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Characterization of antibody response in patients with acute and chronic chikungunya virus disease

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ABSTRACT

Background: Chikungunya virus (CHIKV) is a re-emerging arbovirus capable of causing chronic arthralgia, which can last for months to years. Although neutralizing antibodies have been shown to be important for viral clearance, is it not clear whether the quantitative and qualitative nature of antibodies play a role in progression to chronic disease.
Objectives: To characterize and compare the antibody responses in acute and chronic patients in a prospective observational CHIKV study in Curaçao during the 2014-2015 outbreak.
Study design: We performed virus neutralization tests and ELISA on plasma samples collected from a prospective observational chikungunya study in Curaçao to compare the complement-dependent and –independent neutralization capacity, as well as the antibody avidity index of acute and chronic patients.
Results: We found that there was no significant difference in the virus neutralization titers between patients with acute and chronic chikungunya infection. Furthermore, we found that complement increased the neutralization capacity when large amounts of virus was used. Moreover, we found that patients with acute chikungunya disease had a significantly higher antibody avidity index compared to those with chronic disease.
Conclusions: This study suggests that virus neutralization titers in late convalescent sera do not play a role in chronic chikungunya. However, the median antibody avidity was lower in these patients and may therefore suggest a role for antibody avidity in the development of chronic disease.

1. Background

Chikungunya virus (CHIKV) belongs to the genus alphavirus of the *Togaviridae* family. Following acute infection, 20–50% of patients develop chronic symptoms lasting between weeks to years [1,2]. Both innate and adaptive immunity have been proposed to play a role in the development of chronic disease, but the complete mechanisms are still unclear [3]. Antibodies to CHIKV have been shown to be important for viral clearance and mediate protection against re-infection [4,5]. Although neutralizing antibodies (NABs) have been shown to be important for CHIKV clearance, it is unclear whether the qualitative and quantitative nature of antibodies, such as avidity and neutralization capacity, play a role in protection/development of chronic disease. For instance, studies showed that maturation of antibody avidity after vaccination/natural infection may be important for protection against infection and/or disease. However, correlation of antibody avidity with protection seemed to be virus-dependent [6,7]. Currently, it is unclear whether antibody avidity contributes to clearance or persistence of CHIKV infection.

In many systems, neutralizing antibodies (NABs) correlates with protection. Two types of neutralization assays have been described: complement-dependent and -independent. Most studies evaluate the levels of complement-independent neutralization. However, several studies revealed that complement inhibits infection of herpesvirus [8], West Nile virus (WNV) [9], and Sindbis virus (SINV) [10–12]. While complement activation was associated with protection against SINV [10–12], mice deficient in complement factor 3 developed less severe disease compared to wild-type mice infected with Ross River virus [13].

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Whether complement plays a role in CHIKV infection still remains to be elucidated.

2. Objectives

To characterize the antibody response in plasma of patients with acute and chronic CHIKV disease.

3. Study design

3.1. Patients

This study was a prospective observational study conducted in Curaçao, which is a continuation from a previous report [14]. The selection of patients was based on the availability of clinical symptoms and plasma sample during follow-up. Patients that still experienced chronic symptoms after 3 months (arthralgia and/or myalgia) were defined as “chronic disease” while patients with no symptoms were defined as having had an “acute disease”. IgM/IgG ELISA (IBL, Germany) and qRT-PCR were also performed on the follow-up samples as previously described [14,15].

3.2. Cells and viruses

Vero E6 cells were grown and maintained at 37 °C with 5% CO_2 as described before.[14] The virus strains used in this study consisted of the African prototype S27 strain passage (P)6 and the CHIKV-IND/NL10 (Asian) strain P5. Both strains were grown on Vero E6 cells and virus titers were determined as the 50% tissue culture infective dose ($TCID_{50}$), calculated using the Kärber method [16,17].

