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Assessment of the Immunogenicity and Protective Aspects of a DNA Vaccine Targeting Crimean Congo Hemorrhagic Fever Virus Glycoprotein Gc

Kırım Kongo Kanamalı Ateşi Virüsü Glikoprotein Gc'yi Hedef Alan Bir DNA Aşısının Bağışıklık ve Koruyuculuk Sağlama Özelliklerinin Değerlendirilmesi

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ABSTRACT

Aim: Crimean Congo Hemorrhagic Fever (CCHF) is a lethal, endemic infectious disease in human. For the preventive measures of the disease, there is currently no safe and efficient vaccine, widely for human use. Vaccine development for CCHF virus is an actively researched subject. In this study, we aimed to investigate the immunizing and protective potentials of the CCHF virus surface glycoprotein Gc that is delivered as a single antigen via a DNA based vaccine vector.
Material and Methods: A DNA based vaccine targeting the immunogenic envelope glycoprotein Gc of a CCHF virus isolate with Turkey origin (Ank2) was generated and its immunogenicity and protective capability against lethal challenge in IFNα/βR-/- receptor knock out mice was assessed.
Results: The developed vaccine candidate (pGc) elicited a considerable amount of neutralizing antibody responses in the vaccinated mice. The vaccine candidate significantly induced both antiviral Th1 and B cell activating Th2 immune responses deduced from the cytokine production profiles in the vaccinated mice. However, despite the immune responses elicited post-immunization, the vaccine failed to confer protection against lethal CCHF virus infection.
Conclusion: To the best of our knowledge, this is the first report of a DNA vaccine candidate generated against CCHF virus based on the glycoprotein Gc. The pGc vaccine candidate exhibited antigen-specific immunity in IFN/α/βR-/- mice, but was unable to produce a protection upon lethal challenge with the homologous CCHF virus. Once we comprehensively understand the immune correlates of protection, we will be more eligible to significantly improve the efficacy of vaccines.
Keywords: Crimean Congo hemorrhagic fever virus; DNA vaccine; immune responses; lethal challenge.

ÖZ

Amaç: Kırım Kongo Kanamalı Ateşi (KKKA), insanda ölümcül, endemik bir enfeksiyon hastalığıdır. Hastalığın önleyici tedbirleri için şu anda insanlarda yaygın olarak kullanılmak üzere güvenli ve etkili bir aşı bulunmamaktadır. KKKA virüsü için aşı geliştirilmesi, aktif olarak araştırılan bir konudur. Bu çalışmada, DNA esaslı bir aşı vektörü ile tek bir antijen olarak verilen KKKA virüsü yüzey glikoproteini Gc'nin bağışıklık kazandırıcı ve koruyucu potansiyellerinin araştırılması amaçlanmıştır.
Gereç ve Yöntemler: Türkiye menşeli (Ank2) bir KKKA virüs izolatının immünojenik özellikteki zarf glikoproteini Gc'yi hedefleyen DNA esaslı bir aşı oluşturulmuş ve bu aşı adayının immünojenisitesi ve aşılanmış IFNα/βR-/- farelerde öldürücü doza karşı koruyucu yeteneği değerlendirilmiştir.
Bulgular: Geliştirilen aşı adayı (pGc), aşılanmış farelerde önemli miktarda nötralize edici antikor yanıtı ortaya çıkardı. Aşı adayı, aşılanmış farelerde hem antiviral Th1, hem de B hücresini aktive eden Th2 bağışıklık tepkilerini önemli ölçüde uyardı. Bununla birlikte, aşılama sonrasında ortaya çıkan bağışıklık yanıtlarına rağmen, aşı, ölümcül KKKA virüsü enfeksiyonuna karşı koruma sağlayamadı.
Sonuç: Bildiğimiz kadarıyla bu çalışma, glikoprotein Gc'yi hedef alan KKKA virüsüne karşı oluşturulan bir DNA aşı adayının ilk raporudur. PGc aşı adayı, IFNα/βR-/- farelerde antijene özgü bağışıklık yanıtı oluşturdu, ancak ölümcül dozdaki homolog KKKA virüsüne karşı bir koruma üretemedi. Aşı aracılı korunmanın bağışıklık ile olan ilişkilerini daha detaylı olarak anladığımızda, aşıların etkinliğini önemli ölçüde iyileştirme kabiliyetine sahip olacağız.
Anahtar kelimeler: Kırım Kongo kanamalı ateşi virüsü; DNA aşısı; immün yanıt; ölümcül sınama.

Part of this study was presented as an oral presentation at the 2nd International Conference on Crimean Congo Hemorrhagic Fever (September 10-12, 2017; Thessaloniki, Greece)

INTRODUCTION

Crimean Congo hemorrhagic fever virus (CCHFV) is a negative sense, single-stranded RNA virus belonging to the *Nairoviridae* family of the order *Bunyavirales*. The virus has a tripartite genome consisting of small (S) segment encoding for nucleocapsid protein (NC), medium (M) segment encoding for a polyprotein which is proteolytically processed into two structural glycoproteins (Gn and Gc) and other nonstructural proteins, and the large (L) segment encoding for the RNA dependent RNA polymerase (RdRp) (1). With being the causative agent of the most widely distributed tick-borne viral zoonosis, infection with CCHFV leads to severe hemorrhagic fever syndrome in humans with the case fatality rates ranging from 5% to 80% (2). Although CCHFV infections can be detected in various vertebrates including livestock animals, they do not cause any sign of disease in these organisms and hence humans appear to be the only host for CCHFV in which pathogeny takes place. CCHFV transmission to humans may occur via tick bite or tick crushing by hand and through direct contact with body fluids of an infected animal or patient (2-4). The disease occurs widely in western Asia, southern Russia, the Middle East, much of Africa and parts of Eastern Europe (2,3). The geographical distribution and prevalence of Crimean Congo hemorrhagic fever (CCHF) disease appear to gradually increase in Europe since the last two decades (5,6). Between 2002 and 2017, more than 10000 patients with CCHF were reported in Turkey involving the mortality rate of around 5% (7).

