Chikungunya virus infection: an overview

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**INTRODUCTION**

Chikungunya virus (CHIKV), an arbovirus transmitted by mosquito vectors, is an alphavirus belonging to the *Togaviridae* family. Alphaviruses are small spherical enveloped viruses, with a 60-70 nm diameter. The genome is a single-strand RNA molecule of positive polarity, encoding four non structural (nsP1-4) and three structural proteins (C, E1, E2). Viral replication is initiated by attachment of the viral envelope to host cell receptors (Strauss and Strauss, 1994), followed by clathrin-mediated endocytosis of the attached particle (Lee et al., 2013), low pH-mediated membrane fusion and delivery of the viral nucleocapsid into the cytoplasm (Sorisseau et al., 2007). To date no CHIKV interacting protein has been characterized, but in a very recent study, Wintachai et al. identified prohibitin as CHIKV-binding protein expressed by microglial cells (Wintachai et al., 2012). The replication cycle is fast, taking around 4 hours. Alphaviruses are sensitive to desiccation and to temperatures above 58°C (Strauss and Strauss, 1994; Khan et al., 2002). About 30 species of arthropod-borne viruses are included in the alphavirus genus, antigenically classified into 7 complexes. These viruses are widely distributed throughout the world, with the exception of Antarctica. Besides CHIKV, several arthropod-transmitted alphaviruses cause human disease, characterized by similar clinical presentation: Barmah Forest...
Chikungunya fever (CHIKF) derives its name from Makonde, a language spoken in south Tanzania, and means “that which bends up”, referring to the posture of patients afflicted with severe joint pain characterizing this infection. First isolated in Tanzania in 1952 (Robinson, 1955), CHIKV attracted worldwide attention when it caused a massive outbreak in the Indian Ocean islands (Enserik, 2006). Since 1952, CHIKV has caused a number of epidemics, both in Africa and in Southeast Asia, many of them involving hundreds of thousands of people. After a few years of relative dormancy in La Réunion Island, CHIKV transmission has restarted, renewing concerns about the possibility of renewed autochthonous transmission in Mediterranean countries.

**GEOGRAPHIC DISTRIBUTION**

CHIKF has an epidemiological pattern with both sporadic and epidemics cases in West Africa, from Senegal to Cameroun, and in many other African countries (Democratic Republic of Congo, Nigeria, Angola, Uganda, Guinea, Malawi, Central African Republic, Burundi, and South Africa). Moreover, many epidemics occurred in Asia (Burma, Thailand, Cambodia, Vietnam, India, Sri Lanka, Timor, Indonesia, and the Philippines) in the 1960s and in the 1990s (Pialoux et al., 2007; Jain et al., 2008). Major epidemics appear and disappear cyclically, usually with an inter-epidemic period ranging from 7 to 20 years. The huge outbreak that increased concern about CHIKV started in Kenya in 2004, where the seroprevalence rates reached 75% in Lamu island (Pialoux et al., 2007), before reaching the Comores, Seychelles, and Mauritius islands. The virus reached La Réunion island in March-April 2005, probably

![FIGURE 1 - Geographic distribution of CHIKV shown in the most recent map (May 2012) retrieved from the CDC website (http://www.cdc.gov/chikungunya/map/index.html, last accessed May 2013).](http://www.cdc.gov/chikungunya/map/index.html)
as a result of importation of cases among immigrants from the Comores and rapidly spread to several countries in the Indian Ocean and India (Enserik, 2006; Mavalankar et al., 2007). Compared to earlier outbreaks, this episode was massive, occurred in highly medicalized areas such as La Réunion, and had very significant economic and social impact. Since the beginning of the outbreak in the Indian Ocean region, more than 1,000 imported CHIKV cases have been detected among European and American travellers returning from the affected areas (Fusco et al., 2006; Taubiz et al., 2007), giving rise, in 2007, to the first autochthonous (human-to-mosquito-to-human transmission) European outbreak in Italy (Rezza et al., 2007; Charrel and de Lambellerie, 2008). During the period December 2006-July 2009, no confirmed cases were detected on La Réunion and Mayotte Islands, but new outbreaks were reported in Madagascar. After a few years of relative dormancy in La Réunion, CHIKV transmission restarted in 2009 and 2010, leading to re-importation to Europe (May 2010) (D’Ortenzio et al., 2011).