3.3. Virus neutralization assay

The serostatus of all samples was confirmed using a virus neutralization test (VNT) [16]. Briefly, plasma of patients was heat-inactivated (HI) at 56 °C for 30 min and diluted (1:20-1:10,240) in quadruplicate in 96-wells plates. Subsequently, 100 $TCID_{50}$ of CHIKV-S27 or CHIKV-IND/NL10 was added to each well. To assess the potency of the NABs, the same plasma dilutions were incubated with 10,000 $TCID_{50}$ of CHIKV-S27 or CHIKV/NL10. Neutralization experiments with 100 $TCID_{50}$ of CHIKV were defined as “standard” while experiments with 10,000 $TCID_{50}$ were defined as “potency”. After one hour of incubation at 37 °C, 2×10^4 Vero E6 cells were added per well and plates were incubated for 5 days at 37 °C. The neutralizing titers (VNT_{50}) were determined microscopically and defined as the plasma dilution that reduced CPE by 50% [17].

3.4. IgG antibodies neutralizing capacity

In order to assess the contribution of IgM to the neutralizing capacity, denaturation of IgM was performed using β-mercaptoethanol [18] (Sigma-Aldrich, USA). Briefly, plasma of patients was HI and diluted (1:20 to 1:10,240) in quadruplicate on 96-well plates. Subsequently, the diluted samples were treated for 2 h at 37 °C with β-mercaptoethanol at a final concentration of 250 μM, followed by addition of 100 or 10,000 $TCID_{50}$ of CHIKV-S27 to the designated wells and incubation for 1 h. Neutralization assays were then performed as described above.

3.5. Avidity assay

In order to assess the avidity of the antibodies directed against CHIKV E1 and E2, avidity ELISA was performed. To this end, 0.1 μg of E1 antigen (MyBioSource, USA) or 50 ng of E2 antigen (Immune Technology, USA) was coated overnight at 4 °C on high-binding ELISA plates (Corning, USA). Subsequently, the plates were washed with PBS + 0.05% Tween-20 (PBST) and blocked with PBS + 2% BSA (Sigma-Aldrich) for 1 h at 37 °C. Plasma (HI) was diluted (1:50 to 1:6400) in six replicates and 1.5 M of NH_4SCN^- (Sigma-Aldrich) or PBS was added (each in triplicate) and incubated for 5 min at room temperature. Plates were washed with PBST followed by incubation with rabbit-anti-human antibody (1:6000; Dako, The Netherlands). After one hour of incubation, TMB (Invitrogen, USA) was added and the reaction was stopped after 5 min by addition of 0.5 M of H_2SO_4 (Sigma-Aldrich). Absorbance was measured at 450 nm using an ELISA reader (Tecan, USA). The avidity index (AI) was expressed as the EC_{50} of the OD values obtained using non-linear regression fitting. The AI ratio was calculated as the $EC_{50\ (NH4SCN)}$ / $EC_{50\ (PBS)}$ X 100%.

3.6. Complement-mediated virus neutralization assay

To determine the role of complement-mediated virus neutralization in CHIKV infection, guinea-pig complement was added to the neutralization protocol. To this end, HI-plasma was diluted (1:20 to 1:10,240) in quadruplicate on 96-well plates and 100 or 10,000 $TCID_{50}$ of CHIKV-S27 was added to the wells and incubated for 1 h at 37 °C. Subsequently, 10% guinea-pig complement (MP Biomedicals, USA) was added to the plasma dilutions (1:1 vol/vol) and incubated for 1 h at 37 °C. Neutralization experiments were then performed as described above.

3.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, USA). Mann-Whitney U test was used for comparison between continuous variables. The p-value was adjusted for multiple testing using Bonferroni correction. P-values of ≤ 0.00625 were considered to be statistically significant.

4. Results

4.1. Cohort description

Fifty-two patients were diagnosed with chronic disease and 38 patients with acute disease. The median follow-up time for the sample collection was 12 months (range 3–14 months) for both groups. All follow-up samples were negative for CHIKV RNA and positive for anti−CHIKV IgGs. Three patients showed measurable levels of anti−CHIKV IgM in the follow-up samples, which were taken 12 months (2 patients) and 6 months (1 patient) post-infection. Two patients belonged to the acute group while one patient belonged to the chronic group.

