Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of Crimean-Congo hemorrhagic fever virus

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Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus Nairovirus of the family Bunyaviridae, causes severe disease in humans with high rates of mortality. The virus has a tripartite genome composed of a small (S), a medium (M) and a large (L) RNA segment; the M segment encodes the two viral glycoproteins, GN and GC. Whilst relatively few full-length M segment sequences are available, it is apparent that both GN and GC may exhibit significant sequence diversity. It is unknown whether considerable antigenic differences exist between divergent CCHFV strains, or whether there are conserved neutralizing epitopes. The M segments derived from viral isolates of a human case of CCHF in South Africa (SPU 41/84), an infected tick (Hyalomma marginatum) in South Africa (SPU 128/81), a human case in Congo (UG 3010), an infected individual in Uzbekistan (U2-2-002) and an infected tick (Hyalomma asiaticum) in China (Hy13) were sequenced fully, and the glycoproteins were expressed. These novel sequences showed high variability in the N-terminal region of GN and more modest differences in the remainder of GN and in GC. Phylogenetic analyses placed these newly identified strains in three of the four previously described M segment groups. Studies with a panel of mAbs specific to GN and GC indicated that there were significant antigenic differences between the M segment groups, although several neutralizing epitopes in both GN and GC were conserved among all strains examined. Thus, the genetic diversity exhibited by CCHFV strains results in significant antigenic differences that will need to be taken into consideration for vaccine development.

INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV) causes a haemorrhagic disease in humans with mortality rates that range from 10 to 80 % (Whitehouse, 2004). CCHFV can be isolated from ticks, livestock and humans (Whitehouse, 2004). Infection can occur through the bite of an infected tick, exposure to tissue and fluids from an infected animal or through contact with infected human bodily fluids. CCHFV infection was first described during an outbreak in Russia during the 1940s, when more than 200 cases of severe haemorrhagic fever were reported among agricultural workers and soldiers in the Crimean peninsula (Chumakov et al., 1968, 1970). Since then, the virus has spread or has been recognized throughout many regions of the world, including sub-Saharan Africa (Williams et al., 2000; Wood et al., 1978), Bulgaria, the Arabian Peninsula, Iraq, Pakistan, the former Yugoslavia, northern Greece and north-west China (Chumakov et al., 1970; Hoogstraal, 1979; Olaleye et al., 1996; Onishchenko et al., 2000, 2001a, b).

CCHFV is a member of the genus Nairovirus within the family Bunyaviridae (Schmaljohn, 1996). Members of this enveloped virus family have a tripartite, single-stranded RNA genome of negative polarity. The small segment (S) encodes the viral nucleocapsid, the medium segment (M) encodes the two glycoproteins, GN and GC, and the large segment (L) encodes an RNA-dependent RNA polymerase. The viral glycoproteins, like those of other members of the family Bunyaviridae, are synthesized as a polyprotein...
precursor (Schmaljohn, 1996) that undergoes proteolytic cleavage events to yield the mature glycoproteins (Vincent et al., 2003). The G\textsubscript{N} precursor protein (Pre-G\textsubscript{N}) contains an N-terminal domain with a high proportion of Ser, Thr and Pro residues. This region resembles the mucin-like domain present in the glycoproteins of other viruses, most notably the Ebola virus glycoprotein (Simmons et al., 2002).

The G\textsubscript{N} and G\textsubscript{C} glycoproteins of CCHFV probably influence the host range, cell tropism and pathogenicity of this vertebrate and tick virus, and are the targets for neutralizing antibodies. Studies thus far indicate that portions of G\textsubscript{N} are highly variable compared with other regions of G\textsubscript{N} and with G\textsubscript{C} (Chinikar et al., 2004; Hewson et al., 2004a, b; Morikawa et al., 2002; Papa et al., 2002). However, there is limited sequence information available on CCHFV isolates from regions outside China and the former Soviet Union (Chinikar et al., 2004; Hewson et al., 2004a, b; Morikawa et al., 2002; Papa et al., 2002). We previously described the first neutralizing mAbs to CCHFV (Bertolotti-Ciarlet et al., 2005). In addition, some of these antibodies were shown to be protective in a suckling mouse animal model (Bertolotti-Ciarlet et al., 2005). However, it is not clear whether significant antigenic differences exist between divergent CCHFV isolates or whether conserved neutralizing epitopes are present. This information is important for vaccine development, as the identification of conserved neutralizing epitopes may lead to the development of vaccines and entry inhibitors.

