Prolonged Myalgia in Sindbis Virus Infection: Case Description and In Vitro Infection of Myotubes and Myoblasts

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Background. Sindbis virus (SINV) is a mosquito-borne alphavirus found in Eurasia, Africa, and Oceania. Clinical SINV infection is characterized by febrile rash and arthritis and sometimes prolonged arthralgia and myalgia. The pathophysiological mechanisms of musculoskeletal and rheumatic disease caused by SINV are inadequately understood.

Methods. We studied the muscle pathology of SINV infection ex vivo by examining a unique muscle biopsy obtained from a patient with chronic myalgia and arthralgia 6 months after acute SINV infection and assessed potential genetic predisposing factors by determining the human leukocyte antigen (HLA) and complement factor C4 genes and proteins. In addition, we performed in vitro SINV infections of primary human myoblasts and myotubes.

Results. In the muscle biopsy we found evidence of muscle regeneration due to previous necrotic lesions likely caused by earlier SINV infection. We showed that human myoblasts and myotubes were susceptible in vitro for SINV infection as the cells became immunoreactive for viral antigens and cytopathic effect was observed. The patient was homozygous for HLA-B*35 alleles and heterozygous for HLA-DRB1*01 and HLA-DRB1*03 alleles and had total deficiency of C4B protein.

Conclusions. This study provides new insights concerning pathological processes leading to chronic symptoms in SINV infection and demonstrates for the first time the susceptibility of human myogenic cells to SINV infection.

Mosquito-borne Sindbis virus (SINV) is an enveloped single-stranded RNA virus of the genus Alphavirus, family Togaviridae [1]. SINV is found across Eurasia, Africa, and Oceania, but clinical infections are mainly reported from Northern Europe. The infection is known as Pogosta disease in Finland [2], Ockelbo disease in Sweden [3], and Karelian fever in Russia [4]. Human SINV infection is characterized by joint symptoms, muscle pain, fever, and rash [5–7]. Joint manifestations may persist for years [8] and have considerable public health importance in endemic areas. Other important closely related alphaviruses causing rash and arthritis include Chikungunya virus (CHIKV) and Ross River virus (RRV) [9].

SINV epidemics have an unusual cyclic emergence in Finland and during large outbreaks hundreds or even thousands of cases are reported [10, 11]. Fluctuations of population density in grouse, which are
considered probable natural hosts for the virus, and changes in climatic and weather conditions may be associated with the observed epidemiological pattern [11].

The tissue tropism of SINV and pathophysiological mechanisms of SINV infection are at present inadequately characterized. A recent study showed that SINV can infect human macrophages and that the proinflammatory response produced by macrophages contributes to the pathogenesis of arthritis [12]. In mouse models, SINV replication has been detected within connective tissue adjacent to articular joints as well as in skin and muscle [13-15]. RRV has been shown to target bone, joint, and skeletal muscle tissue in a mouse model [16], and the activation of complement system contributes to inflammatory tissue destruction in RRV infection [17]. Only 1 study has previously investigated the susceptibility of human muscle tissue to alphavirus infection on muscle biopsy specimens taken from CHIKV-infected patients. This study provided evidence that CHIKV is able to infect and cause cytopathic effects on human skeletal muscle satellite cells. Furthermore, CHIKV antigens were detected by immunohistochemistry in the satellite cells of muscle biopsies from 2 patients with myositis syndrome [18].

Our aim was to investigate the factors behind the pathogenesis of myalgia in SINV infection by studying a muscle biopsy specimen obtained from a patient presenting with chronic myalgia and arthralgia after SINV infection, and by in vitro experiments on primary human myoblasts and myotubes. Moreover, because only a minority of SINV infections are symptomatic [11] with certain geographical clustering and because previous chronic joint symptoms can predispose to progressive symptoms in alphavirus disease [7, 19], we assessed possible genetic predisposition factors by determining the human leukocyte antigen (HLA) and complement factor C4 genes and proteins of the case patient.