4.2. CHIKV NABs induced by an Asian genotype cross-neutralize an ECSA genotype

To determine whether NABs in patients infected with an Asian genotype cross-neutralized an East Central South African (ECSA) genotype, we performed VNT with CHIKV strains representing both genotypes. There was no apparent difference in the neutralizing titers against both genotypes (Fig. 1A). In addition to performing a standard VNT, we also used 100-fold more virus to test the potency of the NABs, since it has been shown that potent NABs could block infection at concentrations that result in low occupancy of accessible sites on the virion while weak NABs recognize fewer sites on the virion and require almost complete occupancy to inhibit viral infection [19]. Based on this, we presumed that potent NABs could also neutralize when challenged with a higher amount of virus. We found that NABs elicited by an Asian strain cross-neutralized the ECSA strain at similar levels. However, no significant differences in neutralization titers were found between acute and chronic patients for either the standard (Fig. 1B) or potency VNT (Fig. 1C).

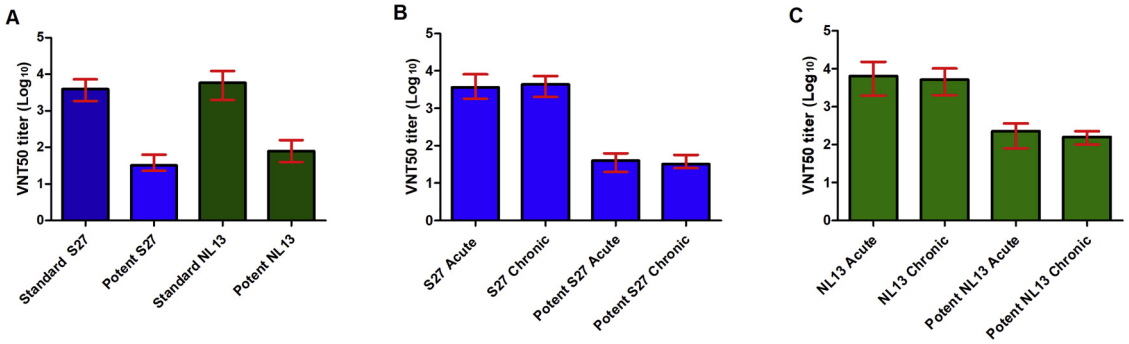

Fig. 1. Neutralization capacity of plasma of patients against ECSA and Asian CHIKV.
(A) Plasma collected from patients during the Asian CHIKV outbreak had similar neutralization titers against CHIKV-S27 (ECSA) and CHIKV/NL10 (Asian) strains in both standard and potency VNTs (virus concentration of 100 and 10,000 $TCID_{50}$, respectively). Neutralization titers of patients with acute and chronic disease were compared for both CHIKV-S27 **(B)** and CHIKV/NL10 strains **(C)**. No significant differences were observed between acute and chronic patients for both virus strains. Data are presented as median with inter-quartile range.

4.3. No differences in IgG neutralization titers between patients with acute and chronic disease

It is known that natural IgM antibodies as well as virus-specific IgM, which can be detected for months post-infection, can contribute to neutralization activity [1,20,21]. To measure the IgG neutralization activity only, IgM denaturation was performed using β-mercaptoethanol. We first determined the optimal concentration of β-mercaptoethanol, defined as the concentration that was not toxic to cells, did not affect virus infectivity and was able to abolish neutralization activity of IgM antibodies. Plasma samples of two patients in the acute phase (5 days after the onset of symptoms with positive IgM, but no detectable IgG antibodies) were used for validation. The neutralization activity was lost at a concentration of ≥250 μM (data not shown). When the samples were tested in the presence of β-mercaptoethanol, only minor differences in the neutralizing titers were found between the standard and potency groups (Fig. 2A). Furthermore, there was no significant difference between acute and chronic groups (Fig. 2B).