To further characterize the genetic diversity of the CCHFV M segment, we cloned and expressed glycoproteins from divergent CCHFV strains that were passaged a limited number of times. Additionally, to assess antigenic differences between CCHFV isolates, we cloned and fully sequenced the open reading frames from five CCHFV isolates obtained from humans or ticks in South Africa, Congo, Uzbekistan and China. Phylogenetic analyses determined the genetic proximity of strains and their antigenic differences between CCHFV isolates, we cloned and expressed glycoproteins from divergent CCHFV strains, or in some way focus the immune response on conserved neutralizing epitopes.

**METHODS**

**Virus strains and cells.** African green monkey kidney fibroblast (CV-1), Vero, Vero E6, human cervix carcinoma (HeLa) and human embryonic kidney (HEK-293T) cells, obtained from the ATCC (Manassas, VA, USA), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Similarly, the human tumour cell line SW-13 (adenocortical carcinoma) was grown in DMEM supplemented with 2.5% FBS. CCHFV strains Hy13, U2-2-002, SPU 41/84, SPU 128/81, SPU 94/87 and UG 3010 were used in this study. All of the viruses were passaged by intracerebral inoculation of 1-day-old mice with each CCHFV isolate, using a dose resulting in the death of 50% of the mice. The mice were killed 24 h post-infection and the brains were harvested. Brains were homogenized to 10% (w/v) with Hanks’ salt solution and clarified by centrifugation at 10,000 r.p.m. in an SW41 rotor for 30 min. CCHFV prototype strain IbAr10200, first isolated in 1976 from ticks (Haemoproteus excavatum) from Sokoto, Nigeria, was grown in African green monkey kidney Vero or Vero E6 cells (Sanchez et al., 2002). Republic of South Africa CCHFV strain SPU 41/84 was isolated from an infected human in 1984 and passaged in suckling mice four times. Republic of South Africa CCHFV strain SPU 128/81 was isolated in 1981 from infected ticks (Haemoproteus marginatum rufipes) and passaged in suckling mice twice. Congolese strain UG 3010 was isolated in 1956. This was one of the first ‘Congo’ strains isolated (Simpson et al., 1967; Woodall et al., 1967). Chinese strain Hy13 was isolated from infected ticks (H. asiaticum) in 1968 and was passaged in suckling mice three times. CCHFV strain U2-2-002/U-6415 from Uzbekistan was isolated from an infected human and passed in suckling mice four times. All work with replication-competent CCHFV was conducted in a biosafety level 4 facility at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).

**RNA purification, RT-PCR and sequencing.** Consensus primers were designed based on an alignment of known full-length M segment sequences available in GenBank. In order to amplify the 5' half of the M segment from each strain, primers CCHF 5' (5'-TCTCAAGAAAGACGGCCGC-3') and CCHF 3519 R (5'-GTACTCTGAGACGTRGARTCAT-3') were designed. CCHF 3235 F (5'-AATGCAATAGAYGCTGARATGCA-3') and CCHF 3519 R (5'-TCTCAAGAWATAGTGCGGAGCCACGCTTCT-3') were designed to amplify the 3' half of the M segment for each strain. Wobble code includes R=A or G, Y=C or T and W=A or T. The two designed amplicons share 1 kb overlapping sequence at the centre of the M segment. This strategy of amplification of the M segment in two halves was utilized for most of the strains. Total RNA was isolated from lyses of SW-13 cells infected with the different CCHFV strains by utilizing TRizol LS (Invitrogen) and removed from bio-contaminant. The samples were chloroform-extracted, followed by high-speed centrifugation and isolation of the resulting aqueous layer. RNA was precipitated by using propan-2-ol and pellets were resuspended in RNase-free distilled water. RNA was further purified through the RNaseasy system (Qiagen) according to the manufacturer’s directions.