MATERIALS AND METHODS

Ethics Statement

Ethical approval for ex vivo studies was obtained from the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (permission number 127/13/03/00/2009). The case patient provided written informed consent. For in vitro experiments, informed consent was obtained from all patients or legal representatives prior to the tissue being donated to the tissue bank, in accordance with the French legislation on bioethics.

Patient With Persistent Symptoms After SINV Infection

The patient was a 51-year-old man from eastern Finland with persistent symptoms after acute SINV infection. His medical history included dyslipidemia with permanent medication. There were no rheumatologic diseases in the family medical history. In early September 2009 the patient presented with papular skin rash, as well as swelling and pronounced tenderness in both wrists and ankles. The patient was earlier exposed to mosquitoes during outdoor activities. Tenderness was present also in knees, elbows, and shoulders at a later stage. Other symptoms included fever, lower back pain, headache, dizziness, and fatigue. The musculoskeletal symptoms resulted in impaired walking and hand function during daily activities. SINV immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody seroconversion, determined with standard enzyme immunoassay (EIA) [20], was seen between paired sera during the acute phase. No SINV RNA was detected in serum. The symptoms in knees, ankles, and elbows subsided after 4 weeks. As the symptoms in wrists continued, the patient was administered intra-articular cortisone injections, which, however, did not alleviate the pain. Oral diclofenac, acetaminophen, and codeine were prescribed. The symptoms in his left wrist subsided after 2 months from onset. As the pain in the right wrist continued, another cortisone injection was administered at 4 months after onset, and 1.5 mL of peritendineal fluid was punctured from dorsal peritoneum but was not available for this study.

At 6 months postinfection the following data and specimens were obtained from the patient: serum samples, leukocyte samples, muscle biopsy specimen taken from deltoid muscle (performed by J. T.), questionnaire, and results from blood parameters. Monocytes were isolated from blood by a previously described method [21]. IgM and IgG titers in serum were determined with endpoint titration.

HLA and Complement C4 Typing

DNA extracted from whole blood was genotyped for HLA-A, HLA-B, HLA-C, and HLA-DR as previously described [22]. Complement C4 allotypes were determined with electrophoresis followed by immunofixation [23] and copy numbers of C4A and C4B genes were determined using isotype-specific genomic real-time Polymerase chain reaction (PCR) amplification (Paakkinen R, Vauhkonen H, Eronen K, Järvinen A, Seppänen M, Lokki ML, unpublished data).

RNA Extraction and Real-time Reverse Transcription PCR

RNA was extracted from cell culture supernatant with QIAamp Viral RNA Mini Kit (Qiagen) and from monocytes and muscle biopsy using RNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was analyzed with sensitive and SINV-specific real-time reverse transcription PCR assay [24].

Cryosectioning and Staining of Muscle Biopsy Specimen

The muscle biopsy was snap-frozen at −170°C in isopentane precooled with liquid nitrogen. Frozen tissue was stored at −80°C prior to cryosectioning. Cryosections were stained with routine histological (hematoxylin and eosin), Herovic,
periodic acid-Schiff, and Oil Red O staining) and histochemical methods (for fiber typing ATPase with preincubations at pH 4.3 and 10.4, and for oxidative enzymes NADH-tetrazolium reductase and cytochrome-C-oxidase + succinate dehydrogenase [COX-SDH]). In addition, immunohistochemical staining for fetal, neonatal, fast, and slow myosin heavy chains (MyHGD, MyHCn, MyHCl, and MyHCs) and inflammatory cells (CD20 for B cells, CD3 for T cells, and CD68 for histiocytes; DAKO) was performed.

For detection of SINV in muscle biopsies, 2 different antibodies were used: a mouse monoclonal anti–Semliki Forest virus directed against a conserved region of the alphavirus nucleocapsid protein (kindly provided by I. Greiser-Wilke [25]) or a mouse polyclonal anti-SINV (kindly provided by M. Grandadam, National Reference Center for Arbovirus, Pasteur Institute, Paris, France).