During the last three years (2011-2013) concerns about Chikungunya outbreaks arose again due to increasing number of CHIKV infections, starting from 2011, when a massive outbreak with more than 11,000 cases occurred in the Republic of Congo (Brazzaville) (ProMED-mail: 20110613.1806). During 2012, 29 cases of CHIKV infection were reported in India (Rajasthan) (ProMED-mail: 20120716.1203694), and two additional outbreaks were recorded: one in Cambodia, with almost 1,500 cases (ProMED-mail: 20120920.1303166) and one in the main island of Papua New Guinea, with a total of 633 suspected cases (ProMED-mail: 20121010.1335814); Bali has also had sporadic outbreaks (ProMED-mail: 20130320.1594512). In Samar (Philippines) 600 cases were recorded in 2012, but in 2013 the infection rate has been increasing, with 500 cases recorded until March; these numbers appear to be increasing day by day (ProMED-mail: 20130128.1518853). Considering the capacity of CHIKV to emerge, re-emerge, and quickly spread in novel areas, heightened surveillance and preparedness seem to be a priority. In particular, travellers act as carriers who inadvertently ferry pathogens between countries. They can thus serve as a sentinel population providing information on the emergence or re-emergence of an infectious pathogen in a source region, and can be used to map the location, dynamics and movement of pathogenic strains (Pistone et al., 2009).

The geographic range of CHIKV is mainly in Africa, Asia and Australia (Figure 1).

**PHYLOGENESIS**

Three lineages of CHIKV, with distinct genotypic and antigenic characteristics, have been identified. Isolates that caused the 2004-06 Indian Ocean outbreak form a distinct cluster within the large eastern/central Africa (ECSA) phylogenetic group, in addition to the Asian and west African phylogenetic groups (Powers et al., 2000; Schuffenecker et al., 2006). The divergence of each distinct lineage reflects, to some extent, the path of global transmission and occasional outbreaks. According to phylogenetic analysis performed by Volk and colleagues (2010), the currently circulating CHIKV strains have an ancestor that existed within the last 500 years. Interestingly, despite their close geographic distance, the two African lineages did not cluster together, indicating limited genetic exchange between the two lineages in Africa. The only exception was a 1963 bat isolate from Senegal, which grouped in the ECSA clade. This finding is the first to suggest that the main West African and ECSA lineages may overlap spatially in the enzootic cycle, at least occasionally (Volk et al., 2010).

Moreover, phylogenetic analysis of CHIKV strains circulating in A. albopictus-human transmission cycles, obtained during outbreaks, 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., 2008a). This mutation, together with M269V and D284E E1gp mutations, have been described as molecular signatures of the Indian Ocean outbreak (Arankalle et al., 2007; Tsetsarkin et al., 2007; Vazeille M, 2007). In particular, the A226V mutation, which was absent in the strains isolated during the initial phases of the outbreak in La Réunion, appeared in >90% of the isolates after December 2005. This change could be related to...
virus adaptation to the mosquito vector species (see below). Together with the lack of herd immunity, this might explain the abrupt and escalating nature of the La Réunion outbreak. The A226V mutation was clearly demonstrated to increase viral fitness in the *A. albopictus* vector (Vazeille *et al.*, 2007; Tsetsarkin *et al.*, 2007) that, in turn, may expand the potential for CHIKV to diffuse to the Americas and Europe, due to the widespread distribution of this vector, particularly in Italy (Knudsen, 1995). In a previous paper we characterized 7 viral isolates (5 imported and 2 autochthonous cases) with respect to the molecular E1 signatures of the Indian Ocean Outbreak, particularly the A226V mutation. These isolates had been obtained from 3 travellers returning from Mauritius in 2006, 2 returning from India in 2006 and 2007, and 2 autochthonous cas-

**FIGURE 2** - Phylogenetic tree of CHIKV strains performed on partial E1 gene. Sequences of a 1013 bp fragment of E1 gene (nucleotide positions 10145-11158, with respect to the reference strain S27). The CHIKV strains isolated from human cases in Italy (3 strains deriving from patients returning to Italy from Mauritius, 2 strains from patients returning from India, 2 strains from patients involved in the 2007 Italian outbreak) are indicated with the strain name in bold. Their GenBank accession numbers are: EU188924 for ITA1_TAM_06; EU190879 for ITA2_BMI_06; EU190881 for ITA3_CGO_06; EU190884 for ITA4_MRA_06; EU272130 for ITA5_JEM_07; EU272132 for ITA7_BI_07; EU272133 for ITA8_VEN_07 (Bordi *et al.*, 2008). The sequences used for comparison are indicated with their GenBank accession number. CHIKV strains carrying the A226V mutation are underlined.
es that occurred during the 2007 Italian outbreak (Bordi et al., 2008) (Figure 2).
All the strains isolated in Italy, both imported and autochthonous, displayed two molecular signatures of the Indian Ocean outbreak (M269V and D284E). The A226V mutation was present in all imported and autochthonous cases, with the exception of the isolate imported from the Indian subcontinent in 2006. The absence of this mutation in the isolate imported in 2006 from India was in agreement with published data (Arankalle et al., 2007), and with available GenBank sequence data indicating that the virus strains circulating in India in 2006 lacked this mutation.

