Hendra virus (HeV) and Nipah virus (NiV) are deadly zoonotic Henipaviruses (HNVs) responsible for recurrent outbreaks in humans and domestic species of highly fatal (50 to 95%) disease. A HeV variant (HeV-g2) of unprecedented genetic divergence has been identified in two fatally diseased horses, and in two flying fox species in regions of Australia not previously considered at risk for HeV spillover. Given the HeV-g2 divergence from HeV while retaining equivalent pathogenicity and spillover potential, understanding receptor usage and antigenic properties is urgently required to guide One Health biosecurity. Here, we show that the HeV-g2 G glycoprotein shares a conserved receptor tropism with prototypic HeV and that a panel of monoclonal antibodies recognizing the G and F glycoproteins potently neutralizes HeV-g2– and HeV G/F–mediated entry into cells. We determined a crystal structure of the Fab fragment of the hAH1.3 antibody bound to the HeV G head domain, revealing an antigenic site associated with potent cross-neutralization of both HeV-g2 and HeV. Structure-guided formulation of a tetravalent monoclonal antibody (mAb) mixture, targeting four distinct G head antigenic sites, results in potent neutralization of HeV and HeV-g2 and delineates a path forward for implementing multivalent mAb combinations for postexposure treatment of HNV infections.

Hendra virus | Nipah virus | antibodies | variants | receptor

Hendra virus (HeV) and Nipah virus (NiV) are highly pathogenic viruses of the Henipavirus (HNV) genus in the Paramyxoviridae family, causing fatal diseases in various mammalian species including horses, pigs, and humans (1). HNVs are enveloped viruses and infect host cells through fusion of the viral and cell membranes. This process is mediated by the concerted action of two glycoproteins on the HNV surface known as G (attachment glycoprotein) and F (fusion glycoprotein). HNV G, also known as the receptor-binding protein, is homotetrameric with an N-terminal transmembrane domain followed by a stalk domain and a C-terminal head domain (2), the latter recognizing ephrin-B2 (EB2) and ephrin-B3 (EB3) as entry receptors (3–7). F is a homotrimmeric class I viral fusion protein, processed by host cathepsin L in the endosomal compartment via a recycling process (8–10). Both G and F are required for HNV infection and are the targets of the neutralizing humoral immune response (2, 11–14). Serum neutralizing antibodies against F and G are a correlate of protection in animals experimentally infected with NiV or HeV (15–18).

F and G are the basis for the design and development of candidate postexposure therapies and vaccines against HNVs (19). A licensed horse subunit vaccine (Equivac HeV) based on the HeV G protein ectodomain (sG) has been used in Australia since 2012 (20). A HeV sG-based human vaccine is also currently being evaluated in a phase 1 clinical trial (21). Several anti-F and anti-G monoclonal antibodies (mAbs) have been identified and shown to have potent neutralizing activity against both NiV and HeV (2, 11, 12, 14, 22–24). The G-specific m102.4 mAb has been administered to 16 individuals as an emergency postexposure therapy on a compassionate basis and has demonstrated desirable safety and immunogenicity properties in a phase 1 clinical trial (25).

A novel Hendra virus variant (HeV-g2) was detected in Australia in horses that succumbed to fatal HeV illness and in two species of flying foxes suffering vasculitis (26, 27). Despite frequent HeV testing in horses in areas with known viral circulation in wildlife, this new HeV-g2 escaped routine PCR-based testing due to much lower sequence conservation than ever detected compared with prototypic HeV. Furthermore, HeV-g2 was detected in regions of Australia previously thought to be at low risk of HeV cross-species transmission (26, 27). HeV-g2 F and G share 95.60 and 92.85% amino acid sequence identity with their counterparts in prototypic HeV, respectively.
Given the marked genetic divergence of HeV-g2 relative to HeV, it is unknown whether this newly discovered virus will share similar receptor usage and antigenic properties relative to prototypic HeV. Here, we set out to investigate the ability of HeV-g2 to utilize EB2 and EB3 as receptors for entry into cells and the likelihood that postexposure therapies and vaccines in development will be effective against this new variant. Here, we show that several HNV F- and G-specific mAbs cross-react with HeV-g2 glycoproteins and inhibit entry into target cells. We identify and characterize a mAb cross-neutralizing HeV and HeV-g2, designated hAH1.3, and determined its structure bound to the HeV G head domain, revealing recognition of an antigenic site distinct from those targeted by all other known HNV G-reactive mAbs. These data delineate a path forward to deploy mAb mixtures with a high barrier to the emergence of escape mutants.