Reverse transcription of the entire M RNA segment was performed by using 5 μl RNA from above, CCHF 3'R (300 ng) and 1 μl of a mixture of the four dNTPs (at μM each) in 12 μl. This mixture was heated to 65°C for 5 min and chilled rapidly on ice. Four microliters of 5× RT buffer, 2 μl 0·1 M dithiothreitol and RNAsin (40 U) were added to the mixture and heated to 42°C for 2 min. Then, 1 μl Superscript II (Invitrogen) reverse transcriptase (RT, 200 U) was added to the reaction mixture and incubated at 42°C for 1 h. The resultant cDNA generated from this reaction was used as a template in subsequent PCRs. PCR was performed by using 2 μl cDNA generated from the RT reaction, 5 μl CCHF primers (10 μM each), 5 μl 10 × PCR buffer, 1:5 μl dNTP mixture, 2 μl MgSO\textsubscript{4} (50 μM) and 0·6 μl Hi-Fidelity polymerase (5 U) in a 50 μl reaction. PCR thermocycler conditions were used as recommended by the manufacturer with an annealing temperature of 45°C. When the consensus primer set was unable to generate a PCR product for one half of the M segment, a gene-specific internal primer was designed based on sequences from the half of the M segment that did yield a product. This was the case with UG 3010;
the 3’ half of the UG3010 M segment was amplified by using a gene-specific internal primer, 3370F (5’-TGAAACAGGCGGCAA-
AAAAATC-3’), in combination with the 3’ external consensus primer CCHF3’R. Resultant PCR products were TA-cloned into pCR4-TOPO
using the TOPO cloning for sequencing system (Invitrogen) according to
the manufacturer’s instructions. Recombinant clones were con-
ﬁrmed by sequencing in both directions. On average, three clones from
two PCRs were sequenced in both directions to generate a sequence for
each M segment half. By using the data from the 5’ and 3’ ends of each M
segment that shared a 1 kb overlap, the sequence of each strain’s
M segment was resolved. These two overlapping fragments were
utilized for cloning a full-length M segment into the expression vector
pcAGGS (Niwa et al., 1991). The sequences have been deposited in
GenBank (accession numbers AY900141–AY900145).

Mapping of mAb 11E7. In order to map the epitope recognized by
mAb 11E7, we constructed expression plasmids that represent frag-
ments of the Gc–ectodomain. Primers were synthesized according to
the published sequence for strain IbAr10200 (Sanchez et al., 2002)
and standard PCR technology was performed to clone the ampiclons
into the pcDNA3.1/D/V5-His-TOPO vector (Invitrogen). The 5’
primers included the CACC sequence at the 5’ end and the start codon
to allow for directional cloning. The 3’ primers did not possess a
stop codon to allow the inclusion of the V5 cassette and polyhistidine
epitope tags at the C terminus of the protein. Cloning was performed
as described by the manufacturer (Invitrogen) and all constructs
were sequenced. All primer sequences are available upon request.

Protein analysis. To analyse protein expression, HEK-293T cells
were infected with recombinant vaccinia virus vTFI.1 expressing T7
polymerase (Alexander et al., 1992) and transfected 40 min later by
using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection,
cell extracts were prepared in 50 mM Tris/HCl (pH 7.4), 1 %
Triton X-100 and Complete Protease Inhibitor cocktail
(Roche Applied Sciences). Cell lysates were incubated at 4
°C for 3 min and then centrifuged at 10,000 g for 10 min. The supernatant
was mixed with sample buffer [0·08 M Tris/HCl (pH 6·8), 2 %
SDS, 10 % glycerol, 5 % β-mercaptoethanol, 0·005 % bromophenol blue]
and incubated at 56 °C for 10 min before electrophoresis in a
 Criterion SDS-PAGE. Western blot analysis was performed by using mouse anti-V5 (Invitrogen) or
mAb 11E7 as primary antibodies and sheep anti-mouse horseradish
peroxidase-conjugated secondary antibody (Amersham Biosciences)
followed by visualization with ECL-Plus Western blotting detection
reagents (Bioscience). In the case of Western blotting developed
with mAb 11E7, samples were not treated with β-mercaptoethanol.