The presence of A226V in the isolate imported from India in July 2007 and in the isolates from the 2007 Italian outbreak (originating from a case imported from India) supports the view that the virus envelope sequence of strains from India changed over time, acquiring the E1 mutation associated with enhanced fitness in A. albopictus after 2006. So it appears that the acquisition and fixation of the A226V mutation may be a common pathway of Chikungunya explosion in epidemic areas, in a parallel interplay with the mosquito vector dynamics. It is noteworthy that the outbreak in Singapore, where the A226V mutation was absent, was rapidly controlled.

VECTOR AND RESERVOIR
Two distinct transmission cycles have been well documented for CHIKV: an enzootic sylvatic cycle and an endemic/epidemic urban cycle. The African sylvatic cycle likely involves several arboreal Aedes mosquitoes species as vectors (A. furcifer, A. vittatus, A. fulgens, A. luteocephalus, A. dalzieli, A. vigilax, A. camptorhynchites) and non-human primates as reservoir/amplifying hosts. In Africa, the enzootic transmission cycle can spill over to infect people who live nearby, and enzootic mosquito vectors may be involved in inter-human transmission during small outbreaks. A. furcifer, probably a principal enzootic vector, is known to enter human villages (Diallo, 1999), where it presumably transmits the virus from monkeys to humans (Peyrefitte et al., 2007; Peyrefitte et al., 2008). Endemic/epidemic transmission cycles were established when the virus was introduced into Asia around 1950, and into the Indian Ocean region, India and then Southeast Asia since 2005. As previously stated, a mutation in the E1gp gene, that results in the A226V amino acid substitution, dramatically increased the infectivity of some epidemic strains for an alternative urban vector, A. albopictus (ProMED archive 20100926.3495). Therefore, the urban transmission cycle relies only on A. aegypti and/or A. albopictus, anthropophilic vectors that can initiate human-mosquito-human transmission, and human amplification hosts. This endemic/epidemic cycle results in high levels of human exposure to mosquito transmission, particularly because these vectors live in close proximity to people. The behaviour and ecology of A. aegypti, in particular, are ideal for epidemic transmission because adult females prefer to feed on humans, often take several blood meals during a single gonotrophic cycle, oviposit in artificial containers as their preferred larval sites, and rest inside houses with ready access to human hosts (Weaver et al., 2012).

A. albopictus is zoophilic and anthropophilic, aggressive, silent, active all-day long, and has a lifespan longer than other mosquitoes (up to 8 weeks). In recent decades it has expanded to several areas previously known to be Aedes-free (Charrel et al. 2007). It seems that most new introductions of A. albopictus have been caused by vegetative eggs contained in timber and tyres exported from Asia throughout the world. Other emerging events also contributed to the introduction of A. albopictus mosquitoes into previously unaffected areas, such as climate change and the increasing use of plastic containers in developing countries. Indeed, climate changes may have several effects on vector biology: increasing temperatures may improve survival at higher latitudes and altitudes, increase the growth rates of vector populations, and alter their seasonality; increased rainfall may have an effect on the larval habitat and population size, and finally an increase in humidity could favourably affect vector survival (Gubler et al., 2001). The use of plastic containers in developing countries, where they are usually not correctly disposed of and remain in the environment for years, has also been linked with the spread of the mosquitoes: acting as rainwater receptacles, and being exposed to sunlight, they can become perfect “incubators” for mosquito eggs, where the ideal conditions of tem-
perature and humidity are achieved easily and naturally. Human beings serve as the main CHIKV reservoir during epidemic periods. In Africa some animals (monkeys, rodents, and birds) constitute the virus reservoir during non-epidemic periods, sustaining virus circulation in the environment in the absence of human cases. Outbreaks might occur in monkeys when herd immunity is low; the animals develop viremia but no pronounced physical manifestations (Wolfe et al., 2001; Inoue et al., 2003). An animal reservoir has not been identified in Asia, where humans appear to be the only host.