Results

**HeV-g2 and HeV Share a Conserved Receptor Tropism.** Comparison of the HeV and HeV-g2 G sequences revealed that there are 33 residue substitutions between the two variants, most of them clustering within the receptor-binding G head domain (SI Appendix, Fig. S1). To understand the effect of these substitutions on receptor usage, we evaluated binding of purified monomeric HeV and HeV-g2 G head domains to immobilized EB2 and EB3 genetically fused to the human immunoglobulin 1 (IgG1) Fc fragment using biolayer interferometry (BLI). Despite the four residue differences between human EB2 (hEB2) and *Pteropus alecto* EB2 (PaEB2), both G head domain variants bound indistinguishably with monovalent binding affinities in the 0.5 to 1 nM range (Fig. 1 A, B, D, and E and SI Appendix, Fig. S2 A–D). The strictly conserved EB3 bound with comparable affinities to HeV and HeV-g2 G head domains, though affinities for EB3 were much lower than for EB2 (Fig. 1 A, C, F, and G and SI Appendix, Fig. S2 E–H). These findings suggest that HeV-g2 recognizes each of the two known human receptor subtypes with similar efficiency to HeV, consistent with the strict conservation of the receptor-binding interface. Furthermore, the small number of (largely conservative) mutations found between hEB2 and PaEB2 and the strict conservation of hEB3 and PaEB3 (SI Appendix, Fig. S2) concur with the indistinguishable binding properties observed here across orthologs, supporting the role of flying foxes as reservoir hosts for both variants of HeV (and NiV).

To investigate the ability of HeV-g2 to utilize hEB2 and hEB3 for cell entry, we generated a green fluorescent protein (GFP)-encoding, replication-competent, Cedar (henipavirus) chimera in which the native glycoproteins are substituted with the HeV-g2 F and G glycoproteins (rCedV-HeV-g2-GFP). Stable expression of hEB2 (HeLa-USU-EB2) or hEB3 (HeLa-USU-EB3) rendered HeLa-USU cells susceptible to rCedV-HeV-g2-GFP, as was the case for the rCedV-HeV-GFP positive control which harbors the prototypical HeV F and G glycoproteins.

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**Fig. 1.** HeV and HeV-g2 share a conserved receptor tropism. (A) Ribbon diagram of a HeV G head domain (gray) in complex with human EB2 (purple). Residues mutated between the HeV G and HeV-g2 G head domains are colored red. The rendering was made using PDB ID code 6PDL. (B and C) Ribbon diagram of human EB2 (B; PDB ID code 6PDL) and EB3 (C; PDB ID code 3D12). Residues that differ between human and *P. alecto* orthologs are colored red. (D–G) BLI binding analysis of 200 nm HeV (red) or HeV-g2 (orange) G head domain to immobilized human EB2-Fc (D), *P. alecto* EB2-Fc (E), human EB3-Fc (F), or *P. alecto* EB3-Fc (G). All ephrin-Fc fusions were immobilized on AHC biosensors. (H and J) EB2 and EB3 are entry receptors for rCedV-HeV-g2-GFP. (Scale bars, 50 μm.) (H and J, Insets) Zoomed-in regions within the yellow boxes. Bright-field, fluorescence, and merged images are shown (Left to Right).
We did not observe any significant differences in the percentage of GFP foci upon rCedV-HeV-g2-GFP infection of HeLa-USU-EB2 (91%) or HeLa-USU-EB3 (98%) cells compared with rCedV-HeV-GFP (set to 100%). No evidence of infection was observed in HeLa cells infected with either rCedV-HeV-g2-GFP or rCedV-HeV-GFP (SI Appendix, Fig. S3). Therefore, HeV-g2 retains the same receptor tropism as HeV and is expected to have a similar tissue tropism upon infection of humans and other animals.

Broadly Neutralizing HNV F-Specific mAbs Inhibit HeV-g2. We previously described and humanized a panel of F-specific mouse mAbs, designated 1F5, 5B3, and 12B2, binding to two distinct, prefusion-specific quaternary antigenic sites and broadly neutralizing NiV and HeV by blocking membrane fusion (11, 12). Therapeutic administration (up to several days after infection) of a humanized 5B3 derivative protected ferrets challenged with lethal doses of NiV or HeV (18), underscoring the importance of F-specific mAbs for treatment of NiV and HeV infections.

The HeV-g2 F glycoprotein differs from HeV F at 25 out of a total of 546 amino acid residues, with 10 substitutions mapping to the predicted signal peptide (residues 1 to 26) (SI Appendix, Fig. S1). To understand the impact on antigenicity of the sequence divergence across HeV-g2 and HeV F glycoproteins, we compared binding of the 1F5, 5B3, and 12B2 mAbs to prefusion-stabilized HeV-g2 and HeV F ectodomain trimers. All three mAbs cross-reacted with identical binding kinetics and affinities with the two variant virus F trimers, consistent with the mutations mapping outside of the antigenic sites recognized by these mAbs, except for the SHeV/TNiV81NHeV-g2 substitution, which is at the periphery of the 1F5/5B3 epitopes and does not interfere with binding (Fig. 2 A–F and G–I).

