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Novel neutralizing monoclonal antibodies protect rodents against lethal filovirus challenges

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ABSTRACT

Filoviruses are the causative agents of lethal hemorrhagic fever in human and non-human primates (NHP). The family of *Filoviridae* is composed of three genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. There are currently no approved vaccines or antiviral therapeutics for the treatment of filovirus infections in humans. Passive transfer of neutralizing antibodies targeting the Ebola virus (EBOV) glycoprotein (GP) has proven effective in protecting mice, guinea pigs and NHP from lethal challenges with EBOV. In this study, we generated two neutralizing monoclonal antibodies (MAbs), termed S9 and M4 that recognize the GP of EBOV or multiple strains of Marburg virus (MARV), respectively. We characterized the putative binding site of S9 as a linear epitope on the glycan cap of the GP_1 subunit of the EBOV-GP. The M4 antibody recognizes an unknown conformational epitope on MARV-GP. Additionally, we demonstrated the post-exposure protection potential of these antibodies in both the mouse and guinea pig models of filovirus infection. These data indicate that MAbs S9 and M4 would be good candidates for inclusion in an antibody cocktail for the treatment of filovirus infections.

1. Introduction

The family of *Filoviridae* is composed of three genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. The *Ebolavirus* genus can be subdivided into 5 distinct species: Zaire (EBOV), Sudan (SUDV), Tai Forest (TAFV), Reston (RESTV) and Bundibugyo (BDBV) viruses. The *Marburgvirus* genus comprises of a single species, *Marburg marburgvirus*, of which several strains have been reported, including its prototype virus Marburg virus (MARV) [1]. Infection with EBOV and MARV results in a rapidly fatal hemorrhagic fever in humans with reported case fatality rates of up to 90% [2,3]. Currently, there is no approved treatment for EBOV or MARV hemorrhagic fever beyond palliative care [4].

Attachment and entry of filoviruses into target cells is mediated by the viral glycoprotein (GP), which is the sole viral protein exposed on the virion surface [5]. The GP is post-translationally cleaved into two subunits, GP_1 and GP_2, both of which form a trimer of heterodimers. The filovirus GP is composed of a heavily glycosylated mucin domain and glycan cap region, which form a deep, heavily glycosylated, chalice-like structure that encloses the putative receptor-binding domain [6].

To date most research evaluating the potential of passive antibody transfer as a therapy for filovirus infections has focused on EBOV infections. Neutralizing antibodies that target the EBOV-GP have proven effective in protecting mice, guinea pigs and more recently non-human primates (NHP) from lethal EBOV challenge [7–12], while neutralizing monoclonal antibodies (MAbs) against MARV GP were shown to confer only partial protection in guinea pigs [13].

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In this study, we characterized 2 novel neutralizing MAbs against EBOV and MARV, and show that both MAbs protect against lethal EBOV or MARV challenge in mice and/or guinea pigs.

2. Materials and methods

2.1. Ethics statement

Approval for animal experiments was obtained from the Institutional Animal Care and Use Committees at Rocky Mountain Laboratories, DIR, NIAID, NIH and the University of Texas Medical Branch. Animal work was performed by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility. Animal housing, care and experimental protocols were in accordance with NIH guidelines.

2.2. Viruses and cells

Wildtype (WT) EBOV (strain Mayinga) was kindly provided by the Special Pathogens Branch of the Centers for Disease Control and Prevention, Atlanta, Georgia, United States, mouse adapted (MA)-EBOV (strain Mayinga) and guinea pig adapted (GPA-) EBOV (strain Mayinga) were kindly provided by United States Army Medical Research Institute of Infectious Diseases, Frederick, MD, US and WT MARV (strain Angola) and GPA-MARV (strain Angola) were kindly provided by Public Health Agency of Canada, Winnipeg, Canada. Generation of recombinant VSV expressing EBOV-GP and MARV-GP has been described previously [14]. All viruses were grown in Vero E6 cells in Dulbeccos modified eagles medium (DMEM) supplemented with 2% Fetal Bovine Serum (FBS) and antibiotics. Hybridoma cells were grown in DMEM supplemented with 20% FBS, antibiotics, L-glutamine, and 10 mM HEPES at 37 °C and 5% CO_2. All work was performed in a class II biological safety cabinet. All work with live EBOV or MARV was performed under biosafety level 4 conditions at the Integrated Research Facility, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana and the Galveston National Laboratory, University of Texas Medical Branch, Galveston, Texas.