Immunofluorescence (IF) microscopy. To determine whether
there were antigenic differences among glycoproteins from different
CCHFV strains and to characterize their localization within cells, we
performed indirect IF microscopy as described previously (Morais
et al., 2003). HeLa cells grown to 50 % conﬂuence on glass coverslips
were transfected with the different pcAGGS plasmids containing the
CCHFV M segments. At 24 h post-transfection, the cells were ﬁxed
with 2 % (v/v) formaldehyde in PBS, permeabilized with 0·5 %
Triton X-100 and stained with ascites containing a Gc– or Gc–
speciﬁc mAb, diluted 1:250 in PBS containing 0·5 % MgCl2 and
4 % FBS. Then, cells were washed with PBS and incubated for 1 h
with the secondary antibody conjugated to Alexa Fluor 488 (goat anti-
mouse) (Molecular Probes) diluted 1:500 in PBS containing 4 %
FBS. Finally, cells were washed in PBS, mounted with Fluoromount-
G (Southern Biotechnology Associates) and examined on a Nikon
E600 microscope at ×60 magniﬁcation utilizing UV illumination.

Sequence analysis. We studied the relationships between the
newly sequenced CCHFV M segments and previously published full-length isolates. The sequence alignments were produced by
using CLUSTAL_X (Thompson et al., 1997) and checked manually for
accuracy. The phylogenetic trees were drawn by using the PHYLIP
package version 3.57c (Felsenstein, 1997). Briefly, the trees were
obtained by using distance methods; SEQBOOT was used to obtain
1000 bootstrap replications of the original sequence alignment. The
bootstrapped alignments were used for construction of a consensus
tree with NEIGHBOR and CONSENSE as described in the package docu-
mentation. Distance between species shown in Fig. 1 was obtained
from the original alignment. Consensus trees were rooted with the
Dugbe strain, using TREEVIEW version 1.6.1 (Page, 1996).

RESULTS AND DISCUSSION

Cloning and expression of M segments from
diverse regions of the world

There is limited sequence information on CCHFV isolates
from regions outside China and the former Soviet Union,
with only one full-length M segment from an African strain
described previously (Chinikar et al., 2004; Hewson et al.,
2004a, b; Morikawa et al., 2002; Papa et al., 2002). It is not
known whether divergent CCHFV strains exhibit signiﬁ-

cant antigenic variability or share neutralizing epitopes –
information that is important for vaccine development. In
addition, only the glycoproteins of the extensively passed
IbAr10200 and Matin strains have been well characterized
with regard to processing and cellular localization (Sanchez
et al., 2002). To deﬁne the genetic and antigenic diversity
of geographically diverse CCHFV strains, we cloned, sequenced
and expressed the M segments from five isolates. Congolese
strain UG3010 was isolated in 1956 from a physician who
became ill after handling blood taken from an infected boy
at the Kisangani Hospital (Simpson et al., 1967; Woodall
et al., 1967). Republic of South Africa CCHFV strain SPU
41/84 was isolated from a patient in South Africa in 1984
(Blackburn et al., 1987), whilst Republic of South Africa
strain SPU 128/81 was isolated from H. marginatum ticks
(Shepherd et al., 1985). Chinese strain Hy13 was isolated from
H. asiaticum ticks in Xinjiang, China, and Uzbekistan
strain U2-2-002/U-6415 was isolated from an infected
human. The viruses were passaged in suckling mice for
between three and 11 times, as described in Methods.