4.4. Complement increased the neutralization capacity when high amounts of virus was used

Normally, neutralization is measured in the absence of complement. To investigate the involvement of complement in the neutralization of CHIKV, we incorporated complement in the assay. First, we determined whether complement itself neutralize CHIKV. We found that the complement concentration that we used did not neutralize CHIKV (data not shown). Next, we found that complement did not increase neutralization when 100 $TCID_{50}$ of virus was used. In contrast, the addition of complement significantly increased the neutralization capacity ($p < 0.0001$) in the potency group (Fig. 3A). This synergistic effect of complement on neutralization was measured in both acute ($p < 0.0001$) and chronic ($p < 0.0001$) patients (Fig. 3C). These results imply that complement-mediated neutralization of CHIKV may play a role in the presence of a high virus load. Nonetheless, no major differences were seen between acute and chronic patients in either the standard or potency complement-mediated neutralization assay using a high viral load.

4.5. Patients with acute disease had higher antibody avidity compared to chronic disease

To determine whether avidity of the antibodies differed between acute and chronic patients, we compared the antibody AI between these groups. We found a significant difference in the AI of both the anti-E1 ($p < 0.0001$) and anti-E2 ($p = 0.0003$) antibody response between these groups, which indicates that acute patients had higher antibody avidity against E1 and E2 glycoproteins compared to chronic patients (Fig. 4). Despite this difference, the median AI of anti-E2 antibodies was still relatively high in the chronic group (EC_{50} = 81.6%), compared to anti-E1 (EC_{50} = 52.8%).

5. Discussion

This manuscript describes several properties of the antibody response in a cohort of CHIKV patients. Firstly, our data revealed that NABs produced against one genotype of CHIKV are cross-reactive against another genotype. This result is in agreement with the other

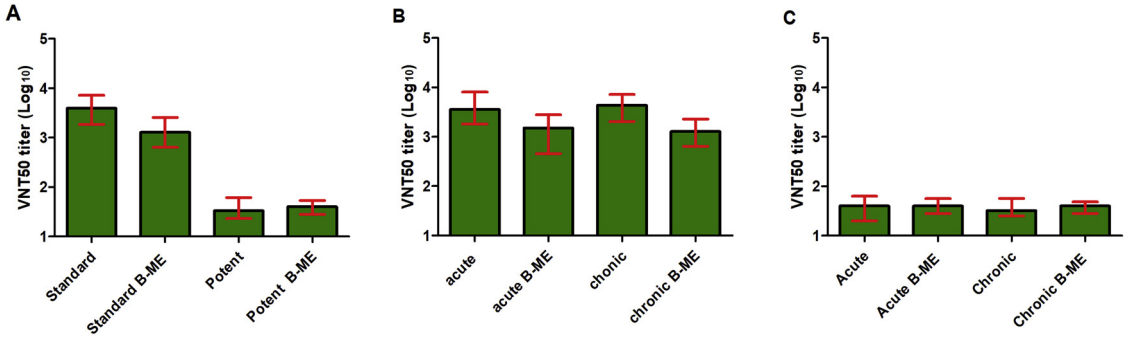

Fig. 2. IgG neutralization titers in patients with acute and chronic CHIKV infections.
(A) IgG neutralization titers of plasma samples were compared in the presence and absence of denaturation agent (B-ME). No significant differences were observed for both standard (100 $TCID_{50}$) and potency (10,000 $TCID_{50}$) virus challenge groups. Neutralization titers of patients with acute and chronic disease were compared in both the standard **(B)** and potency **(C)** groups. No major differences were observed for both groups. Data are presented as median with inter-quartile range. B-ME: β-mercaptoethanol.

