MAJOR ARTICLE

Clinical Sindbis Alphavirus Infection Is Associated With *HLA-DRB1*01* Allele and Production of Autoantibodies

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Background. Sindbis virus (SINV) is a mosquito-borne alphavirus found in Eurasia, Africa, and Oceania. Clinical SINV infection, characterized by arthropathic disease that may persist for years, is primarily reported in Northern Europe where the disease has considerable public health importance in endemic areas. The aim of this study was to investigate the role of genetic factors in the susceptibility and outcome of SINV infection and to elucidate the association between SINV infection and autoimmunity.

Methods. The study included 49 patients with serologically confirmed symptomatic SINV infection who were followed for 3 years after acute infection. Human leukocyte antigen (HLA) genes known to be associated with rheumatic and infectious diseases and complement *C4* genes were determined in 35 patients. Furthermore, a set of autoantibodies was measured at the acute phase and 3 years after infection in 44 patients.

Results. The frequency of *DRB1*01* was significantly higher among patients with SINV infection than in the reference population (odds ratio, 3.3; 95% confidence interval, 1.7–6.5; $P = .003$). The *DRB1*01* allele was particularly frequent in patients who at 3 years postinfection experienced joint manifestations. The frequency of rheumatoid factor at 3 years postinfection was 29.5% and had increased significantly ($P = .02$) during the 3-year period. In addition, antinuclear and antimitochondrial antibodies were present in serum 3 years postinfection with frequencies of 15.9% and 6.8%, respectively.

Conclusions. Our data show that symptomatic SINV infection is associated with the HLA system and that autoantibody titers are elevated in patients 3 years postinfection. These findings indicate that SINV-induced arthritis shares features with autoimmune diseases.

Several alphaviruses in the family *Togaviridae* are major causative agents of arthropathic disease worldwide [1, 2]. In recent years, thousands of cases of Ross River virus (RRV) have been reported annually in Australia [1], and Chikungunya virus (CHIKV) emerged as a major outbreak in islands of the Indian Ocean and India in 2004–2009, during which several millions of cases were reported [3]. Mosquito-borne Sindbis virus (SINV) is the causative agent of febrile rash-arthritis, particularly in Northern Europe, where larger outbreaks of hundreds or even thousands of cases occur cyclically [4]. The vast majority of clinical SINV infections are diagnosed in Finland where the disease is known as Pogosta disease. All arthritogenic alphaviruses, namely, RRV, CHIKV, SINV, Mayaro virus, O'nyong-nyong virus, and Barmah Forest virus, can cause a persisting form of illness characterized by joint symptoms that can last for years [1, 5, 6].

The pathophysiological mechanisms of rheumatic manifestations caused by arthritogenic alphaviruses are inadequately understood. Recent studies suggest that macrophage recruitment and subsequent secretion of proinflammatory cytokines and chemokines play a

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major role in the development of arthritis in alphavirus infection [7–11]. In addition, the activation of complement system seems to contribute to inflammatory tissue destruction in RRV infection [12]. Few studies have characterized host genetic determinants associated with alphavirus infections. The prevalence of human leukocyte antigen (HLA)-DR7 was found to be higher among RRV patients than controls [13], and a study of chronic CHIKV patients showed that *HLA-DRB1*01* and *DRB1*04* alleles were frequently found among the patients who developed rheumatoid arthritis after the infection [14]. *HLA* alleles *B*27* and *DRB1*04* were previously determined in a study of 21 patients with SINV infection, but no association was found [15]. However, genetic factors influencing the occurrence or consequence of SINV infection have not been further characterized.

We recently described a patient with persistent SINV infection to be homozygous for *HLA-B*35*, heterozygous for *HLA-DRB1*01* and *DRB1*03* alleles, and with total deficiency of C4B protein [16]. In the current study, our aim was to further investigate the role of genetic factors in the susceptibility and outcome of SINV infection. We analyzed a set of *HLA* alleles reported to have an association with rheumatic and infectious diseases [17], as well as the number of *C4* genes in patients with SINV infection. Furthermore, we aimed to study the association between SINV infection and autoimmunity by measuring different autoantibodies at the acute phase and 3 years after infection.