M segment phylogeny

Hewson et al. (2004b) thoroughly described CCHFV
phylogeny, revealing the existence of four M segment
groups termed M1, M2, M3 and M4. We found that
Chinese strain Hy13 clustered with group M1, along with several
other Chinese strains and Pakistan strain Matin (Fig. 1a).
South African strains SPU 41/84 and SPU 128/81 and
Uzbekistan strain U2-2-002 clustered with group M2, along
with previously described strains from China, Uzbekistan,
Pakistan, Iraq and Nigeria (Hewson et al., 2004b; Morikawa
et al., 2002; Sanchez et al., 2002). Congo strain UG3010
clustered with group M3, which contains two previously
described Chinese strains (Fig. 1a) (Morikawa et al., 2002).
As noted previously, whilst there is some geographical
clustering of CCHFV strains, there are also examples of
geographically distant but genetically closely related virus
isolates, perhaps reflecting trade in livestock or dispersal of infected ticks by migratory birds (Hewson et al., 2004b).

We repeated the phylogenetic analysis of the strains using different regions of the M segment (the mucin-like domain or P35 domains of G_N, G_N lacking these domains, and G_C). The same phylogenetic tree was obtained in all cases (data not shown), even when only the highly variable mucin-like domain was used (Fig. 1b). This indicates that sequencing only a small portion of the M segment should make it possible to categorize new CCHFV isolates accurately.

**Pairwise analysis of M segments sequences**

The five completed M segment sequences had lengths ranging from 1684 to 1699 aa. The CCHFV glycoprotein precursor has been described to contain 78–80 cysteine residues on average, suggesting the presence of an exceptionally large number of disulfide bonds and a complex secondary structure. Cysteine residues were highly conserved, as were the sequences at the predicted proteolytic cleavage sites that have been described previously (Vincent et al., 2003). The number of potential N-linked glycosylation sites ranged from nine to 14. The M segments of the newly described strains were aligned with published sequences by using the CLUSTAL_X program (Jeanmougin et al., 1998; Thompson et al., 1997), and an identity matrix was constructed by using the program BioEdit (Tippmann, 2004). The G_N precursor protein (Pre-G_N) contains a highly variable domain at its N terminus that contains a high proportion of serine, threonine and proline residues, and it is predicted to be heavily O-glycosylated, thus resembling a mucin-like domain (Table 1) (Hewson et al., 2004a, b; Morikawa et al., 2002; Sanchez et al., 2002). When the identity values for the M segments were calculated based only on the mucin domain, the M1, M2, M3 and M4 strains were clearly distinct, consistent with the phylogenetic analyses (Table 1). When the same type of comparison was performed by using the full-length sequences or other portions of G_N or G_C, distinctions between the subgroups were not as obvious (data not shown), although the M3

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**Fig. 1.** Phylogenetic trees showing the relationships between CCHFV M segments. The bootstrap values shown are percentages of 1000 replications of the original dataset. All sequences were retrieved from GenBank. Strains marked in bold were sequenced as part of this study (GenBank accession numbers AY900141–AY900145). (a) Phylogenetic tree constructed by utilizing the full-length M segment sequence. The branch length for the Dugbe sequence (outgroup) was cropped for presentation purposes. The small tree at the bottom left of the figure shows the correct branch-length relationship between Dugbe and the remaining sequences. (b) Phylogenetic tree constructed with only the mucin-like domain sequence. Bars, 0–1 substitution per base position.
Table 1. Complete M segment deduced amino acid identities of CCHFV virus strains

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**Full-length sequence**

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group was the best defined and differentiated of the four subgroups (Table 1).