**Electronmicroscopy**
A selected fragment of the muscle biopsy was fixed in 3% phosphate-buffered glutaraldehyde and processed routinely to be embedded in Epon. Toluidine blue–stained semithin sections were used to select the regions for thin sectioning. Thin sections were examined with a JEOL JEM 1400 electron microscope.

**Virus Culture From Muscle Biopsy**
Virus isolation was attempted from the muscle biopsy specimen using a previously described method [2]. In brief, frozen muscle tissue was cut into small pieces, homogenized in a mortar with sterile sand, and suspended in 150 µL of Dulbecco’s modified Eagle’s medium (D-MEM) plus 0.2% bovine serum albumin. A volume of 100 µL of this suspension diluted in 500 µL of culture medium was added to confluent Vero cells. Cells were examined daily for cytopathic effects. After 8 days, supernatant was collected and RNA was extracted. The cells were studied with immunofluorescence assay with SINV IgG-positive serum as previously described [2].

**Muscle Cell Cultures and In Vitro Infection**
The human myogenic precursor cells, myoblasts, were originally isolated from the quadriceps from 3 different healthy donors, referred as KMSC25, SC180C15, and CHQ (aged 25 years, 15 years, and 5 days, respectively, as previously described [26]). Cells were provided by the AFM Tissue Bank (Paris). Myoblasts were cultivated in Ham’s F-10 with Glutamax medium (Gibco) with 50 µg/mL gentamycin and 20% fetal calf serum (FCS). Myoblasts were differentiated into myotubes by replacing the growth medium by differentiation medium, that is, D-MEM with Glutamax (Gibco) supplemented with gentamycin and insulin (10 µg/mL; Sigma) and transferring (100 µg/mL; Sigma) for 3–5 days.

We assessed the sensitivity of primary human myoblasts and myotubes to SINV infection at multiplicities of infection (MOI) 10, 1, and 10⁻¹. Two Finnish SINV strains were used for the experiments, which had been previously isolated from 2 different patients in Finland [2], called Ilomantsi-2002B and Ilomantsi-2002C (GenBank accession numbers AY532326 and AY532324, respectively). The viruses had been passaged 3 times in Vero cells prior to experiments on myoblasts and myotubes and titers of 10⁶.5 (Ilomantsi-2002B) and 10⁷ DCP50/ mL (Ilomantsi-2002C) were obtained in the last passage. At different days postseeding, (1 day for myoblasts, 6–7 days for myotubes), cells were incubated for 2 hours with viral strains and washed once in phosphate-buffered saline (PBS), and culture medium (F10 with 20% FCS for myoblasts, D-MEM without FCS for myotubes) was added. At different days postinfection, cultures were processed for viral antigen detection by immunofluorescence or viral titration. For immunofluorescence, cells were fixed for 20 minutes in 4% paraformaldehyde, incubated for 30 minutes in PBS with 10% normal goat serum (Vector), and then permeabilized with 0.1% Triton X-100 (Sigma). Permeabilization medium was removed and primary antibodies (anti-alphavirus nucleocapsid in dilution 1/50 or polyclonal anti-SINV in dilution 1/100) were incubated on cell cultures for 90 minutes at room temperature. After 3 washes in PBS, secondary antibody (horse antimouse antibody, coupled to FITC, rat adsorbed, 1/100; Vector) was incubated for 90 minutes at room temperature. Muscle cell identification was performed with a rabbit polyclonal anti-desmin antibody (dilution 1/200, Molecular Probes). After 3 washes, cultures were mounted in Fluoromount G medium (Southern Biotech). Preparations were observed with a Zeiss Axiovision fluorescence microscope, and image acquisition was performed with a Zeiss Axiocam camera.

At different times postinfection, supernatants from cell cultures were collected and plaque-titrated on Vero cells. Culture supernatants were also collected at 8 and 24 hours postinfection for cytokine production studies. The concentration of 5 cytokines and 1 chemokine (interleukin 6 [IL-6], interleukin 8 [IL-8], tumor necrosis factor α [TNF-α], interferon α [IFN-α], interferon γ [IFN-γ], and monocyte chemotactant protein 1 [MCP-1]) was simultaneously assessed using Luminex assay (Cytokine Human Singleplex, Invitrogen), according to the manufacturer’s instructions.