**TREATMENT AND PREVENTION**

There are no specific drugs against CHIKV and patients are symptomatically treated with non-steroidal anti-inflammatory drugs, fluids, and medicines to relieve symptoms of fever and aching, such as ibuprofen, naproxen, acetaminophen, or paracetamol. Steroids have occasionally been used but their efficacy was not significant (Taubitz et al., 2007). Some time ago chloroquine, a drug useful for prophylaxis and treatment of malaria, showed promising results for treating chronic Chikungunya arthritis (Brighton, 1984), even if a further trial conducted on La Réunion Island proved that there was no justification for the use of chloroquine to treat acute Chikungunya disease (de Lamballerie et al., 2008b); overall, the usefulness of chloroquine treatment remains unclear. Ribavirin (200 mg twice a day for seven days) given to patients who continued to have crippling lower limb pains and arthritis for at least two weeks after a febrile episode, seems to be effective against CHIKV, leading to faster resolution of joint and soft tissue manifestations (Ravichandran and Manian, 2008). Briolant and colleagues (2004) screened various active antiviral compounds against viruses of the alphavirus genus in vitro and demonstrated that 6-azauridinet was more effective than ribavirin against CHIKV. Moreover, the combination of interferon (IFN)-α2b and ribavirin had a synergistic antiviral effect on CHIKV (Briolant et al., 2004). Since inhibitors of chemokine pathways associated with monocyte/macrophage recruitment may be a promising approach in humans, to be further explored. It is widely recognized that passive vaccination is an appropriate preventive and therapeutic option for many viral infections in humans, including those spread by viral vertical transmission, especially when no alternative therapy is available (Dessain et al., 2008). CHIKV infection seems to elicit long-lasting protective immunity, and experiments performed using animal models have shown a partial cross-protection among CHIKV and other alphaviruses (Hearn and Rainey, 1963; Edelman et al., 2000). Since human polyclonal immune globulins, purified from plasma samples obtained from donors in the convalescent phase of CHIKV infection, exhibited high neutralizing activity in vitro and a powerful prophylactic and therapeutic efficacy against CHIKV infection in vivo mouse models (Couderc et al., 2009), it could be used in humans for prevention and treatment, especially in individuals at risk of severe CHIKV disease, such as neonates born to viremic mothers and adults with underlying conditions. Polyclonal immune globulins present the advantage of a broad reactivity but the therapeutic intervention is limited, due to the short viremia in the acute phase of CHIKV infection: thus the only benefit this treatment has to offer would be to help reducing viremia faster (Kam et al., 2009). As an alternative approach, more specific human monoclonal antibodies (MAbs) could be used. In a recent study two unique human MAbs, specific for the CHIKV E1gp, strongly and specifically neutralized CHIKV infection in vitro (Warter et al., 2011).

To date a number of CHIKV vaccines have been developed, but none have been licensed. While a number of significant questions remain to be addressed related to vaccine validation, such as the most appropriate animal models (species, age, immune status), the dose and route of immunization, the potential interference from multiple vaccinations against different viruses, and lastly, the practical cost of the vaccine, since most of the epidemic geographical regions belong to the developing countries, there is real hope that a vaccine to prevent this disease will not be too long in arriving. Although no licensed vaccines are currently available for CHIKV, potential vaccine candidates...
have been tested in humans and animals with varying success. Several vaccine strategies have been undertaken:
1. whole inactivated virus preparation;
2. attenuated live vaccines;
3. recombinant proteins or virus-like particles;
4. DNA vaccination.

Due to the ease of preparation, the first developed vaccines were formulations of whole-virus grown in cell cultures and inactivated either by formalin or tween-ether (Harrison et al., 1967; Eckels et al., 1970; Harrison et al., 1971; White et al., 1972). Further vaccines focused on attenuated strains of CHIKV obtained after serial passages in cell cultures (Levitt et al., 1986; Edelman et al., 2000). One of these promising candidates is TSI-GSD-218, a serially passaged and plaque-purified live CHIKV vaccine, tested for safety and immunogenicity in human Phase II trials by the US Army Medical Research Institute (Edelman et al., 2000). Some chimeric candidate vaccines were developed using either Venezuelan Equine Encephalitis virus (VEEV) attenuated vaccine strain TC-83, a naturally attenuated strain of Eastern Equine Encephalitis virus, or SINV as a backbone and the structural protein genes of CHIKV. Vaccinated mice were fully protected against disease and viremia after CHIKV challenge (Wang et al., 2008). Traditional attenuation approaches, relying on cell culture passages, typically result in attenuation that depends only on small numbers of attenuating point mutations. In addition to the risk of reactogenicity, attenuation based on small numbers of mutations can also result in residual alphavirus infectivity for mosquito vectors. This risk, underscored by the isolation of the TC-83 VEEV vaccine strain from mosquitoes in Louisiana during an equine vaccination campaign designed to control the 1971 epidemic (Pedersen et al., 1972), is especially high when a vaccine that relies on a small number of point mutations is used in a non-endemic location that could support a local transmission cycle.