Research on developing preventive measures for CCHF have been significantly hindered due to the requirement of laboratories with adequate biosafety containment levels for handling the virus and the absence of proper animal models of the disease. Recently, two lethal mouse models which reproduce at least some of the disease symptoms due to having deficiencies in their interferon pathways were generated (8,9). The generation of these two CCHF model, a type I interferon α/β receptor knockout (IFN/α/βR-/-) mice and STAT1 knockout mice paved the way for testing the efficacy of newly developed CCHF vaccine candidates.

To date, the only available vaccine used to immunize human is a suckling mouse brain-derived, chloroform and heat inactivated virus subsequently formulated with aluminum hydroxide adjuvant (10). This inactivated virus vaccine has been used in Bulgaria to vaccinate people from risk groups since 1974 (10). Due to its crude preparation which causes concerns for safety, along with the absence of controlled human studies and the laboratory assessments of the efficacy of this vaccine, it is unlikely to gain approval by the international regulatory bodies. Therefore, there is currently no internationally approved, safe and effective vaccine against CCHFV available for widespread human use.

In regards to the CCHFV vaccine research, there are some recently reported studies on the development of vaccine candidates against CCHFV exhibiting protective efficacy in the IFN/α/βR-/- mice (11-18). These vaccine research studies mostly focus on recombinant virus based and DNA based vaccines development approaches (11-13,16-18). Recent approaches used on the development of vaccines against CCHFV were comprehensively reviewed elsewhere (19).

Here, we aimed to examine the immunizing properties and the protective abilities of a DNA based vaccine targeting the glycoprotein Gc antigen of a CCHFV isolate with Turkey origin (Ank2) by means of measuring the humoral and cell-mediated immune responses and conducting a challenge assay in IFN/α/βR-/- mice. To our knowledge, this is the first study reporting on the immunogenic characteristics and the disease protective properties of a CCHFV Gc antigen expressed via a DNA based vaccine candidate.

MATERIAL AND METHODS

Cells and Virus

Baby hamster kidney (BHK-21-C13) and Scott and White No. 13 (SW-13) cells were used in this study. Both cells were cultured in their suitable mediums supplemented with relevant concentrations of fetal bovine serum and antibiotics and maintained at 37°C in a humidified incubator containing 5% CO_2 as described previously (20). CCHFV clinical isolate Ank2 from the virus collection of Ankara University, Faculty of Veterinary Medicine, Department of Virology was used for all experiments. The third passage of this virus propagated in SW-13 cells was used in this study. All experiments related to CCHFV was carried out in the biosafety level 3 plus (BSL3+) and animal biosafety level 3 plus (ABSL3+) facilities of the Virology Department at Veterinary Faculty of Ankara University.

Animals

Five to nine weeks old, female, inbreed BALB/c mice and IFN/α/βR-/- mice with AG129 background were used in this study. Mice housing and handling procedures were followed according to the ethical rules of The Republic of Turkey Ministry of Food, Agriculture, and Livestock. All the *in vivo* experimental protocols were carried out under The Ankara University Ethical Committee's approval and in ABSL3+ animal facility at Virology Department of the Faculty of Veterinary Medicine. The study was approved by the Local Ethics Committee for Animal Experiments of Ankara University (17.12.2014, 23/155).

Construction of DNA Vaccine

pVAX1 (Thermo Fischer Scientific, USA) and pEGFP-C1 (Clontech, USA) plasmid vectors were used for the phylogenetic analysis of the cloned Gc ORF of CCHFV Isolate Ank2 and the construction of DNA vaccine vector, respectively. Plasmid isolations were carried out using the GeneJET Plasmid Maxiprep Kit (Thermo Scientific, USA). SLiCE (Seamless Ligation Cloning Extract) cloning method, which works through homologous recombination was used for all cloning experiments (21). Bacterial transformation and selection of the right clones were carried out according to the standard protocols (22). In the cloning of Gc ORF into pVAX1, viral RNA was extracted from the original stock of CCHFV Isolate Ank2 using the standard procedure of QIAamp Cador Pathogen Mini Kit (QIAGEN, Germany) and reverse transcribed with random hexamers using the standard procedure of Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The Gc ORF was then PCR amplified from the full-length M segment cDNA using the standard protocol of MyTaq HS DNA Polymerase (BIOLINE, UK) with the oligonucleotide primer couple: SliceF1:

5'ACGACTCACTATAGGGAGACCCAAGCTGGCTA GCGTTTAAACTTAGCCACCATGTTCTTGGA CAGTATAGTTAAAGG3' and SliceR1: 5'AATTCCACCACACTGGACTAGTGGATCCGAGCT CGGTACCAAGCTTTAGCCAATGTGTGTTTTTGTG G3' and cloned into pVAX1 plasmid vector linearized with HindIII restriction enzyme (New England Biolabs, UK) and blunt-ended with Phusion DNA polymerase (Thermo Scientific, USA) beforehand using the SLiCE cloning method. This construct was named as pVAX1-Gc. pEGFP-Gc and pGc plasmid vectors were constructed using the pEGFP-C1 vector backbone and used for the *in vitro* expression and *in vivo* immunization experiments respectively. For the generation of pEGFP-Gc vector, the Gc ORF of isolate Ank2 was PCR amplified from the pVAX1-Gc vector using the standard protocol of MyTaq HS DNA Polymerase (BIOLINE, UK) with the oligonucleotide primer couple: SliceF2: 5'GAGCTGTACAAGTCCGGACTCAGATCTCGAGCT ATGTTCTTGGACAGTATAGTTAAAGG3' and SliceR2: 5'GTATGGCTGATTATGATCAGTTATCTAGATCCG GTTTAGCCAATGTGTGTTTTTGTGGAGAAC3' and cloned into pEGFP-C1 vector linearized with BamHI and HindIII restriction enzymes (New England Biolabs, UK) before, using the SLiCE cloning method. By this way, in the pEGFP-Gc vector, the cloned Gc ORF was designed to be expressed in-frame with EGFP as a C terminal fusion product under the control of the Cytomegalovirus (CMV) promoter. pGc vector was derived from the pEGFP-Gc vector by replacing the EGFP ORF with 8X Histidine Tag. For this purpose, the pEGFP-Gc vector backbone was PCR amplified by excluding the EGFP ORF using the standard protocol of MyTaq HS DNA Polymerase (BIOLINE, UK) with the oligonucleotide primer couple: EGFP RemovalF:5'CACCATCATCACCACCACCATCACTT CTTGGACAGTATAGTTAAAGGTATGAAAAATTTG C3' and EGFP RemovalR: 5'GTGATGGTGGTGGTGATGATGGTGCATGGTGG CGACCGGTAGCGC3' and then the amplicon was circularized using SLiCE. Thus, in the pGc vector, the Gc ORF was planned to be expressed in-frame with N-terminal 8X His Tag under the control of the CMV promoter. For the negative control experiments, an empty vector was generated from the pEGFP-C1 vector by excision of the CMV promoter from the vector backbone. For this purpose, the CMV promotor site was cut out from the vector using the NdeI and NheI restriction enzymes (New England Biolabs, UK) and the remaining vector backbone was circularized simply by the "alternative end joining" mechanism of *E. coli* (9). The vector map images were created using the SnapGene Viewer 4.1.6 Software (GSL Biotech LLC). The nucleotide sequences of each vector were confirmed with both next-generation sequencing (NGS) (Ion Torrent Platform) and Sanger dideoxy chain termination DNA sequencing methods prior to their use. In the latter method, the following sequencing primers were used:
CMV Forward: 5'CGCAAATGGGCGGTAGGCGTG3'
GcSeq1: 5'CCGACAACCACTACCTGAGCAC3',
GcSeq2: 5'GTGGTTGCACATCATCAACCTGC3',
GcSeq3: 5'GGATGTCCTGGGATGGTTGTGAC3' and
GcSeq4: 5'AAACTTGAGCAGCCACAGAGC3'.