**RESULTS**

**Case Patient: Clinical Observations**
The onset of symptoms of the patient coincided with the time of the diagnosis of acute SINV infection and thereafter uninterruptedly continued. A consultant rheumatologist (T. H.) examined the patient at 6 months postinfection. At this time,
the patient was unable to work due to continuous arthralgia and myalgia. In the clinical examination, tenderness was observed in the right metacarpophalangeal joints II–V. The right wrist was moderately swollen, and a dorsal swelling on the wrist within the tendon sheaths of the extensor muscles was observed. Pronounced tenderness was observed during extension and flexion, with a considerably weakened handgrip. Tenderness was also observed in the right acromioclavicular joint, and myalgia particularly in the area of right supraspinatus insertion. Otherwise the rheumatological status was normal. The following blood parameters at 6 months after infection were within normal range: hemoglobin, platelet count, C-reactive protein, erythrocyte sedimentation rate, K⁺, Na⁺, Ca²⁺, creatinine, albumin, myoglobin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, urate, rheumatoid factor, cyclic citrullinated peptide, antinuclear antibodies, and C3 complement level. The following parameters were slightly elevated: leukocyte count (11.5 × 10⁹/L), neutrophil count (6.93 × 10⁹/L), and C4 complement level (0.36 g/L). In addition, a persisting positive SINV-IgM response was observed. The endpoint titers of IgM and IgG antibodies were 77 and 434, respectively.

**HLA Typing and Complement Analysis**

The patient was homozygous for HLA-A*03 and HLA-B*35 alleles and heterozygous for HLA-DRB1*01 and HLA-DRB1*03 alleles. C4 analysis showed a total deficiency of C4B genes and proteins. Increased quantity of C4A proteins was observed.

**Histology, Immunohistochemistry, and Electron Microscopy of Muscle Biopsy**

The variation of myofiber size was within normal limits, and no significant fiber atrophy was encountered. ATPase staining for fiber typing showed that the distribution of type 1 and type 2 fibers was somewhat uneven but not fulfilling the criteria of fiber type grouping (Figure 1A). Several medium-dark type 2C (immature) fibers of the same size as the rest of the fibers were observed (Figure 1A). No necrotic fibers, phagocytosis, or inflammatory cell infiltrates were detected, but there was an increased number of internal nuclei (Figure 1B). In the COX-SDH staining, 5 COX-negative fibers (≤1% in the biopsy) were encountered (Figure 1C). Immunohistochemical staining to show regeneration did not disclose any MyHCd-positive fibers, but several MyHCn-positive fibers, also.
indicative of regeneration, of approximately normal size were observed (Figure 1D). Immunostaining for B and T lymphocytes and phagocytes gave negative results. SINV antigen was not detected immunohistochemically in the muscle biopsy. Electron microscopy did not reveal any structures suggestive of viral particles, but showed increased quantity of glycogen in the sarcoplasm of the myofiber (data not shown).

**Virus Isolation and Detection of SINV RNA**

Virus isolation from the muscle biopsy was not successful as no cytopathic effects was observed, cells were SINV antibody negative by immunofluorescence assay, and no SINV RNA was detected in muscle biopsy or in cell culture supernatant. Monocytes were also negative for SINV RNA.

**In Vitro Infection**

In human myoblasts as well as in myotubes infected with the Ilomantsi-2002B strain (at an MOI of 10), viral antigens could be detected as early as 12 hours postinfection (data not shown), and at 24 hours postinfection myoblasts as well as myotubes were found immunoreactive for viral antigens with an antibody detecting the capsid protein (green) and desmin (red). A, Mock-infected cells. B, Undifferentiated cells (myoblasts) infected. C, Differentiated cells (myotubes) infected. D, Myoblasts infected and labeled with a specific polyclonal mouse anti-SINV antibody. Upper line, Staining for viral antigens. Middle line, Staining for desmin. Lower line, Merge. Original magnification, ×400. E and F, Infection rates of cells from different donors. Muscle cells were infected by SINV (Ilomantsi-2002B and -2002C strains) at an MOI of 10 and labeled 24 h postinfection for viral capsid and desmin. Infection rates were assessed by the proportion of cells immunoreactive for both antibodies vs desmin-immunoreactive cells. For each experiment, 300 cells were counted. Data represent the average of 2 independent experiments (bar = SD). E, Myoblasts. F, Myotubes.