In 2012, the United States Army developed and tested a live attenuated strain of CHIKV, CHIKV181/25 for vaccine application. CHIKV181/25 demonstrated an excellent immunogenic profile, however, transient arthralgia was observed in about 8% of vaccine recipients. Sharma and colleagues tried to inactivate CHIKV181/25 with 1,5 iodonaphyl azide (INA), a photoactive hydrophobic azide molecule that they used in a previous study (Sharma et al., 2007) to completely inactivate VEEV, in addition to UV irradiation. The INA-inactivated CHIKV181/25 formulation may address the issue of residual virulence associated with live attenuated CHIKV181/25, but the INA-inactivation results in a relatively weaker binding capacity of CHIKV181/25 to the neutralizing polyclonal anti-CHIKV E2 glycoprotein (E2gp) so that further investigations are necessary (Sharma et al., 2012). Alternative genetic strategies such as viral chimeras offer the promise of more stable attenuation (Kennedy et al., 2011). For instance, a recent study showed that chimeric alphaviruses, encoding CHIKV-specific structural genes (but no structural or nonstructural proteins capable of interfering with development of cellular antiviral response), induce protective immune response against subsequent CHIKV challenge (Wang et al., 2011).

A novel CHIKV vaccine candidate, CHIKV/IRES (internal ribosome entry site), was generated by manipulation of the structural protein expression of a wt-CHIKV strain via the encephalomyocarditis virus IRES, and exhibited a high degree of murine attenuation that was not dependent on an intact IFN type I response, highly attenuated and efficacious after a single dose (Plante et al., 2011). Another approach, recently undertaken by Akata and colleagues (2010), was the use of virus-like particles (VLPs) expressing CHIKV structural proteins that resemble replication-competent alphaviruses (Akahata et al., 2010). Immunization of monkeys with these VLPs elicited neutralizing antibodies against envelope proteins from different CHIKV strains that could confer passive protection against lethal CHIKV challenge into new mice. The last frontier in the approach of CHIKV vaccine design is the DNA vaccine strategy. An adaptive constant-current electroporation technique was used to immunize mice (Muthumani et al., 2008) and rhesus macaques (Mallilankaraman et al., 2011) with an intramuscular injection of plasmid coding for the CHIKV-capsid, E1 and E2. Vaccination induced robust antigen-specific cellular and humoral immune responses in both cases. Kumar and colleagues (2012) aimed to develop candidate vaccines following two different strategies: one based on recombinant E2gp; the other based on chemically inactivated whole virus, both...
with promising results (Kumar et al., 2012). Since a vaccine is not currently available, protection against mosquito bites and vector control are the main preventive measures. Individual protection relies on the use of mosquito repellents and measures to limit skin exposure to mosquitoes. Bednets should be used during the night in hospitals and day-care facilities but Aedes mosquitoes are active all-day long. Control of both adult and larval mosquito populations uses the same model as for dengue and has been relatively effective in many countries and settings. Breeding sites must be removed, destroyed, frequently emptied, and cleaned or treated with insecticides. Control of A. aegypti has rarely been achieved and never sustained (Reiter et al., 2006). Recent data show the different degrees of insecticide resistance in A. albopictus and A. aegypti (Cui et al., 2006). Large-scale prevention campaigns using dichlorodiphenyltrichloroethane have been effective against A. aegypti but not A. albopictus. However, vector control is an endless, costly, and labor-intensive measure and is not always well accepted by local populations, whose cooperation is crucial. Control of CHIKV infection, other than use of drugs for treatment of disease, development of vaccines, individual protection from mosquitoes and vector control programs, also involves surveillance that is fundamental for early identification of cases and quarantine measurement. A model used in investigation of the transmission potential of CHIKV in Italy has proven useful to provide insight into the possible impact of future outbreaks in temperate climate regions and the effectiveness of the interventions performed during the outbreak (Poletti et al., 2011).