**Antigenic analysis of G\textsubscript{N} and G\textsubscript{C}**

Antigenic variation of arboviruses is of relevance because it may provide clues on the possible directions of epidemics or endemic spread. Little is known about antigenic relationships among CCHFV strains, in part because of the lack of adequate reagents. Early studies have shown that strains from diverse parts of the world have close antigenic relationships (Tignor et al., 1980). However, these studies were performed by utilizing polyclonal serum obtained from animals inoculated with infected mouse-brain tissue, which usually results mainly in antibodies directed against the nucleocapsid (Blackburn et al., 1987). Indeed, with the exception of a recent report from our laboratory (Bertolotti-Ciarlet et al., 2005), the CCHFV mAbs described thus far are directed against the nucleocapsid (Blackburn et al., 1987). The viral glycoproteins might exhibit a degree of higher antigenic variability than the nucleocapsid protein as a result of immune selection and the adaptation needed to efficiently bind to and enter diverse cell types. Therefore, we determined antigenic differences between G\textsubscript{N} and G\textsubscript{C} from different strains, utilizing a panel of eight mAbs to G\textsubscript{N} and nine mAbs to G\textsubscript{C} (Bertolotti-Ciarlet et al., 2005). These mAbs bind to conformation-dependent epitopes and so were characterized for their ability to recognize the different G\textsubscript{N} and G\textsubscript{C} proteins by IF microscopy utilizing constructs expressing only one of the glycoproteins (Bertolotti-Ciarlet et al., 2005). The G\textsubscript{N} and G\textsubscript{C} proteins from each of the five strains were recognized by a subset of the mAbs and were localized to both the endoplasmic reticulum and the Golgi, consistent with correct processing and transport (Table 2 and Fig. 2) (Andersson & Pettersson, 1998; Andersson et al., 1997a, b; Chen & Compans, 1991; Chen et al., 1991; Gerrard & Nichol, 2002). The Golgi localization was confirmed by IF microscopy using a marker for TGN46 (Serotec), a sheep antibody specific for a heavily glycosylated protein localized primarily in the trans-Golgi network (data not shown). The M segments from each of the five virus strains appeared to be expressed at similar levels, as they were all recognized well by mAb 11E7 (see Supplementary Fig. S1, available in JGV Online). In addition, by using a rabbit polyclonal serum, we were able to show that the G\textsubscript{N} glycoproteins from each of the five CCHFV strains were expressed and processed properly (see Supplementary Fig. S2, available in JGV Online). With regards to mAb reactivity, two of the M2 group strains (SPU 128/81 and U2-2-002) were virtually identical to IbAr10200, which itself is an M2 group strain. However, the closely related SPU 41/84 M2 strain was not recognized by two of the G\textsubscript{C} mAbs or by two of the G\textsubscript{N} mAbs (Table 2). On the other hand, the M1 group strain Hy13 was recognized by seven of the eight G\textsubscript{N} mAbs, but by only three of nine G\textsubscript{C} mAbs. The M3 strain UG 3010, which was genetically the most distantly related to IbAr10200, shared a high degree of antigenic similarity with this prototype CCHFV strain.

**Table 2. Reactivity of IbAr10200 mAbs with different CCHFV strains**

The characterization of neutralization and protection has been described previously (Bertolotti-Ciarlet et al., 2005). Neutralization is shown as the plaque-reduction neutralization titre (PRNT 80%) and protection data as the number of surviving mice compared with the total number of mice treated. +, Positive signal by IF; −, negative result. The identity of the antibodies was determined by IF analysis using constructs that contain G\textsubscript{N} or G\textsubscript{C} alone (Bertolotti-Ciarlet et al., 2005).

<table>
<thead>
<tr>
<th>Target</th>
<th>mAb</th>
<th>Neutralization</th>
<th>Protection (%)</th>
<th>Hy13</th>
<th>IbAr10200</th>
<th>U2-2-002</th>
<th>SPU 128/81</th>
<th>SPU 41/84</th>
<th>UG 3010</th>
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 Altogether, the mAbs exhibited eight different reactivity patterns, including some mAbs that recognized only M2 virus strains and others that recognized all strains tested. Of the seven mAbs known to neutralize IbAr10200 potently in vitro (Bertolotti-Ciarlet et al., 2005), only 11E7 bound to all six virus strains. Of the five mAbs described previously to be able to protect at least 70% of suckling mice challenged with IbAr10200 (Bertolotti-Ciarlet et al., 2005), 11E7 and 8F10 could bind to all six virus strains. It is important to note that, in each experiment, we used the parental IbAr10200 strain as a positive control (as it was recognized by all of the mAbs) and mock-transfected cells as a negative control (see Supplementary Fig. S3, available in JGV Online). These results suggest that there are significant antigenic differences between CCHFV strains that may not correlate well with genotypic or geographical characteristics. In addition, a number of epitopes to which neutralizing or protective mAbs can be directed are not highly conserved. However, at least one broadly cross-reactive, potently neutralizing mAb that can protect mice from a lethal CCHFV challenge (11E7) was identified.