2.3. Generation of antibody producing hybridomas

The neutralizing MAbs were generated through a project originally designed to induce cross-reactive MAbs. Therefore, groups of BALB/c mice were vaccinated with 3×10^5 plaque forming units (PFU) rVSV/EBOV-GP. Three weeks after vaccination, mice were boosted with 3×10^5 rVSV/SUDV-GP or rVSV/MARV-GP to induce a memory response against cross-reactive epitopes. Three days after boost the mice were sacrificed, spleens harvested, and hybridomas generated as described previously [15]. Briefly, mouse plasma cells were fused with SP2/0-Ag14 myeloma cells (ATCC). Hybridomas were selected using HAT/HT selection medium. Monoclonal hybridomas were isolated by two rounds of limiting dilutions in 96 well flat bottom tissue culture plates. Isolated hybridomas were screened for secreted antibodies using a soluble transmembrane deleted trimeric glycoprotein of EBOV, SUDV, RESTV, TAFV, BDBV and MARV via indirect ELISA as previously described [16]. Plasmids for soluble GP were kindly provided by Dr. Ayato Takada.

2.4. Monoclonal antibody purification

Antibody was purified from hybridoma cell culture supernatant by concentration using Amicon plus-70 30,000 MWCO centrifugal concentrators followed by protein A/G purification columns (Thermo Scientific) according to manufacturer's guidelines. Antibody was buffer exchanged into PBS using an Amicon 15 ml centrifugal spin concentrator. Purified antibody concentrations were determined by Bradford Protein Assay Reagent (Thermo Scientific).

2.5. Plaque reduction assay

MAbs were serially diluted twofold in DMEM and added to 100 plaque forming units (PFU) of rVSV/EBOV-GP, rVSV/MARV-GP, WT EBOV or WT MARV and incubated for 1 h at 37 °C. The MAb/virus mix was used to infect monolayers of VeroE6 cells for 1 h at 37 °C. Following adsorption, the inoculum was removed and the cells were overlaid with 0.8% agarose/MEM/10% FBS. Cells were incubated for two days with rVSV or 5–7 days or WT MARV or EBOV, respectively, after which cells were stained with crystal violet and plaques counted. Neutralization was calculated as the % reduction in plaques as compared to untreated virus.

2.6. Western blotting

Purified soluble EBOV- and MARV-GP samples were analyzed by SDS–PAGE using 10% acrylamide gel and transferred onto Hybond-P PVDF Membrane (GE Healthcare) utilizing a Bio-Rad, Trans-Blot SD Semi-Dry Transfer Cell following the manufacturer's instruction. The membrane was blocked with 5% non-fat milk PBS/0.1% tween overnight at 4 °C and incubated with 2 μg/ml S9 or M4 MAb diluted in 5% non-fat milk/PBS-T for 1 h. The membrane was then washed three times in PBS-T and incubated with anti-mouse HRP antibody (1:25000; Jackson Immunoresearch), for 1 h at room temperature. Following a final washing step the binding of antibody to the membrane was detected by ECL Plus Western Blotting Detection Reagent and Hyperfilm ECL (GE Healthcare, Amersham).

2.7. Escape mutants

Escape mutants were generated by incubating 10^5 $TCID_{50}$ rVSV/EBOV-GP or rVSV/MARV-GP with a sub-neutralizing amount (5 μg/ml) of MAb S9 or M4 for 1 h at 37 °C. The mixture was then inoculated onto a monolayer of Vero E6 cells for 1 h at 37 °C. Cultures were checked for CPE and viruses that escaped neutralization were then passaged two additional times in the presence of MAb until resistant viruses were obtained. Neutralization escape mutants were plaque purified in the presence of MAb, and RNA from six individual plaques was isolated using TRIzol Reagent (Life Technologies) according to the manufacturer's guidelines. The GP gene was amplified from the viral RNA using primers flanking this gene in the rVSV backbone by RT-PCR and sequenced.

2.8. Peptide epitopes

Overlapping synthetic pin peptides for the complete amino acid sequence of EBOV-GP (GenBank accession number NP_066246.1) were synthesized on pins as 15-mer peptides with a 10-mer overlap of each peptide (PepScan Presto). The pins were used to determine the linear binding epitope of antibodies. Briefly, pins were blocked with 1% Skim milk (Bio-Rad) + 1% Tween-20 (Fisher) in 0.01 M PBS in a Nunc round-bottom 96 well plate for 2 h at room temperature then washed in 0.9% w/v NaCl and 0.05% v/v Tween-20 in PBS (pH 7.2). Concentrated hybridoma supernatant for mAbs were diluted 1:1 in 0.1% Skim milk + 0.1% Tween-20 in 0.01 M PBS and added to a new flexible Falcon 96 well plate and the pins were incubated over night at 4 °C in the antibody solution. Next, the pins were washed as described above and an appropriate dilution of a goat anti-mouse IgG-HRP secondary antibody (Southern Biotech) in 0.1% Skim milk + 0.1% Tween-20 was incubated with the pins