CLINICAL MANIFESTATIONS

After infection with CHIKV, there is a silent incubation period lasting about 2-4 days (range 1-12 days) (Lam et al., 2001). Clinical onset is abrupt, with high fever, headache, back pain, myalgia, and arthralgia; the latter can be intense, affecting mainly the extremities (ankles, wrists, phalanges) but also the large joints (Robinson, 1955; Lam et al., 2001; Hochedez et al., 2006; Quatresous, 2006; Saxena et al., 2006). Skin involvement is present in about 40-50% of cases, and consists of a pruriginous maculopapular rash predominating on the thorax. The clinical presentation may also involve facial oedema and, in children, a bullous rash with pronounced sloughing, localised petechiae and gingivorrhagia (Fourie and Morrison, 1979; Brighton et al., 1983). Radiological findings are normal, and biological markers of inflammation (erythrocyte sedimentation rate and C-reactive protein) are normal or moderately elevated (Fourie and Morrison, 1979; Kennedy et al., 1980). Iridocyclitis and retinitis are the most common ocular manifestations associated with CHIKF; less frequent ocular lesions include episcleritis. All ocular manifestations have a benign course with complete resolution and preservation of vision. Retinitis shows gradual resolution over a period of 6 to 8 weeks (Mahendradas et al., 2008). Erratic, relapsing, and incapacitating arthralgia is the hallmark of Chikungunya, although it rarely affects children. These manifestations are normally migratory and involve the small joints of hands, wrists, ankles, and feet with pain on movement. The symptoms generally resolve within 7-10 days, except for joint stiffness and pain: up to 12% of patients still have chronic arthralgia three years after onset of the illness. Arthralgia experienced by CHIKV patients closely resembles the symptoms induced by other viruses like RRV and BFV (et al., 2002; Jacups et al., 2008). Neurological complications such as meningo-encephalitis were reported in a few patients during the first Indian outbreak in 1973, and during the 2006 Indian outbreak (Chatterjee et al., 1965 a, b; Ravi, 2006). Moreover, during the 2006 Indian-Ocean outbreak, rare cases of Guillain-Barré syndrome associated with CHIKV infection have been described (Wielanek et al., 2007; Lebrun et al., 2009). The possible mechanisms underlying these processes remain unknown, even if it was found that mouse CNS tissues such as the choroid plexi could also be targets of CHIKV, lending more credence to the fact that CHIKV infections do affect CNS cells and tissues (Couderc et al., 2008). Other rare complications described after CHIKV infection are mild hemorrhage, myocarditis, and hepatitis (Lemant et al., 2008). CHIKV is not generally considered a life-threatening disease. Usually the clinical course is fairly mild, but fatal cases directly or indirectly linked to infection with CHIKV were observed during the Indian-Ocean outbreak (Josseran et al., 2006). The main evidence of a mortality linked to
CHIKF epidemics was obtained in La Réunion, Mauritius, and India, by comparing expected and observed mortality data. In all cases, during the months when the epidemics were raging, the observed mortality significantly exceeded the expected rate. In particular, in La Réunion the monthly crude death rates in February and March 2006 were respectively 34.4% and 25.2% higher than expected. This corresponded to 260 excess deaths (an increase of 18.4%) with a rough estimate of the case-fatality rate for CHIKF of $\approx 1/1,000$ cases. The case-fatality rate calculated on increased crude death rates in Mauritius and Ahmedabad, India, is substantially higher than that calculated in La Réunion: approximately 4.5% (15,760 confirmed or suspected cases and 743 excess deaths) and 4.9% (60,777 confirmed or suspected cases and 2,944 excess deaths), respectively (Beesoon et al., 2008; Mavalankar et al., 2008). These differences may be attributed to many factors (greater disease severity, preexisting patient conditions, different patient management, or coincident excess deaths from other causes) but may also be due to a different efficacy of the surveillance systems for CHIKF, that probably worked poorly in Mauritius and India, leading to underestimation of the total number of cases (Fusco et al., 2010). The possible link between CHIKV infection and multiorgan failure is still under investigation.

VIRUS DISSEMINATION AND TARGET ORGANS

Following intradermal inoculation by infected mosquitoes, CHIKV directly enters the subcutaneous capillaries where its replication starts immediately (Figure 3) with some viruses infecting susceptible cells in the skin, such as macrophages or fibroblasts and endothelial cells. Local viral replication seems to be minor and limited in time, with the locally produced virus probably being transported to secondary lymphoid organs close to the site of inoculation, where infected migratory cells produce new viruses which can, in turn, infect susceptible resident cells. Even if the host is mounting a response to control the virus in the skin dermis, the virus disseminates quite rapidly to the blood circulatory system. Viruses produced in the draining lymph nodes are released into the lymph circulation and then to the blood through the thoracic duct. Once in the blood, the virus will have access to various parts of the body, including the liver, muscle, joints and brain. In these tissues, the infection is associated with a marked infiltration of mononuclear cells, including macrophages, that can be considered Trojan horses for virus spread to sanctuary body sites. The pathological events associated with tissue infection are mostly subclinical in the liver (hepatocyte apoptosis) and lymphoid organs (adenopathy), whereas mononuclear cell infiltration and viral replication in the muscles and joints are associated with very strong pain, with some patients presenting arthritis (Dupuis-Maguiraga et al., 2012).

During the first week of CHIKV infection, viremia can reach very high levels (viral loads of $3.3 \times 10^9$ copies/ml) (Parola et al., 2006). Thus, it remains unclear if the virus detected in the blood is released from virus-infected peripheral blood mononuclear cells, or is spilled out from other replication sites.

IMMUNOPATHOGENESIS

The innate immune response is the first barrier against viruses, being able to inhibit viral replication through cytolitic and non-cytolytic mechanisms. The IFN system plays an important role in limiting virus spread at an early stage of infection. In vitro growth of all alphaviruses can be greatly suppressed by the antiviral effects of IFN-α/β when it is added to cells prior to infection (Sourisseau et al., 2007; Courderc et al., 2008; Schilte et al., 2010). The finding that aberrant Type I interferon signalling in mice led to severe forms of CHIKF (Courderc et al., 2008) further highlighted the important roles cytokines play in the pathology of CHIKV infection.