METHODS

Patients and Control Groups

The study population consisted of 49 patients with a serologically confirmed symptomatic SINV infection in 2002, who were followed over 3 years (2002–2005) [5]. The main symptoms at the acute phase included arthritis, fever, rash, and myalgia as described elsewhere [18], and the diagnostic criteria for inclusion of the patients for the study were seroconversion in paired serum samples and/or positive immunoglobulin M (IgM) result in enzyme immunoassay (EIA) in a single serum sample [19]. The specificity of EIA result was further confirmed with hemagglutination inhibition test. The patients were mainly female (70%), and all were recruited from 11 healthcare centers in North Karelia and Kuopio University Hospital, located in eastern Finland. The median age for the patients was 54 years (range, 7–78). Serum samples from the cohort were available from the acute phase, 6 months and 3 years postinfection. Whole blood specimens were obtained at the acute phase, when possible. At 3 years postinfection, as previously described, the patients were classified into 4 clinical categories (A–D) according to objective findings in a clinical examination conducted by a rheumatologist, as well as subjective joint symptoms reported in a standardized interview [5]. In the rheumatological examination, 72 joints were assessed for tenderness and 70 for swelling, according to the European League Against Rheumatism handbook of clinical assessments [20]. The clinical categories were defined as follows: (A) arthritis, defined as swelling and, in addition, pain on palpation or tenderness in joint movement assessed by a rheumatologist; (B) objective joint pain, defined as pain on palpation or tenderness in joint movement assessed by a rheumatologist; (C) subjective joint pain, defined as joint pain reported in the standardized interview; and (D) no joint symptoms that could be associated with SINV infection [5].

Data available on 90 Finns from bone marrow donor registry were used as a reference population [21] for *HLA* allele frequency comparisons. The reference group for the comparison of *C4* gene numbers was composed of 150 voluntary subjects participating in a health survey prior to accepting a new occupational post [22]. Ethical approval for this study was obtained from the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (permission number 127/13/03/00/2009). Written informed consent was received from the patients.

HLA and Complement C4 Typing

Adequate volume of whole blood for the *HLA* and *C4* gene analyses was available from 35 of 49 patients. DNA extracted (NucleoSpin Tissue kit, Macherey-Nagel) from whole blood was genotyped for *HLA-B*27*, *-B*35*, *-DRB1*01*, *-DRB1*03*, *-DRB1*04*, and *-DRB1*15* alleles using genomic real-time polymerase chain reaction (PCR). We used unlabeled primers with SYBR green QPCR (Stratagene, Cedar Creek, Texas) or Absolute QPCR SYBR Green Mix (Abgene, Epsom, UK) according to the manufacturers' instructions with minor modifications. Primers were based on published major histocompatibility complex (MHC) gene sequences (http://www.ebi.ac.uk/imgt/hla/probe.html) with minor modifications used in the laboratory with European Federation for Immunogenetics accreditation for HLA class I and class II low- and high-resolution histocompatibility testings. Copy numbers of *C4A* and *C4B* genes were determined using isotype-specific genomic real-time PCR amplification (Paakkanen R et al, unpublished data).

Analysis of Autoantibodies

Of the 49 patients, 44 had serum collected both at the acute phase and 3 years postinfection. Nuclear (ANA), mitochondrial (AMA), smooth muscle (SMA), and parietal cell (PCA) antibodies were determined by the indirect immunofluorescence technique. For ANA, serum samples were screened at a dilution of 1 : 80 and titrated further at 4-fold dilution steps using HEp-2 slides (Nova Lite HEp-2 assay, INOVA Diagnostics Inc, San Diego, California) as substrates. For tissue antibodies, unfixed 5-μm cryostat sections of tissue blocks of rat kidney,

Table 1. Human Leukocyte Antigen Allele and *C4* Gene Deficiency Frequencies in 35 Patients (70 Alleles) With Confirmed Sindbis Virus Infection and Reference Population

HLA Alleles and *C4* Deficiency	SINV Patients	Reference Population	OR (95% CI)	*P* Value (Corrected)
HLA alleles	(n = 70 alleles)	(n = 180 alleles)[a]		
*B*27*	5/70 (7.1)	15/180 (8.3)	0.8 (.3–2.4)	.76
*B*35*	13/70 (18.6)	23/180 (12.8)	1.6 (.7–3.3)	.24
*DRB1*01*	23/70 (32.9)	23/180 (12.8)	3.3 (1.7–6.5)	.003
*DRB1*03*	4/70 (5.7)	16/180 (8.9)	0.6 (.2–1.9)	.4
*DRB1*04*	6/70 (8.6)	38/180 (21.1)	0.4 (.1–0.9)	.1
*DRB1*15*	10/70 (14.3)	26/180 (14.4)	1 (.5–2.2)	.97
C4 genes	(n = 35 patients)	(n = 150 patients)[b]		
C4B deficiency (<2 genes)	18/35 (51.4)	59/150 (39)	1.5 (.7.3.2)	.25
C4B total deficiency	5/35 (14.3)	15/150 (10)	1.5 (.5–4.5)	.46
C4A deficiency (<2 genes)	3/35 (8.6)	27/150 (18)	0.4 (.1–1.5)	.17
C4A total deficiency	0/35	2/150 (1.3)	NA	1

Data are no. (%) unless otherwise indicated.

