** Dose-dependent reduction of viral RNA and virus infectivity yield, respectively, by recombinant IFN- α . One representative experiment is shown for each panel. Symbols: ■ = 226wt; ○ = A226V; continuous line: viral RNA (Log cp/ml); dotted line: viral infectivity (Log TCID₅₀/ml).

new infectious diseases, and may therefore play important roles in this regard. Changes in the envelope glycoproteins of CHIKV have been described to affect infectivity in different mosquito species. In particular, a single change at position 226 of E1 gene has been associated with the adaptation of the virus to more efficiently infect and be transmitted by *A. albopictus* vector, although not affecting the virus replication efficiency in insect cells *in vitro* (10). Our results suggest that the presence of A226V mutation also does not influence the replication kinetics on primate cells, both using single and multiple replication cycle conditions. Moreover, the time course of appearance of CPE and of cells immunostained with CHIKV-specific antiserum, is very similar for both the isolates, as is the shape of the virus-positive multicellular foci, thus suggesting a similar mechanism of spread of the virus in the infected cell cultures.

During the very early phases of infection, a pivotal role in the outcome of virus infection is currently attributed to all the immediate response mechanisms that the host may deploy. Among immune response mechanisms, the innate compartment surely deserves special consideration, given its ability to respond immediately and effectively to virus infections.

Overall, our results do not support the concept that A226V mutation confers a replicative advantage in primate cell cultures, nor do they support the possibility that partial resistance to the inhibitory action of IFN- α could account for the explosive spread of the mutated strain in the human population in the countries where this mutation had occurred (8).

In fact, the isolate carrying the A226V mutation is more inhibited by recombinant IFN- α with respect to the wild type one in terms of viral RNA, infectivity and reduction of CPE. A stronger inhibition of virus infectivity for the mutated strain is also supported by the fact that the RNA/TCID₅₀ ratio is significantly enhanced in virus progeny replicated in the presence of IFN- α for this strain, while it is virtually unaffected for the wt strain. Major limitations of the present study are the use of only two primary isolates, and the lack of information on their genetic background. In fact, genetic divergence between the two isolates in genome positions other than E1226, as well as phenotypic or epigenetic characteristics, or *in vitro*

selection bias, may have contributed to the observed differences in sensitivity to exogenous IFN- α . However, we tried to reduce the selection bias due to *in vitro* isolation and propagation as much as possible using virus stocks with identical passage history and culture conditions.

Overall, it is not possible, at present, to draw clear evidence that the A226V mutation is actually contributing to enhanced pathogenicity in primate hosts (including humans), and targeted experiments introducing the A226V mutation in a common genetic background are warranted to clarify this point.

In addition, the possibility that the interplay between the virus and the innate defence system may act at different levels of the virus/host interaction is to be taken into consideration, by exploring, for instance, other steps of the IFN response activation.

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