Moreover, in a recent study Wauquier and colleagues (2011) demonstrated that CHIKV infection in humans elicits strong innate immunity involving the production of numerous proinflammatory mediators. Interestingly, high levels of Interferon IFN-α were consistently found. Production of interleukin (IL), IL-4, IL-10, and IFN-γ suggested the engagement of the adaptive immunity. This was confirmed by flow cytometry of circulating T lymphocytes that showed a CD8+
T lymphocyte response in the early stages of the disease and a CD4+ T lymphocyte-mediated response in the later stages (Wauquier et al., 2011). CHIKV interactions with monocytes and with other blood leukocytes induced a robust and rapid innate immune response with the production of specific chemokines and cytokines, including IFN-α. The involvement of monocytes during the early phase of CHIKV infection in vivo is massive, and infected monocyte/macrophages migrate in the synovial tissues of chronically CHIKV-infected patients, where they contribute to the inflammation process. This may explain the persistence of joint symptoms despite the short duration of viremia (Her et al., 2010). Infected monocyte/macrophages may be the main cells responsible for viral dissemination in other sanctuary body sites, such as the nervous system, and, in turn, may contribute to the development of clinical manifestations mediated by excess immune response. Usually, CHIKF is a self-limiting disease, with a defined duration of clinical course (7-10 days). Recovery is associated with a vigorous immune response, that may confer protection from re-infection. However, in some cases, chronic disease (arthralgia) may be established. Chronic symptoms may persist even after clearance of the virus from the blood, but it is possible that an active viral reservoir persists locally in the joints. Five studies have tried to identify the factors associated with chronic Chikungunya disease in groups of patients in Singapore (Chow et al., 2011), La
Réunion (Hoarau et al., 2010), Dakshina Kannada (India) (Manimunda et al., 2010; Chaaitanya et al., 2011), and Emilia Romagna (Italy) (Kelvin et al., 2011).

Regulatory mechanisms silencing the vigorous (even localized) inflammatory response seem to be required to prevent the establishment of chronic disease weeks or even months after viral clearance from the blood. The absence of such mechanisms leads to chronic arthralgia. In fact, in patients from the La Réunion study, various markers of inflammation (IFN-α, IL-6, monocyte chemotactic protein-1/CCL-2, IL-8, and matrix metalloproteinase 2) were detected in the synovial fluid of a patient suffering from chronic pain, but not in patients who fully recovered (Hoarau et al., 2010). The persistence of a local reservoir of CHIKV in joints may therefore be characteristic of chronic disease, consistently with findings in the macaque model, in which CHIKV was detected after up to 90 days especially in joint tissues, leading to chronic local inflammation (Labadie et al., 2010). Moreover, Hoarau et al. (2010) reported high plasma concentrations of IL-12 and IFN-α mRNA in blood mononuclear cells after the convalescent phase in patients with chronic disease between 6 months and 1 year after infection. In patients from Singapore, the concentrations of these two cytokines, measured by alternative techniques, peaked in the acute phase and returned to normal levels at 2-3 months, even in patients who still had clinical symptoms. According to these findings, Chaaitanya and colleagues (2011) and Kelvin and colleagues (2011) reported high levels of Th1-type cytokines in the blood of patients with chronic disease (Chaaitanya et al., 2011; Kelvin et al., 2011). Thus, despite certain discrepancies, the available studies suggest that chronic disease is associated with a de-regulation of inflammation during the acute and convalescence phases. This lack of regulation results in a deleterious inflammatory process that persists for ≥ 1 year after the first clinical signs (Dupuis-Maguiraga et al., 2012).

Concerning the possible implication of viral factors in the pathogenesis, attention has focused on the A226V mutation, that has been associated with enhanced replication and fitness of CHIKV in A. albopictus vector, and has also been shown to modulate the cholesterol requirement for infection of insect cells (Tsetsarkin et al., 2007). A recent study by our group investigated the possible involvement of A226V mutation in enhancing human pathogenesis by testing the replication competence in primate cell cultures of two isolates, differing for the presence or absence of this mutation (Bordi et al., 2011). We observed that the presence of A226V mutation did not influence the replication kinetics on primate cells. Moreover, the two isolates displayed very similar time course of cytopathic effect onset, number and extent of CHIKV antigen-positive cells, as well as the t-shape of the virus-positive multicellular foci, thus suggesting a similar mechanism of spread of the virus in the infected cell cultures.

In addition, we considered the possibility that the A226V mutation could be associated with partial resistance to the antiviral activity of recombinant IFN-α in classical experiments of virus replication inhibition. Surprisingly, the A226V-carrying strain was more susceptible than the wt virus to the antiviral action of IFN-α.