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; NA, not applicable; OR, odds ratio; SINV, Sindbis virus.

[a] Ninety historic controls, 180 *HLA-B/DRB1* alleles [21].

[b] One hundred fifty unselected voluntary subjects coming to a health survey before accepting a new occupational post [22].

rat stomach, mouse liver, and mouse stomach were used asz described elsewhere [23]. Anti-human immunoglobulin G (IgG) antibodies coupled with fluorescein isothiocyanate (Dako, Glostrup, Denmark) were used as secondary antibodies.

Extractable nuclear (ENA) and cyclic citrullinated peptide antibodies (CCP) were measured by fluorescence enzyme immunoassay (ImmunoCap250, Phadia, Uppsala, Sweden) and rheumatoid factor (RF) using N LatexRf Kit and BN ProSepc nephelometer (Siemens, Munich, Germany). The following reference titers or units were used as cutoffs: ANA (<320), AMA (<50), RF (<20 IU/mL), CCP (<7 U/mL), SMA (<50), and ENA (<0.7 U). These cutoff values are used in routine, accredited autoantibody diagnostics at Helsinki University Hospital Laboratory.

Statistical Analyses

Two-tailed χ^2 or Fisher exact test was used for comparison of *HLA* allele, *C4A/C4B* deficiency (0 or 1 allele), and autoantibody frequencies between different groups. McNemar test was used to compare paired proportions (autoantibodies at different time points). Statistical significance was considered at 5% level. Holm-Bonferroni correction of *P* value was made for multiple testing when appropriate.

RESULTS

Comparison of *HLA* Allele and *C4* Gene Deficiency Frequencies With the Reference Population

HLA allele and *C4* deficiency frequencies of patients with SINV infection and the reference population are presented in Table 1. Altogether, 18 of 35 patients were carrying the *DRB1*01* allele, of whom 5 were homozygous for *DRB1*01*. Among those carrying the *DRB1*01* allele, 12 also had the *HLA-B*35* allele and *C4B* deficiency (0 or 1 allele). The frequency of *DRB1*01* was significantly higher in SINV-infected patients than in the reference population (corrected *P* = .003, odds ratio [OR], 3.3; 95% confidence interval [CI], 1.7–6.5). The frequency of *C4B* or *C4A* deficiency did not significantly differ between those with SINV infection and the reference group. Of the patients with SINV infection, 13 of 35 patients (37%) had 3 copies of the *C4A* gene, but the difference from the reference group (36 of 150 [24%]) was not significant.

Comparison of *HLA* Allele and *C4* Gene Deficiency Frequencies Between Patient Categories

In this comparison, patients who experienced persistent joint pain 3 years postinfection and were classified into clinical category A, B, or C were considered 1 group and patients from category D another group. The differences in the *HLA* allele or *C4B* deficiency distribution between the groups were not statistically significant (Table 2). However, there was an apparent trend toward *DRB1*01* being more frequent among patients in categories A–C (*P* = .07). Both patients (n = 2) from category A (arthritis) had *B*35* and *DRB1*01* alleles (1 of them was homozygous for *DRB1*01*), as well as partial *C4B* deficiency.

Autoantibodies in SINV Infection

The findings on autoantibodies at the acute phase and 3 years postinfection are shown in Table 3. Seroconversion of RF was observed in 11 of 44 (25%) of the patients (*P* = .022),

Table 2. Human Leukocyte Antigen Allele and *C4* Gene Deficiency Frequencies in Different Patient Categories

HLA Allele/*C4* Deficiency	Clinical Categories A–C (n = 18)[a]	Clinical Category D (n = 52)[b]	*P* Value (Uncorrected)
*B*27*	2/18 (11.1)	3/52 (5.8)	.45
*B*35*	4/18 (22.2)	9/52 (17.3)	.64
*DRB1*01*	9/18 (50)	14/52 (26.9)	.07
*DRB1*03*	1/18 (5.5)	3/52 (5.7)	.97
*DRB1*04*	0/18 (0)	6/52 (11.5)	.13
*DRB1*15*	2/18 (11.1)	8/52 (15.4)	.7
C4B deficiency (<2 genes)	6/9 (66)	12/26 (46)	.29
C4B total deficiency	2/9 (22.2)	3/26 (11.5)	.43
C4A deficiency (<2 genes)	1/9 (11)	2/26 (7.7)	1

Abbreviation: HLA, human leukocyte antigen.