Phylogenetic Analysis

Phylogenetic analysis of the Gc ORF of CCHFV Isolate Ank2 was performed based on its amino acid sequence using the MEGA7 software (23). Thirteen representative CCHFV mature Gc sequences of different strains from various geographical regions including Turkey were obtained from the NCBI GenBank database and used for the phylogenetic analysis. The phylogenetic tree was constructed based on the Neighbor-Joining algorithm (24). The bootstrap values higher than 50 were indicated for each branch based on 1000 replicates (25).

Transient and Stable Transfections of BHK-21-C13 Cells

pEGFP-Gc vector was used for the *in vitro* validation of Gc expression in the transfected BHK-21-C13 cells. For all transient and stable transfection experiments, standard protocol of Lipofectamine 3000 (Thermo Scientific, USA) transfection reagent was used. In the transient transfection experiments, the circular form of the pEGFP-Gc vector was used to transfect BHK-21-C13 cells. Transfected cells were then subjected to downstream analyses at the 48-h post-transfection time point. In the stable transfection experiments, both vectors were linearized with AlwNI restriction endonuclease enzyme (New England Biolabs, UK) before transfection and starting from the 48 h post-transfection, cells were treated with 800 µg of geneticin (G418, Thermo Scientific Fisher, USA) per ml of media during three weeks for the selection of stably transfected BHK-21-C13 cells.

Fluorescence Microscopy

Expression of the EGFP-Gc fusion product was demonstrated by viewing the EGFP-Gc expressing, transiently transfected BHK-21-C13 cells under an inverted fluorescence microscope (Zeiss Axio Vert.A1, Germany) using the blue light (488 nm) as the excitation source.

Western Blot

Following the three-week long geneticin selection process, the stably transfected BHK-21 cells were lysed with Pro-Prep Protein Extraction Solution (İNtRON Biotechnology). Cell lysates were mixed 1:1 with 2X Laemmli's sample buffer (Sigma-Aldrich) and heated at 95°C for 5 minutes. Proteins were separated by SDS-PAGE in Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad, USA) and transferred to Trans-Blot Turbo Mini PVDF membranes (BioRad, USA). The membranes were blocked with 1x Tris Buffered Saline (1x TBS) (Sigma) supplemented with 0.1% Tween 20 (BioRad, USA) (TBST) and 5% BSA bovine serum albumin (Sigma, USA). Blocked membranes were probed for EGFP with 1/1000 GFP tag mouse monoclonal antibody (GF28R) (Invitrogen) prepared in 1x TBST and incubated at +4°C overnight. The membranes were washed 5 times with 1x TBST and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Advansta, USA) diluted in 1x TBST at room temperature for 1 hour. The membranes were washed 5 additional times with 1x TBST and imaged with enhanced chemiluminescence (ECL) assay using the ChemiDoc XRS+ imaging system (BioRad, USA).

RT-PCR

For the demonstration of Gc expression at the transcription level, total RNA of BHK-21-C13 cells stably transfected with pEGFP-Gc vector was isolated using the Trizol reagent (26) (Thermo Scientific, USA) and following a DNase I (New England Biolabs, UK) treatment, the

isolated RNA was purified with the standard protocol of sodium acetate/ethanol precipitation followed by washing of the RNA pellet with 70% ethanol. The mRNA content of the purified total RNA was reverse transcribed with oligo(dt) 18 primer using the standard procedure of Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and the cDNA of Gc transcripts were then PCR amplified using MyTaq HS DNA Polymerase (BIOLINE, UK) with the primer couple: GcSeq4: 5'AAACTTGAGCAGCCACAGAGC3' and SLICER2: 5'GTATGGCTGATTATGATCAGTTATCTAGATCCG GTTTAGCCAATGTGTGTTTTTGTGGAGAAC3' targeting the downstream site of the Gc transcript.

Vaccination Protocol

Two groups of four Balb/c and four IFN/α/βR-/- mice were vaccinated in the medial thigh muscle with 100 µg of either the pGc vaccine vector or the empty vector dissolved in 50 µL of non-pyrogenic physiological saline solution using the 25-gauge syringes (BD, USA). All mice were vaccinated two times with two weeks intervals. Blood samples were collected via tail vein bleeds prior to each vaccination on days 0, 14 and 28.