**Figure 2.** Susceptibility of cultured human muscle cells to Sindbis virus (SINV) infection. A–C, Visualization of viral antigens and desmin. Cells were infected by SINV (Ilomantsi-2002B strain) at a multiplicity of infection (MOI) of 10 and labeled at 24 h postinfection with specific monoclonal antibody for the alphavirus capsid protein (green) and desmin (red). A, Mock-infected cells. B, Undifferentiated cells (myoblasts) infected. C, Differentiated cells (myotubes) infected. D, Myoblasts infected and labeled with a specific polyclonal mouse anti-SINV antibody. Upper line, Staining for viral antigens. Middle line, Staining for desmin. Lower line, Merge. Original magnification, ×400. E and F, Infection rates of cells from different donors. Muscle cells were infected by SINV (Ilomantsi-2002B and -2002C strains) at an MOI of 10 and labeled 24 h postinfection for viral capsid and desmin. Infection rates were assessed by the proportion of cells immunoreactive for both antibodies vs desmin-immunoreactive cells. For each experiment, 300 cells were counted. Data represent the average of 2 independent experiments (bar = SD). E, Myoblasts. F, Myotubes.
infected myoblasts and myotubes did not exhibit any significant changes in production of IFN-α or IFN-γ, TNF-α, IL-6, IL-8, or MCP-1 at 8 hours or 24 hours postinfection with both viral strains although a slight increase in MCP-1 production was observed 24 hours postinfection in myotubes (data not shown).

DISCUSSION

This study provided a unique opportunity to extensively investigate a rare tissue specimen as access to such material from SINV-infected patients with persistent symptoms is restricted and difficult to obtain. Previously, a similar study targeted CHIKV based on 2 human muscle biopsies [18]. We present an ex vivo examination of a muscle biopsy taken from a patient with chronic SINV infection as well as new in vitro data regarding susceptibility of human myogenic cells to SINV infection. The findings on the muscle biopsy, including the presence of MyHCn-positive cells as well as type 2C fibers together with an increased number of internal nuclei, suggest muscle regeneration after previous necrotic lesion. Moreover, the increased amount of glycogen in the sarcoplasm indicated regeneration. Since no major signs of neurogenic disease (atrophic fibers or definite fiber type grouping) were observed, reinnervation as the cause of these findings is unlikely. Virus isolation attempt from the muscle tissue was negative, SINV antigen was not detected with immunohistochemistry, and no SINV RNA was detected with PCR demonstrating the absence of viral antigens in the tissue. Thus, we hypothesize that the observed regeneration due to earlier necrotic lesions may have been caused by the previous SINV infection in muscle cells but no active viral replication was detectable at this time, 6 months postinfection. Because of the long time after the infection, the regeneration process was already in an advanced stage. The necrosis of muscle tissue may also have been caused by secondary immune-mediated response triggered by the virus infection.

We acknowledge that factors other than SINV infection could have at least partly caused these findings on regeneration and more extensive studies on muscle biopsies, if available, would be needed. In addition, lack of healthy control tissue is a further limitation; however, obtaining such tissue samples properly matched to our case patient is not feasible, particularly due to ethical reasons.

Our in vitro data showed that both human myotubes and myoblasts were susceptible for SINV infection and could yield infectious viruses. Although the virus yield was somewhat higher in myoblasts than in myotubes for reasons that remain to be determined, similar results were obtained for the 2 different viral strains on 3 different donors, suggesting susceptibility of human muscle cells (differentiated or not) to field variants of SINV. These observations are somewhat different from a previous in vitro study on CHIKV as myotubes were refractory to CHIKV infection [18]. This observation, taken together with the fact that a fraction of undifferentiated and differentiated human muscle cells remain refractory to infection by alphaviruses, suggests a possible role for differentiation in susceptibility.