[a] Patients with persistent joint symptoms 3 years postinfection.

[b] No joint symptoms that could be associated with Sindbis virus infection.

2 patients were positive for both RF at the acute phase and at 3 years postinfection, and 2 patients had RF antibodies only at the acute phase. The RF levels 3 years postinfection ranged from 21 IU/mL to 179 IU/mL (median, 32). One patient had CCP antibodies both at the acute phase and 3 years postinfection. One patient became positive for AMA and 3 patients for ANA during the 3-year follow-up, but the frequency of seroconversion for these antibodies was not significant within the study population.

Although AMA positivity was found, antibodies to pyruvate dehydrogenase indicating primary biliary cirrhosis were not found in any of the cases. All AMA-positive patients had titers of 250. The titers in the ANA-positive patients were 320 (n = 2), 1280 (n = 3), and >5000 (n = 2). In the 2 patients who were positive for ANA already at the acute phase, the titer increased over 3 years from 320 to 1280 and from 1280 to 5000, respectively.

Both patients in category A were positive for ANA 3 years postinfection, and 1 patient was positive for RF. Of the patients in categories A–C, 5 of 12 (41.7%) were positive for RF compared with 8 of 32 (25%) in category D, but the differences in frequency of autoantibodies between the patient categories were not statistically significant ($P = .3$).

Data on both *HLA* and *C4* genes and autoantibodies were available for 31 patients. Patients with *C4B* deficiency had seroconversion of RF more often than patients without *C4B* deficiency ($P = .05$). Of the RF-positive patients 3 years postinfection, 8 of 10 had *C4B* deficiency and 7 of 10 had *DRB1*01* allele.

Table 3. Patients With Autoantibodies at Acute Phase and at 3 Years After Sindbis Virus Infection

Autoantibodies	Acute Phase	3 Years Postinfection	*P* Value[a]
Mitochondrial antibody	2/44 (4.5)	3/44 (6.8)	1
Rheumatoid factor	4/44 (9)	13/44 (29.5)	.022
Antinuclear antibody	4/44 (9)	7/44 (15.9)	.25
Cyclic citrullinated peptide antibody	1/44 (2.3)	1/44 (2.3)	1
Parietal cell antibody	3/44 (6.8)	3/44 (6.8)	1
Smooth muscle antibody	0/44	2/44 (4.5)	.5
Extractable nuclear antibody	0/44	0/44	1

Data are no. (%) unless otherwise indicated.

[a] Determined by McNemar test.

DISCUSSION

Our novel data demonstrate that symptomatic, clinical SINV infection shows strong association with the markers of the HLA system. The carrier frequency of *HLA-DRB1*01* (51.4%) in patients with SINV infection was remarkably high and the allele frequency significantly higher than in the reference population. Comparison of allele frequencies in patient groups classified according to severity of joint symptoms showed no statistically significant differences, possibly owing to the small number of patients. However, the *DRB1*01* allele was notably frequent in patients who at 3 years postinfection experienced joint manifestations, which were found attributable to previous SINV infection [5]. These data suggest that HLA association may be particularly related to long-term sequelae of SINV infection. Data on autoantibodies showed significant increase in RF frequency during the 3-year follow-up period and also demonstrated that ANAs and AMAs are present with considerably higher prevalence than in the normal population in serum 3 years postinfection. The CCP antibodies, highly specific markers of rheumatoid arthritis, were detected only in 1 patient who was also positive for RF.

The major strength of this study is the availability of a unique, prospective follow-up material from patients with SINV infection. However, the following limitations should be considered when interpreting the results. Reference populations were not from the same area of residence as our patients with SINV infection. Regional differences in HLA antigen frequencies are known to exist in Finland [24], and especially the frequency of *B*35* seems to be higher in eastern Finland [24]. However, *DRB1*01* allele does not display similar frequency deviations, and thus the validity of our findings is not threatened. The reference populations were not tested for SINV

antibodies resulting in possible misclassification bias, which, however, would have made detecting an association more difficult; it is known that the SINV seroprevalence in Finland is on average 5%, and many seroconvert without clinically diagnosed disease [25]. Furthermore, we could not perform more detailed analyses of *HLA* genes owing to the limited amount of DNA available from the only whole blood samples collected during the beginning of the study.