Virus Neutralization Assay (VNA)

The presence and titer of the anti-CCHFV neutralizing antibodies in the sera of mice received two consecutive pGc DNA vaccinations were determined by micro virus neutralization assay (MVNA). The serum samples were initially heat inactivated at 56°C for 30 minutes. Two-fold serial dilutions were prepared starting from 1/8 to 1/532 and mixed with equal volumes of CCHFV Ank2 isolate at a 100TCID50 doses and incubated at 37°C for 1 hour. After the incubation, each reaction mixture was added to the 50,000 SW-13 cells adhered on a 96-well plate with four replicates and incubated for 1.5 hours at 37°C in a humidified cell culture incubator containing 5% CO2. The reaction mixtures were later replaced with fresh Leibovitz's l-15 medium (Gibco, USA) supplemented with 2% heat-inactivated FBS (Gibco, USA) and 1% Penicillin/streptomycin (Gibco, USA) and cells were incubated for one week under the same culture conditions described above. Cells were later fixed with 3.7% formaldehyde (Sigma, USA) and stained with 1% crystal violet (Sigma, USA) solution prepared in 20% ethanol (Sigma, USA) before being visualized by naked eye and under a simple light inverted microscope (Olympus, Japan).

Detection of Cytokines in the Sera of Vaccinated Mice

To examine the Th1 and Th2 type immune responses, cytokine production levels were determined in the serum samples of vaccinated mice using the LEGENDplex Mouse Th1/Th2 Panel (8-plex, Bead assay by flow cytometry) kit (BioLegend, USA, https://www.biolegend.com/en-us/legendplex). All the bead-based cytokine assays were conducted according to the manual of the manufacturer. The reactions were evaluated after reading FacsCanto II FlowCytometer (BD Bioscience, USA) using the LEGENDplexTM Data Analysis Software.

Challenge Assay of IFNα/βR-/- Mice

The CCHFV Ank2 strain, which was previously demonstrated as lethal for IFNα/βR-/- mice (11), was used in the intra-peritoneal challenge assay. Two weeks after the final vaccination, all mice (4 mice per group) were intraperitoneally inoculated with the virus (third passage in SW-13 cell) at a dose of 100LD50 (1000TCID50) prepared in 300µL of Leibovitz's l-15 medium (Gibco, USA). The negative control group consisted of four mice received only sterile physiological saline solution. The assay was continued for 13 days. Daily observations of the clinical signs of the disease including the appearance change on fur such as erectile hairs, weight loss, nasal or ocular discharge, depression, and death were noted.

Statistical Analysis

After performing the Shapiro-Wilk test for normality assumption, the cytokine assay data obtained from BALB/c and IFN/α/βR-/- mice were analyzed using the Mann-Whitney U test. The p values less than 0.05 were regarded as statistically significant. All analyses were performed using the SPSS software v.22.0.

RESULTS

Maps of the pEGFP-Gc, and pGc Vectors

Vector maps depicting the features of pEGFP-Gc and pGc plasmids including the regulatory sequences and protein coding regions were shown in Figure 1a and 1b respectively. While the pVAX1-Gc vector was used for the phylogenetic analysis of the cloned CCHFV Gc ORF and aided to the generation of pEGFP-Gc and pGc vectors, the pEGFP-Gc vector was used for the *in vitro* expression studies. Finally, the pGc vector was used for the mouse immunization experiments.

Phylogenetic Analysis of the CCHFV (Ank2) Glycoprotein Gc

The nucleotide sequence of cloned CCHFV Ank2 Gc ORF was determined using the NGS approach (Ion Torrent platform) and deposited to the NCBI GenBank under the accession number: MG969426. The amino acid sequence of the virus glycoprotein Gc was phylogenetically analyzed using the Gc sequences of a total of thirteen representative CCHFV strains from different geographical areas including Turkey. The phylogenetic tree was constructed based on the Neighbor-Joining algorithm using the MEGA7 software and presented in Figure 2. The CCHFV Ank2 was found to be closely related to the CCHFV strains (e.g. Kelkit06 and Turkey200310849) reported previously from Turkey in terms of Gc sequences (Figure 2).

***In Vitro* Expression of CCHFV (Ank2) Gc**

In order to confirm *in vitro* expression of CCHFV (Isolate Ank2) Gc, BHK-21 cells were transiently transfected with pEGFP-Gc vector and analyzed with fluorescence microscopy at 48 h post-transfection for the detection of EGFP-Gc fusion product. A considerable number of live cells emitting green fluorescent light were apparent as shown in Figure 3b. The expression of EGFP-Gc fusion product was later demonstrated with western blot analysis using the anti-GFP monoclonal antibody in the protein extracts of BHK-21 cells stably transfected with pEGFP-Gc vector (Figure 3d). Additionally, the expression of EGFP-Gc ORF was confirmed at the transcript level with RT-PCR analysis in the mRNA pool of BHK-21 cells stably transfected with pEGFP-Gc vector (Figure 3c).

Neutralizing Antibody Responses of Vaccinated Mice

Anti-CCHFV neutralizing antibody responses were evaluated in the serially diluted serum samples of both immunocompetent BALB/c and IFN/α/βR-/- mice received two consecutive pGc DNA vaccinations using the

micro virus neutralization assay (MVNA). Briefly, the SW-13 cells were subjected to 100 TCID50 of CCHFV (Isolate Ank2) pretreated with various dilutions of sera collected from the vaccinated mice and later, cells were checked for the occurrence of cytopathic effects (CPE) over a one-week period. The apparent formation of CPE in SW-13 cells infected with CCHFV is a well-known phenomenon (30). As a result, all four vaccinated mice in each mouse models responded with considerable amount of neutralizing antibody production with the mean titers of 6.8 and 7.5 Log2 for IFN/α/βR-/- and BALB/c mice respectively (Figure 4).