Overall, our result did not support the concept that A226V mutation confers a replicative advantage in primate cell cultures, nor did it 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. However, 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.

At the moment, understanding CHIKV immunobiology is still in its infancy and there is a long way to go before answers related to the interaction between virus and host immunity are obtained. These will certainly be important in designing novel antiviral control strategies against the spread of CHIKV infection.

**DIAGNOSIS**

Chikungunya infection is diagnosed on the basis of clinical, epidemiological and laboratory criteria. An acute onset of fever and severe arthralgia or arthritis that is not explained by other medical disorders is considered a possible CHIKV case.
The case becomes probable if the patient has lived in or visited epidemic areas in a time frame consistent with the incubation period (WHO Guidelines for prevention and control of Chikungunya fever http://www.searo.who.int/LinkFiles/Publication_SEA-CD-182.pdf (accessed Aug 01, 2011).

However, laboratory confirmation is crucial, because the case should be distinguished from various disorders with similar clinical manifestations, such as dengue fever, other alphaviruses and arthritic diseases and also endemic malaria.

The interpretation of laboratory findings is dependent on knowledge of the kinetics of viremia and antibody response in human beings. The detection of viral nucleic acid or of infectious virus in serum samples is useful during the initial viremic phase, at the onset of symptoms and normally for the following 5-10 days, when CHIKV RNA reaches very high levels (viral loads of $3.3 \times 10^9$ copies/ml) and can be easily detected. Afterwards, the diagnosis is based mainly on the detection of specific immune response by serological methods.

Molecular assays constitutes a rapid and sensitive technique for diagnosis of CHIKV infection during the early stages of illness before an antibody response is evident. Conventional RT-PCR (Hasebe et al., 2002; Pfeffer et al., 2002) are available, together with real time loop-mediated RT-PCR (Parida et al., 2007) and real time TaqMan RT-PCR assay targeting the envelope E1 gene (Pastorino et al., 2005) or the non-structural the nsP1 gene (Carletti et al., 2007). Moreover a one-step SYBR green-based real time assay targeting the non-structural nsp2 gene was described more recently (Ho et al., 2010).

Viral isolation can be performed from serum of infected patients on insect or mammalian cell lines (i.e. C6/36 or Vero E6) or by intracerebral inoculation of 1-day-old mice during the early phase of the disease when the viral load is very high and the immune response is still not detectable. In fact, the presence of early antibody seems to prevent isolation of the virus, hence virus isolation has been shown to be successful largely in antibody-negative samples obtained on or before day 2 of illness (Panning et al., 2006). Moreover, viral isolation is useful for epidemiology or pathogenesis studies or for thorough molecular characterization (Fusco et al., 2010).

The detection of CHIKV-specific immune response is based on serological methods such as enzyme-linked assays (ELISA), indirect immunofluorescence assays (IFA), hemagglutination inhibition (HI) and micro-neutralization (MNT).

IFA and ELISA are rapid and sensitive techniques for detection of CHIKV-specific antibodies, and can distinguish between IgG and IgM. IgM are detectable 2-3 days after the onset of symptoms and persist for several weeks, up to 3 months (Sam and AbuBakar, 2006; Litzba et al., 2008). Rarely, IgM can be detected for longer periods, up to 1 year. CHIKV-specific IgG appear soon after IgM antibodies (2-3 days) and persist for years. Various in-house ELISA techniques using whole antigen or recombinant capsid or envelope antigens have been described (Cho et al., 2008). Commercial serological assays are available and results obtained from a comparison of the assays suggested that the sensitivity for detection of an early antibody response before day 5 is dependent on the strain of the virus used for the assay or the source of the antigen; assays based on recombinant antigens might be too specific with regard to mutations (Cho et al., 2008; Litzba et al., 2008; Yap et al., 2010).

Testing of a couple of sera collected in the acute and convalescent phases of the disease is mandatory for the identification of recent infection using serological methods that cannot distinguish IgG Ab from IgM Ab (i.e. HI and MNT). It is also very useful to confirm results obtained with other methods, especially taking into account the possibility of rare persistence of IgM antibodies. Moreover, rapid bedside tests are commercially available, but their sensitivity and specificity are poorly established, and the possibility of false-positive reactions resulting from cross-reactivity with other arthropod-borne alphaviruses has to be considered (Blackburn et al., 1995). In fact, CHIKV is a member of the SFV antigenic complex, and is most closely related to ONNV. In this respect, diagnosis based exclusively on CHIKV-specific serological testing is useful only for travellers returning from a geographic area affected by epidemic CHIKV diffusion (Pile et al., 1999), while in other cases differential diagnosis is necessary, taking into account the most common viruses circulating in the region where the infection has presumably been acquired.
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