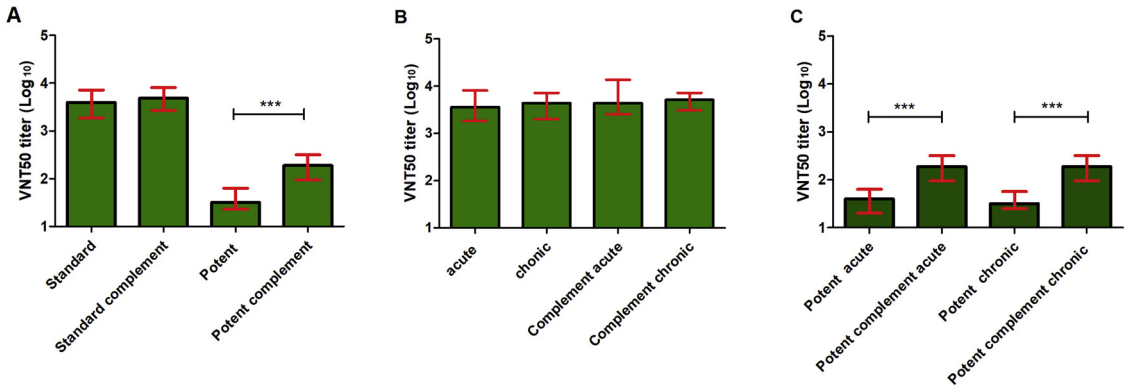

Fig. 3. Complement-mediated enhancement of CHIKV neutralization.
(A) Neutralization titers were compared between plasma samples in the presence and absence of complement. Significant differences were observed for the potency (10,000 $TCID_{50}$ virus) group. Neutralizing titers of patients with acute and chronic disease were compared in both the standard **(B)** and potency **(C)** groups. Increased neutralization was observed for samples that were incubated with 10,000 $TCID_{50}$ of virus (potency) group in comparison to the standard group (100 $TCID_{50}$). Data are presented as median with inter-quartile range. The Mann-Whitney U test was used for group comparisons. *** $p < 0.001$.

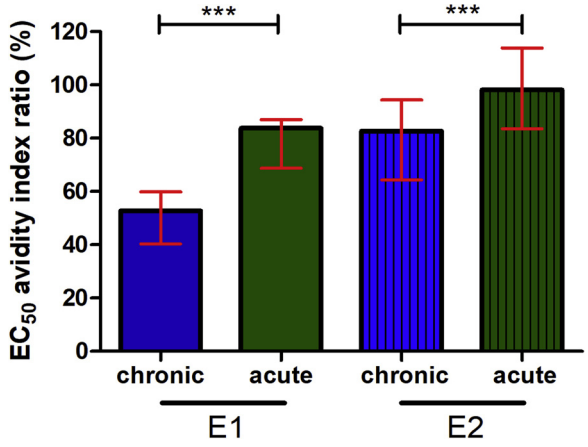

Fig. 4. E1 and E2 protein antibody avidity index in CHIKV patients with acute and chronic infections.
Antibody avidity index ratios for both E1 and E2 protein were significantly higher in patients with acute disease compared to chronic disease (E1: $p = < 0.0001$; E2: $p = 0.0003$). Data are presented as median with inter-quartile range. The Mann-Whitney U test was used to compare the groups. *** $p < 0.0001$.

studies showing complete cross-neutralization [22,23]. The high percentage of amino acid similarity among all CHIKV strains [24] together with minimal differences in vaccine-NAB responses observed among genotypes [25] indicate that CHIKV NABs to one genotype are highly effective against the other genotypes.

Next, we characterized the neutralizing IgG antibodies. Our results showed that NABs were detected in all the patients, but no apparent differences in VNT_{50} between acute and chronic patients were detected. Consistently, previous study showed that the neutralizing titers of IgG3, the dominant IgG subclass found in the study, were equal for both groups of patients at 2–3 months post-infection [26]. This study also found that early induction of anti−CHIKV IgG3 antibodies was associated with protection against chronic arthralgia. As the aforementioned study only looked at shorter time post-infection, we decided to use a longer follow-up time in our study since it has been shown that antibody kinetics can differ over time [7]. Lastly, our data is coherent with the data of Kam et al. [26], suggesting that acute and chronic patients developed similar levels of VN titers. As such, quantitative aspects of antibodies may therefore not play a role in the development of chronic disease. If antibodies measured in late convalescent sera are not associated with chronic disease, it is plausible that differences in antibody kinetics early in infection may be important instead.