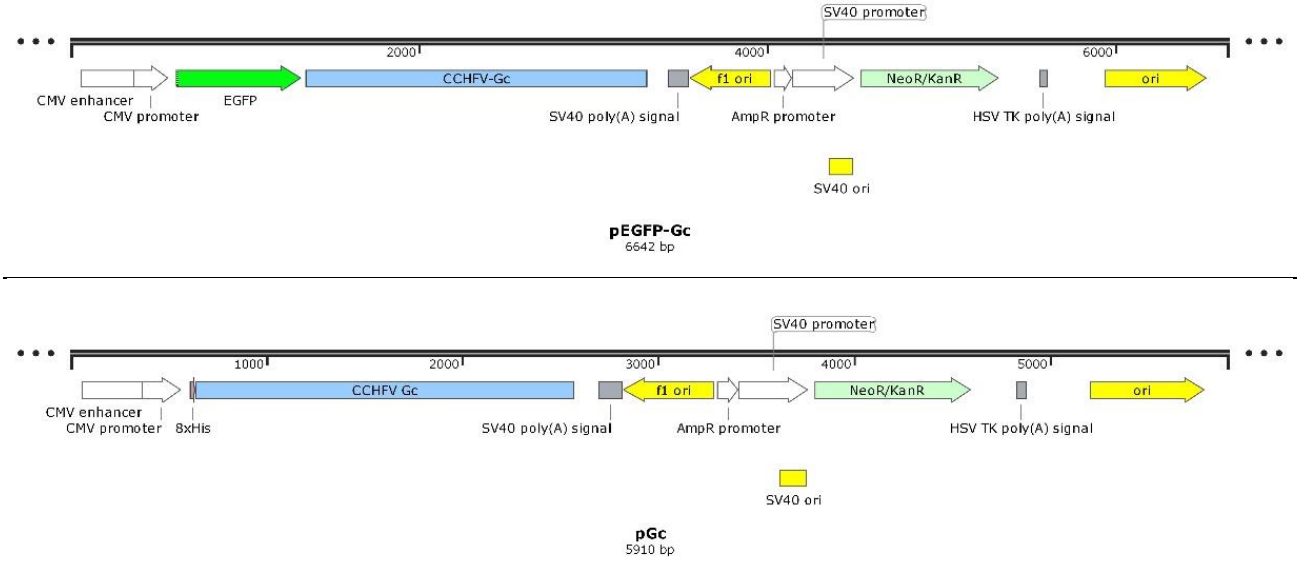

Figure 1. Maps of the **a)** pEGFP-Gc and **b)** pGc plasmids

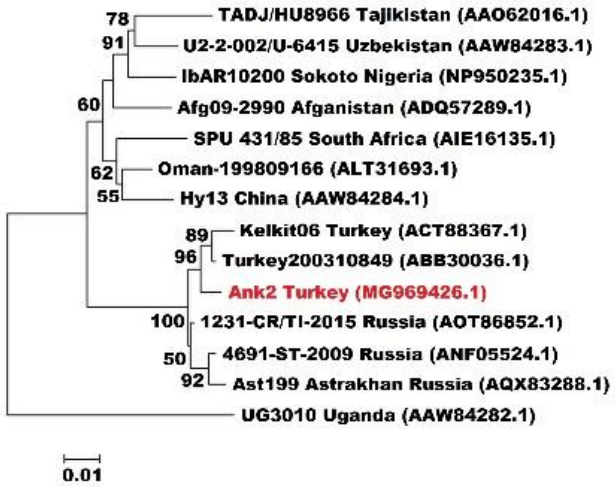

Figure 2. Phylogenetic tree of the CCHFV (Isolate Ank2) glycoprotein Gc constructed based on the Neighbor-Joining algorithm in MEGA7 software. The Genbank accession numbers of each sequence were given in brackets. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.28116628 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and were in the units of the number of amino acid substitutions per site. The analysis involved 14 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 644 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

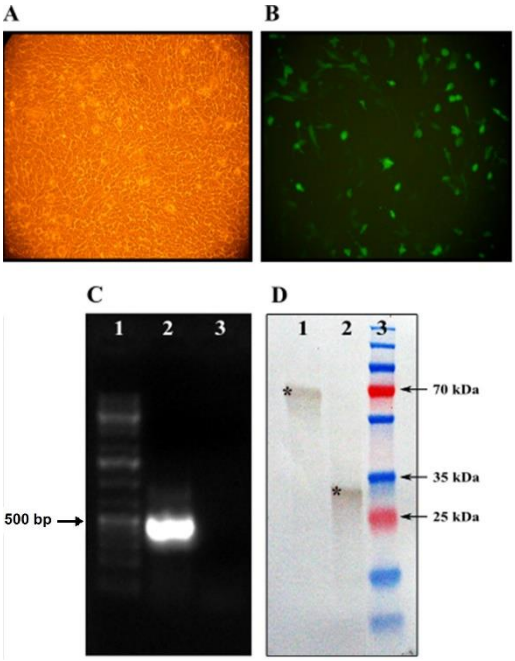

Figure 3. *In vitro* expression of pEGFP-Gc vector in BHK-21 cells **a)** simple light microscope and **b)** fluorescence microscope images of BHK-21 cells transiently transfected with pEGFP-Gc vector, **c)** Gel images of Gc transcript-specific RT PCR analysis conducted with RNA extracts of BHK-21 cells stably transfected with pEGFP-Gc vector, 1: DNA marker, 2: Amplicon of the Gc mRNA specific RT-PCR, and 3: RNA PCR control **d)** Western blot membrane images of EGFP-Gc fusion protein in protein extracts of BHK-21 cells stably transfected with pEGFP-Gc vector, 1: EGFP-Gc fusion product in cells stably transfected with pEGFP-Gc vector (indicated with asterisk) and 2: EGFP product in cells transiently transfected with pEGFP-C1 vector (indicated with asterisk), 3: Protein Marker

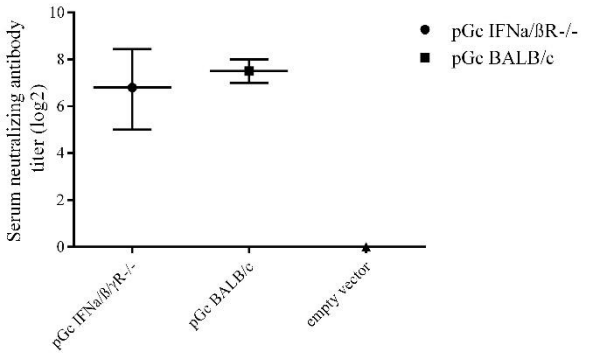

Figure 4. Determination of the neutralizing antibodies in the sera of BALB/c and IFN/α/βR-/- mice vaccinated with pGc vector using the micro-neutralization assay: Figure shows the lowest, the highest and the mean log2 neutralizing antibody titers observed in the sera of four BALB/c and four IFN/α/βR-/- mice received two consecutive pGc vaccine.