Although robust parallel data on autoantibodies from healthy Finnish reference population were not available and therefore a statistical comparison was not performed, the prevalence of ANAs (15.9%), AMAs (6.8%), and RF (29.5%) in SINV-infected patients 3 years postinfection is clearly higher than in healthy individuals. The frequency of ANAs in healthy individuals determined with the same method and cutoff dilution as in our study has been reported to be 3.3% [26], whereas 0.5%–0.9% of the general population has been reported as AMA positive by enzyme-linked immunosorbent assay or immunofluorescence assay [27]. Two percent of a representative Finnish population was previously found to be RF positive by using a different laboratory method [28].

HLA association is a hallmark of autoimmune diseases [29], and *DRB1*01* has been linked to rheumatic diseases such as juvenile idiopathic arthritis [30] and rheumatoid arthritis [31, 32]. The same allele is also associated with symptomatic parvovirus B19 infection [33]. Parvovirus B19 infection shares the predominant clinical features with SINV infection such as rash and arthralgia and has been implicated as the causative agent of several autoimmune disorders including rheumatoid arthritis [34]. Another hallmark of autoimmune diseases includes the occurrence of autoantibodies in serum, indicating ongoing tissue destruction and autoinflammation. Autoantibodies, particularly RF, are frequently detected in acute stage of viral infections as well [35–37], but the appearance is often transient. We detected autoantibodies in serum samples of patients with SINV infection at 3 years postinfection, and actually several patients seroconverted to RF positivity during the follow-up period. A typical further feature of autoimmune diseases is female predominance [38], which is also observed among patients with SINV infection [39]. Thus, our data suggest that similar genetic predisposing factors may contribute to the development of SINV-induced and autoimmune arthritides resulting in disease with similar features although different etiologies are involved. The question whether SINV or arthritogenic alphaviruses in general can trigger the development of autoimmune diseases warrants further investigations. It would also be worthwhile to study cytokine/chemokine profiles and kinetics in patients infected with SINV, as previous studies have shown that rheumatoid arthritis and alphavirus-induced arthritis share remarkable similarities in cytokine profiles such as upregulation of macrophage migration inhibitory factor expression [7, 8].

It is known that *HLA-B*35* is frequently detected together with *HLA-DRB1*01* and *C4B* deficiency [40]. Twelve patients (34%) in our study population, including the 2 patients diagnosed with arthritis 3 years postinfection (category A), had *HLA-B*35* and *-DRB1*01* alleles and *C4B* deficiency. Patients in category A were previously shown to have persisting IgM antibodies 3 years postinfection, suggestive of active replication of virus somewhere in the body, possibly in joint tissue [5]. The same genetic background and IgM persistence was observed in our recent case study on a patient with persistent arthralgia and myalgia 6 months after acute SINV infection [16]. Thus, the combination of the *HLA-B*35* and *-DRB1*01* alleles and *C4B* deficiency may be associated with more prominent or persistent forms of illness, although only *DRB1*01* remains as a statistically significant risk factor in the overall patient group. Recently, the frequency of *C4B* deficiency was reported to be increased in seropositive rheumatoid arthritis patients [41]. However, because *HLA-B*35*, *HLA-DRB1*01*, and *C4B* deficiency are revealed as a haplotype in the Finnish population, we cannot exclude the influence of other MHC genes not determined in this study [42].

The molecular mechanism of the observed HLA association with SINV infection remains unknown. The *DRB1*01* allele may be associated with altered presentation of the SINV epitopes influencing the T- and B-cell interactions and the subsequent inflammatory immune response. Autoimmunity in SINV infection may be induced through molecular mimicry as, for example, suggested for parvovirus B19 [34]. However, the results indicate that *HLA* genes in combination with other genetic and environmental factors contribute to the increased susceptibility to symptomatic SINV infection. The host genetic factors may also explain, at least partly, the high incidence of clinical SINV infections in Finland as compared to other countries, such as Sweden, where SINV also evidently circulates.

In conclusion, our study shows that symptomatic SINV infection is associated with the HLA class II allele *DRB1*01* and that autoantibody titers are elevated in serum of patients 3 years postinfection. These findings indicate that SINV-induced arthritis shares similar features and/or predisposing genetic determinants with autoimmune diseases. Future studies should elucidate the possibilities of employing therapeutic strategies used for autoimmune diseases in the treatment of SINV-induced arthritis.

Notes

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