CHIKV antigen has been detected in satellite cells from muscle biopsies of 2 patients with myositis. The biopsies were taken during the acute phase of CHIKV infection and 3 months postinfection [18]. The authors observed that the sections of biopsy taken in the chronic stage of disease contained fewer satellite cells immunoreactive for CHIKV but more infiltrating inflammatory cells when compared with the biopsy taken in the acute phase [18]. As the muscle biopsy from the patient with chronic SINV infection was taken considerably later, it seems likely that the pathogenic virus had already been eliminated and the inflammatory reaction had faded at this time. Notably, an earlier study on RRV-infected mice showed presence of internal nuclei in muscle cells, indicating muscle regeneration when inflammation and pathology had already resolved [16].

We also observed several COX-negative fibers in the biopsy that indicate deletions in mitochondrial DNA (mtDNA). These deletions generally increase in number with age but in our patient the finding was pathological with respect to the patient’s age. It has been speculated that chronic persistent viral infection may be a triggering factor for sporadic inclusion

Figure 3. Production of infectious virions in Sindbis virus (SINV)-infected muscle cells. Muscle cells were infected by SINV (Ilomantsi-2002B and -2002C strains) at a multiplicity of infection of 10 and the culture supernatant was collected and plaque-titrated on Vero cells at 0, 24, 48, 72, and 144 h postinfection. The data represent the average of 2 independent experiments (bar = SD). Insert, viral-induced cytopathic effects. Infected myoblasts (visualized by viral immunoreactivity; green) show disorganization of actin cytoskeleton (labeled by rhodamine phalloidin). Original magnification, ×100.
body myositis where COX-negative fibers are frequently seen [27]. However, whether these mtDNA deletions are caused by previous SINV infection remains unclear and warrant further studies.

HLA and C4 analyses showed that the patient carried HLA-B*35 (homozygous), HLA-DRB1*01, and HLA-DRB1*03 alleles and had a total deficiency of complement C4B protein. Serum C4 level was slightly elevated, showing that the lack of C4B proteins is compensated by increased concentration of C4A proteins. The 2 C4 isotypes, C4A and C4B, differ in their chemical reactivities. C4B shows higher affinity toward hydroxyl group–containing antigens whereas C4A displays stronger affinity for amino group–containing antigens [28].

Previous studies on the influence of HLA-B*35 allele on the disease susceptibility and prognosis have identified HLA-B*35 as a risk factor for infectious as well as rheumatic diseases [29]. It is also known that HLA-DRB1*01 and C4B deficiencies are associated with rheumatic diseases, particularly with rheumatoid arthritis [29, 30]. Underlying medical conditions affecting joint or connective tissue have been reported in patients with SINV infection [7]. Also, many patients with persistent arthralgia following CHIKV infection have had a history of chronic joint symptoms prior to CHIKV infection [19]. There may be common genetic factors that are associated with both SINV-induced infectious arthritis and autoimmune arthritides; to further investigate the role of HLA system and C4 genes in the susceptibility and outcome of SINV infection, more extensive studies are warranted and ongoing.

The patient showed persisting IgM antibodies in serum, which possibly indicates active viral replication somewhere in the body, with joint tissue being the most likely site. The IgM persistence in SINV infection has been observed in earlier follow-up studies [5, 31], as well as in studies on CHIKV infection [19, 32], but no clear association to prolonged arthralgia has been established.

Taken together, we present comprehensive analyses of a case patient with prolonged arthralgia and myalgia after acute SINV infection. We further showed in this study that SINV can infect human muscle cells, causing cytopathic effects. These in vitro findings support our speculation that earlier SINV infection could have caused necrosis leading to regeneration visible in muscle biopsy.

**Notes**

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