Cytokine Responses of Vaccinated Mice

The sera of mice of the immunization groups were examined for their Th1 and Th2 cytokine profiles. BALB/c mice vaccinated with pGc exhibited levels of the Th1 type cytokines IFN-γ, interleukin (IL-2), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) which were significantly higher than the mice received only the empty vector (Table 1, Figure 5a, 5b, 5f, 5h). Besides, in the vaccinated BALB/c mice, also significantly higher levels of Th2 type cytokines IL-4, IL-5 and IL-10 were observed compared to the mice received only the empty vector (Table 1, Figure 5c, 5d, 5e). On the other hand, IFN/α/βR-/- mice vaccinated with pGc responded with increased levels of the Th1 type cytokines IFN-γ and IL-6 which were significantly higher than the mice received only the empty vector (Table 2, Figure 6a, 6f). Also, in the vaccinated mice, significantly higher levels of Th2 type cytokines IL-4, IL-5 and IL-10 were observed compared to the mice received only the empty vector (Table 2, Figure 6c, 6d, 6e). In the light of these cytokine responses, it appears evident that our DNA vaccine vector elicited a balanced Th1/Th2 cellular response in both immunocompetent BALB/c and IFN/α/βR-/- mice.

Protective Efficacy of the pGc Mediated Immunization

IFN/α/βR-/- mice (n=4 per group) were vaccinated with the pGc vaccine two times at a dose of 100 µg DNA per each vaccination two weeks apart. Two weeks after the last vaccination, mice were challenged with a lethal dose (100LD50) of CCHFV-Ank2. Despite the generation of Gc specific humoral and cellular immunity after the vaccination schedule, all mice were defeated by lethal disease between days 3 and 6 post-challenge (Figure 6).

DISCUSSION

CCHF is the most geographically widespread tick-borne viral zoonosis. Expansion of the endemic areas and emergence of new foci, together with the increasing number of cases put this disease to the forefront with regards to an urgent need for developing effective preventive measures in order to reduce its impact on public health. Apart from the fact that the effective treatment of CCHF inevitably requires developing specific therapeutic agents, vaccination appears to be the most plausible strategy for controlling the disease. Recent reports on the development and efficacy of experimental vaccines against CCHF reveal promising results (11-18). However, currently there is no widely accessible, internationally approved, safe and efficient vaccine for CCHF and thus, in the context of revealing the immunizing and protective aspects of different vaccination approaches, vaccine development against CCHF is at present an actively studied research subject.

In the present study, we generated a DNA vaccine candidate (pGc) encoding for the envelope glycoprotein Gc of a cell adapted local CCHFV isolate (Ank2) and evaluated its immunizing and protective properties in both immunocompetent BALB/c and IFN/α/βR-/- mice by undertaking the homologous prime-boost vaccination regime.

Table 1. Cytokine data summary obtained from BALB/c mice received pGC vaccine or empty vector

Groups	Min	Q1	Median	Q3	Max	Mean	SD	p
IFN-γ (Vaccination)	54.4	54.4	54.4	68.82	83.24	64.01	16.65	**0.043**
IFN-γ (Empty Vector)	19.99	19.99	19.99	23.41	26.84	22.27	3.95	
IL-2 (Vaccination)	19.37	19.37	23.35	28.70	32.82	24.72	6.57	**0.031**
IL-2 (Empty Vector)	4.07	7.41	10.75	10.75	10.75	8.52	3.85	
IL-4 (Vaccination)	18.24	19.64	20.11	22.88	31.19	22.41	5.91	**0.019**
IL-4 (Empty Vector)	3.51	6.21	10.47	13.82	13.82	9.56	5.12	
IL-5 (Vaccination)	23.44	23.44	31.45	39.46	39.46	31.45	9.24	**0.026**
IL-5 (Empty Vector)	8.53	8.53	9.24	9.96	9.96	9.24	0.82	
IL-10 (Vaccination)	87.67	87.67	99.57	133.18	198.29	121.27	52.55	**0.031**
IL-10 (Empty Vector)	11.41	11.41	17.10	14.25	17.10	13.30	3.28	
IL-6 (Vaccination)	21.59	21.59	25.85	33.59	44.03	29.33	10.59	**0.028**
IL-6 (Empty Vector)	6.21	6.21	6.85	8.94	13.27	8.29	3.37	
IL-13 (Vaccination)	8.85	8.85	11.35	13.85	13.85	11.35	2.88	0.300
IL-13 (Empty Vector)	2.20	2.20	5.62	9.05	9.05	5.62	3.95	
TNF-α (Vaccination)	35.17	35.17	41.93	52.34	63.3	45.58	13.42	**0.019**
TNF-α (Empty Vector)	3.64	9.58	15.58	19.61	19.61	13.60	7.65	

Min: minimum; Q1: first quartile; Q3: third quartile; Max: maximum; SD: standard deviation

Table 2. Cytokine data summary obtained from IFN/α/βR-/- mice received pGC vaccine or empty vector