Serum and cellular factors that intermingle with antibodies have the potential to modulate neutralization activity [27]. Our data revealed that complement increased neutralization significantly in the presence of high virus concentration. This effect was equal between the acute and chronic patients, indicating that the neutralization capacity is similar between both groups. Complement is one of the factors that has been shown to play a role in neutralizing several viruses [8,9,11]. Although there are several possibilities to explain the results in our study, it is likely that in face of high viral titers fewer antibodies per virus are available, making complement activation necessary to reduce viral dissemination and consequently levels of viremia.

Another parameter that could influence the protective capacity of antibodies is avidity. Several studies showed that avidity correlates with protection against infection [6,28]. We found a higher AI against both E1 and E2 glycoproteins in patients with acute disease, which indicates a good immune response maturation. Antibodies produced in the early phase of infection usually show a low avidity for the antigen, which then increases continuously over time following contact with an immunogen. This hypothesis is supported by studies on several viruses, such as measles virus (MV) and CMV [7,29]. Our results are different from another CHIKV study, which reported that antibody avidity was comparable in both arthralgia-positive and -negative patients at 36 months post-infection [2]. As the quality and quantity of antibodies can differ over time [7], this discrepancy might be explained by different time intervals used between the studies. Moreover, differences in the protocols may also account for this variance as we used NH_4SCN^- as a chaotropic agent, and several antibody dilutions, while Schilte et al. [2] used urea, and only one antibody dilution.

Interestingly, the AI_{50} values for the antibodies against E1 for the acute group were higher (> 80%) compared to the chronic group (< 60%). Nevertheless, the AI for the antibody response against the E2 protein in the chronic group was still relatively high (> 80%). Therefore, it is unclear whether certain thresholds are needed for protection against chronic disease, or whether a highly avid response against certain epitopes/proteins is more important than others. Although higher levels of avidity were correlated with protection [6,30], the criteria for defining protective levels of AI is not consistent and could also be virus-specific [7,31]. In our study found similar VNT_{50} was measured in the potency test between both groups, indicating that the differences in avidity were not translated into differences in neutralization potency.

Several reports showed that formalin-inactivated measles vaccine (FIMV) produced low avidity antibodies that bind, but do not neutralize MV, leading to formation of complexes of MV antigen, antibody, and

complement in the tissue of macaques [32,33]. In contrast, higher-avidity antibodies were more protective by mediating complement-dependent opsonophagocytosis of both 6B and 23 F pneumococci compared to lower-avidity antibodies [34]. Whether interaction of antibody avidity and complement contributes to CHIKV neutralization warrants further studies.

Our study has several limitations. We could not assess the whole spectrum of the clinical symptoms, and there was a variation in the follow-up times. Moreover, we did not have sequential samples that would allow measurement of antibody kinetics over time. Although several studies showed that guinea pig complement is comparable to human complement [35,36], we cannot rule out that the results that we observed are species specific.

6. Conclusions

Our results indicate that VNT_{50} was not different between patients with acute and chronic chikungunya. However, complement increased the neutralization capacity against CHIKV when high amount of virus was used. Finally, the average AI against E1 and E2 glycoproteins was higher in the acute compared to chronic group.

Credit author statement

FA and BEEM conceptualized and designed the study. FA, SML and SF performed the experiments. RW contributed in the sample collections and reagents. FA, SML, SF, RW, ADMEO and BEEM all contributed in the planning of the manuscript, data analysis and interpretion, and critical review and approval of the manuscript.

Ethical approval

Ethical clearance for this study was obtained from the Medical Ethics Committee of Curaçao (ref. no. 2014-003). Written informed consent was obtained from enrolled patients.

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