Mapping of the 11E7 mAb epitope

As the neutralizing mAb 11E7 was able to recognize GC by Western blot under non-reducing conditions, we were able
to partially map its epitope by testing its ability to recognize fragments of Gc produced in HEK-293T cells. This is of relevance because mAb 11E7 protects mice in vivo from challenge with CCHFV strain IbAr10200 (Bertolotti-Ciarlet et al., 2005). Passive immunization can be effective for the treatment of CCHFV infection in humans, emphasizing the importance of identification of neutralizing antibodies and the epitopes to which they bind (Vassilenko et al., 1990).

We found that a Gc construct lacking the transmembrane and cytoplasmic domains was recognized by mAb 11E7 (Fig. 3). Therefore, we constructed three fragments that covered the length of the Gc ectodomain (C1, C2 and C3). All fragments contained a V5 epitope tag at the C terminus to allow detection of the fragment and to confirm their expression (Fig. 4). Most of the constructs, when expressed, formed SDS-resistant oligomers to some extent (Fig. 4). However, the relevance of this oligomerization is not clear, as the fragments represent only small portions of the protein and may therefore aggregate. Nonetheless, of these three fragments, only construct C3, located at the C terminus of the Gc ectodomain, was recognized by 11E7. Therefore, we focused our attention on this area, further dividing it into three new fragments (C3A, C3B and C3C). The antibody recognized none of these fragments. Next, we decided to divide the C3 fragment into two overlapping regions (C3.1 and C3.2); however, this resulted in disruption of the 11E7 epitope (Figs 3 and 4). Therefore, we performed a small deletion within the C3 C terminus (C3-T1). The antibody recognized this construct. Additionally, a small deletion of the N terminus of the C3 region also yielded a fragment recognized by mAb 11E7 (C3-T2) (Figs 3 and 4). Therefore, we conclude that the neutralizing epitope of mAb 11E7 is contained between aa 1443 and 1566 of the M segment of IbAr10200 strain, a highly conserved region of the protein (Figs 3 and 4).

**Conclusion**

In summary, we report the first description of CCHFV glycoprotein antigenic structure and relatedness, as well as initial mapping of a cross-reactive neutralizing epitope present on divergent CCHFV strains. CCHFV strains can exhibit considerable genetic variability, with the mucin-like domain in Gn in particular being highly divergent. We also found a considerable amount of antigenic variability, which may not follow phylogenetic groupings of CCHFV strains. Even the highly conserved Gc protein exhibited antigenic

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![Fig. 3. Mapping of the epitope recognized by neutralizing mAb 11E7. A schematic representation of the different IbAr10200 Gc fragments utilized to map the 11E7 epitope is shown. All of the constructs were expressed in mammalian cells and included a V5 epitope tag at the C terminus to control for expression. The numbers at the end of each construct represent the amino acid numbers based on the full-length IbAr10200 M segment.](image)

![Fig. 4. Western blot analyses for mapping of the mAb 11E7 epitope. Western blotting was performed by using lysates of HEK-293T cells transfected with some of the constructs shown in Fig. 3 and developed by using mAb 11E7 in parallel with a mAb for the V5 tag (Invitrogen). Some of the smaller fragments ran as both monomers and oligomers in SDS-PAGE. Molecular markers are shown in kDa (Prestained SDS-PAGE standards, broad range; Bio-Rad). GFP, Green fluorescent protein.](image)
variability, suggesting that CCHFV glycoproteins are subject to immune selection. Nonetheless, at least some epitopes to which neutralizing and/or protective antibodies bind are conserved between divergent CCHFV strains, and definition of these antibody-binding sites may be useful for vaccine design.

ACKNOWLEDGEMENTS

We thank Aura Garrison, Louis Altamura and Donald Pijak for expert technical assistance. This work was supported in part by training grant NIH T32 AI055400, Department of Defense Peer Reviewed Medical Research Program grant PRMRP PR033269 and R21-AI-063308.

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