Groups	Min	Q1	Median	Q3	Max	Mean	SD	p
IFN-γ (Vaccination)	835.78	896.23	1288.03	1659.68	1659.68	1267.88	453.60	**0.027**
IFN-γ (Empty Vector)	722.34	722.34	722.32	822.30	822.30	772.32	57.71	
IL-2 (Vaccination)	11.98	11.98	13.68	15.38	15.38	13.68	1.96	0.659
IL-2 (Empty Vector)	5.50	11.38	13.34	13.49	13.94	11.53	4.029	
IL-4 (Vaccination)	64.22	64.80	65.00	76.46	110.86	76.27	23.06	**0.027**
IL-4 (Empty Vector)	49.20	49.20	52.99	56.78	56.78	52.99	4.37	
IL-5 (Vaccination)	23.36	25.82	30.49	34.34	34.34	29.67	5.55	**0.028**
IL-5 (Empty Vector)	11.56	11.95	12.09	14.56	22.00	14.43	5.049	
IL-10 (Vaccination)	157.90	157.90	181.75	205.60	205.60	181.75	27.53	**0.026**
IL-10 (Empty Vector)	91.66	91.66	96.34	101.02	101.02	96.34	5.40	
IL-6 (Vaccination)	14.64	14.64	15.46	16.28	16.28	15.46	0.94	**0.026**
IL-6 (Empty Vector)	12.40	12.40	12.76	13.13	13.13	12.76	0.42	
IL-13 (Vaccination)	77.02	91.48	145.03	193.76	193.76	140.21	62.33	0.663
IL-13 (Empty Vector)	73.77	106.78	118.42	128.33	156.16	116.69	33.69	
TNF-α (Vaccination)	31.56	32.49	36.61	40.42	40.42	36.30	4.78	0.105
TNF-α (Empty Vector)	30.00	30.00	31.30	32.61	32.61	31.30	1.50	

Min: minimum; Q1: first quartile; Q3: third quartile; Max: maximum; SD: standard deviation

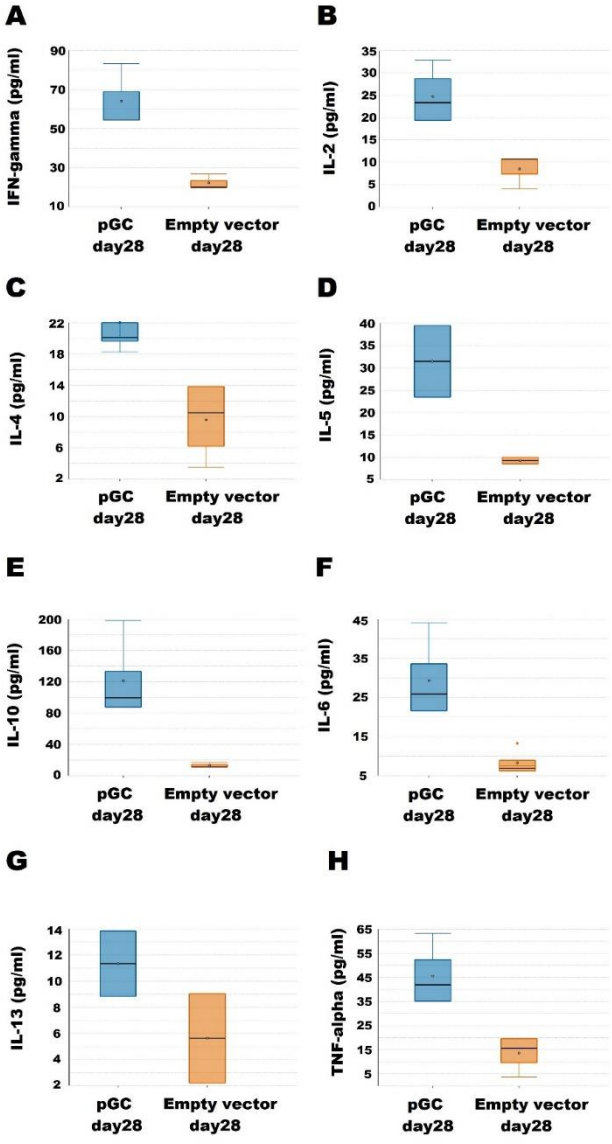

Figure 5. The Th1/Th2 cytokine levels in the sera of BALB/c mice vaccinated with the pGc vector: Levels of **a)** IFN-γ, **b)** IL-2, **c)** IL-4, **d)** IL-5, **e)** IL-10, **f)** IL-6, **g)** IL-13, and **h)** TNF-α

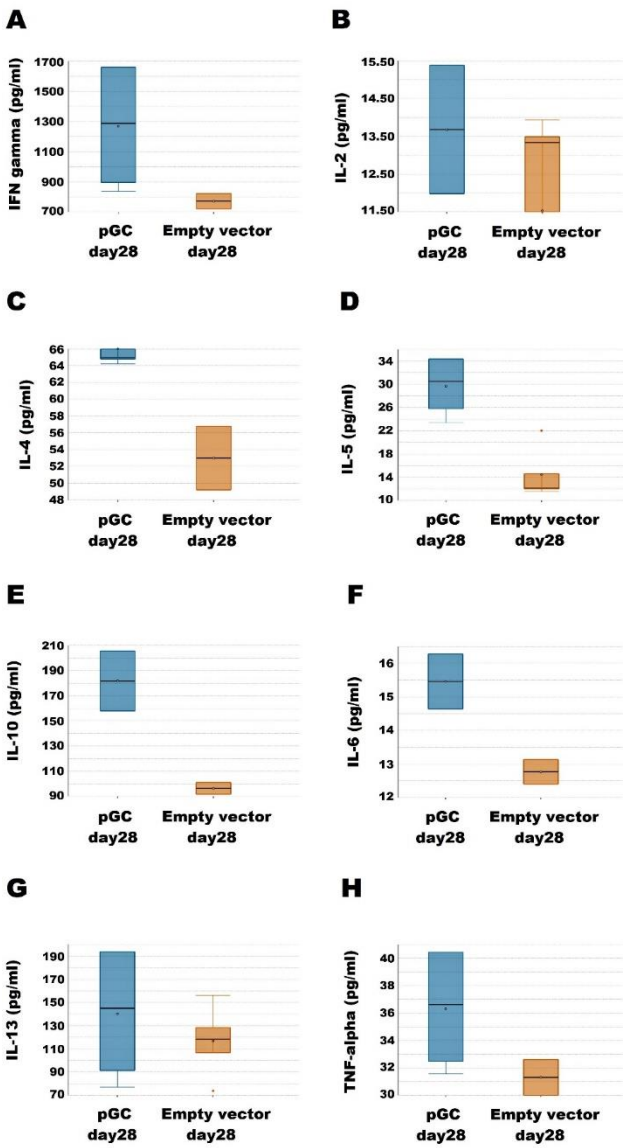

Figure 6. The Th1/Th2 cytokine levels in the sera of IFN/α/βR-/- mice vaccinated with the pGc vector: Levels of **a)** IFN-γ, **b)** IL-2, **c)** IL-4, **d)** IL-5, **e)** IL-10, **f)** IL-6, **g)** IL-13, and **h)** TNF-α

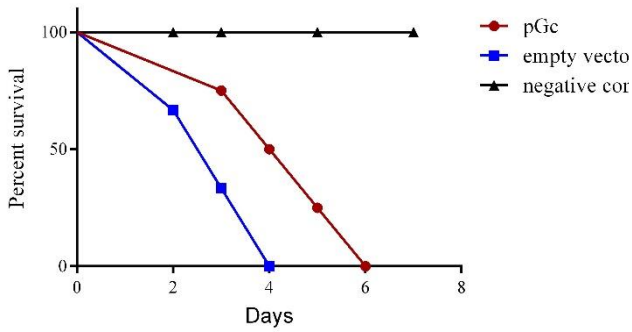

Figure 7. Efficacy of pGc vaccine in IFN/α/βR-/- mice challenged with CCHFV (Isolate Ank2): IFN/α/βR-/- mice were challenged with 100LD50 doses of CCHFV 14 days after the second vaccination with pGc (solid circles) and empty vector (solid squares). The control group received only saline solution was shown with solid triangles

For the immunogenicity of the pGc vaccine, we examined the antibody and cell-mediated immune responses in the immunocompetent BALB/c mouse model and got the similar results with those obtained in IFN/α/βR-/- mice (Figure 4, 5 and 6). Other researchers have also reported similar findings in studies with CCHFV viral vaccines expressing nucleoprotein (27).

In regards to the cytokine responses, mice received two consecutive doses of pGc yielded a cellular response with increased levels of both Th1 type and Th2 type cytokines which were evident from the cytokine profiles of the serum samples in both rodent models. Generation of a Th1 biased or balanced Th1/Th2 responses may be dependent on the antigen type used in the immunization process (28). For instance, studies on the development of DNA vaccines against respiratory syncytial virus (RSV) revealed that while DNA vaccines targeting the fusion (F) glycoprotein of RSV elicit a Th1 biased cellular responses, DNA vaccine constructs designed to express attachment (G) glycoprotein of RSV yield a balanced Th1/Th2 cellular responses in vaccinated mouse models (29,30). The protective aspects of the balanced Th1/Th2 responses primed by DNA based vaccines targeting different pathogen antigens were demonstrated in various *in vivo* infection settings (31-33). Thus it appears that the fail of pGc vaccine vector in conferring protection against lethal infection could not be solely attributed to the generation of balanced Th1/Th2 responses.

Two consecutive vaccinations of both BALB/c and IFN/α/βR-/- mice with pGc vector were found to elicit considerable amount of neutralizing antibody response, which was inferred from the micro virus neutralization assay. However, while the display of the glycoproteins on the surface of virions makes them a convenient target for the induction of neutralizing antibodies, antibody-mediated virus neutralization observed *in vitro* does not always correlate with the *in vivo* protection of antibodies specific to CCHFV (34). Nevertheless, the prolonged survival observed in some individuals of the vaccinated group in comparison to empty vector group might be a consequence of the presence of neutralizing antibodies.

Different than wild type mice, IFN/α/βR-/- mice mostly do not generate swift responses to infection and, furthermore, CCHFV infections create higher viral loads in IFN/α/βR-/- mice compare to the wild type mice (35). Increased obstacle in protecting IFN/α/βR-/- mice from infection appears associated with the deficiency in the immune responses, which is most likely caused by the insufficient cross-presentation of antigens by dendritic cells (36). Therefore, efficacy studies of vaccines in this disease model can be quite difficult as vaccine candidates must generate convenient immune responses by overcoming the IFN/α/βR-/- weakened antigen presentation in order to elicit protection. Furthermore, protective vaccines must trigger an effective adaptive immune response that can compensate the deprivation of the type I interferon mediated antiviral state activation in this disease model. Thus, IFN/α/βR-/- mice should be regarded as a higher bar to cross for efficacy studies of vaccines than the immunocompetent mouse models (18).

CCHFV vaccine development gets further difficult due to the scarcity of knowledge on involvement of both B and T cell epitopes for the establishment of an effective immune response; and the types of immune responses required for the disease protection. The virus like particles (VLP), modified vaccinia virus Ankara (MVA) and plasmid DNA vaccine platforms have exhibited success in protecting murine models from lethal CCHFV challenge, and the yielded protection was shown to be dependent on both arms of the adaptive immunity (13,15-17,27). Furthermore, all these platforms postulates that antibody and/or T cell mediated immune responses developed against the CCHFV glycoprotein precursor are crucial for protection in murine models (13-17,27,37).

Should the vaccine have exhibited any degree of protection, further work would have been done, for instance, by using a codon-optimized version of the pGc vaccine and/or electroporation assisted DNA vaccine delivery, which at least in part might have played a role in the better expression of the antigen *in vivo*. Besides, co-delivery of a relevant adjuvant and the pGc vaccine might have also improved the adaptive immune responses and consequently elicited a protective effect via the stimulation of the innate immunity. Although vaccination approaches that focus on a single antigen could be successful, an elegant vaccine candidate may effectively stimulate immune responses against multiple antigens and give protection with few doses. To this end, the pGc vaccine would be coupled with DNA vaccines or with other vaccine platforms that target CCHFV antigens different than Gc.

CONCLUSION

To the best of our knowledge, this is the first report of a DNA vaccine intended to target CCHFV based on the glycoprotein Gc. The pGc vaccine candidate exhibited antigen-specific immunity in IFN/α/βR-/- mice, but failed to confer a protection upon lethal challenge with the homologous CCHF virus. Once we gain more insight into the immune correlates of protection, the better we will have the chances to significantly improve the efficacy of vaccines.

Ethics Committee Approval: The study was approved by the Local Ethics Committee for Animal Experiments of Ankara University (17.12.2014, 23/155).

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