Fig. 2. Broadly neutralizing HNV F-specific mAbs inhibit HeV-g2. (A–C) Ribbon diagram of HeV F (PDB ID code 7KI6) ectodomain (gray) with the 1F5-interacting surface (blue), 5B3-interacting surface (red), or 12B2-interacting surface (green) highlighted. Residues that are mutated in HeV-g2 F relative to HeV F are colored red. A and B correspond to views along the plane of the viral membrane (F viewed from the side) whereas C corresponds to a view normal to and looking toward the viral membrane (F viewed from the top). (D–F) BLI binding analysis of 300 nm HeV (red) or HeV-g2 (orange) F ectodomain trimer to 1F5 IgG (D), 5B3 IgG (E), or 12B2 IgG (F) immobilized at the surface of AMC biosensors. (G–I) 1F5- (G), 5B3- (H), and 12B2-mediated (I) neutralization of rCedV-HeV-GFP (black) or rCedV-HeV-g2-GFP (blue). The limit of detection for the neutralization assay was 50 fluorescent foci. The differences of IC50 values for each mAb between rCedV-HeV-GFP and rCedV-HeV-g2-GFP were not statistically significant (Student’s t test) with P values of 0.110, 0.255, and 0.333 for 1F5, 5B3, and 12B2, respectively. Error bars: SD. Moreover, we observed Pearson correlations of 0.9732, 0.9718, and 0.9897 for the 1F5, 5B3, and 12B2 IC50 values, respectively, between rCedV-HeV-GFP and rCedV-HeV-g2-GFP with P values smaller than 0.0001.
Side-by-side comparison of mAb-mediated neutralization of rCedV-HeV-g2-GFP and rCedV-HeV-GFP showed that 1F5, 5B3, and 12B2 inhibited viral entry with virtually identical potencies against each of the two variants (Fig. 2 G–I and SI Appendix, Table S2). Our results demonstrate that these three F-specific broadly neutralizing HNV mAbs potently inhibit HeV-g2 and are resilient to the HeV and NiV genetic diversity sampled thus far.

**Broadly Neutralizing HNV G-Specific mAbs Inhibit HeV-g2.**

Several G-targeted neutralizing mAbs have been described and shown to protect against lethal HeV and NiV challenge in a postexposure setting (2, 13, 16, 17, 23, 24, 31). Here, we investigated whether three potent and noncompeting neutralizing mAbs, m102.4, HENV-32, and nAH1.3, can cross-react with HeV-g2 G and neutralize rCedV-HeV-g2-GFP. Only 1 out of 33 residues that are mutated between HeV-g2 and prototypic HeV maps to an epitope recognized by one of these mAbs: The conservative R201K substitution is found within the HENV-32 binding site and recapitulates the NiV G K201 residue, explaining that HENV-32 broadly neutralizes NiV, HeV, and HeV-g2 (23) (Fig. 3 A–C). Indeed, we found that m102.4 and HENV-32 bound to each variant indistinguishably whereas nAH1.3 recognized the two variants with roughly comparable efficiencies by BLI (Fig. 3 D–F). Enzyme-linked immunosorbent assays (ELISAs) also indicated that each mAb has comparable affinity for the HeV and NiV G head domains, including the neutralizing mAbs HENV-103 and HENV-117 (SI Appendix, Fig. S5) (24). The neutralization potency of m102.4, HENV-32, and nAH1.3 was comparable against each of the two viruses (rCedV-HeV-GFP and rCedV-HeV-g2-GFP) with nAH1.3 exhibiting an order of magnitude greater neutralizing activity.

![Fig. 3](https://doi.org/10.1073/pnas.2122769119)
than the other two mAbs (Fig. 3 G–J). These data show that broadly neutralizing HNV mAbs recognizing three distinct G head domain antigenic sites are potent inhibitors of the recently described HeV-g2 variant.

The hAH1.3 Neutralizing mAb Defines a HeV/HeV-g2 G Antigenic Site. The mouse hAH1.3 mAb was previously reported to potently neutralize HeV but not NiV (31). We determined a crystal structure of the hAH1.3 Fab fragment in complex with the HeV G head domain at 2.75-Å resolution (SI Appendix, Table S4). hAH1.3 interacts with an antigenic site located opposite of the head–head dimerization interface and thus of the HENV-32 epitope (23), which is also distinct from the receptor binding site (4, 6) or the nAH1.3 epitope (2) (Fig. 4A). As a result, hAH1.3 does not compete with the hEB2 receptor for binding to the HeV G head domain (as opposed to m102.4) and is unlikely to disturb the G head–dimerization interface (in contrast to HENV-32) (Fig. 4A and SI Appendix, Fig. S6). hAH1.3 binding does not induce major conformational changes of the HeV G head domain. All six hAH1.3 complementary determining regions participate in the paratope through shape complementarity and hydrogen bonding burying an excess of 1,000 Å² at the interface with HeV G and involving contacts with the HeV G polypeptide and N-linked oligosaccharides at positions N378, N481, and N529 (Fig. 4B and SI Appendix, Figs. S7 and S8). The sole residue substitution in the hAH1.3 epitope (H1386N, HeV-g2) does not prevent cross-reactivity with both HeV and HeV-g2 G head domains, as observed using BLI (Fig. 4 C and D and SI Appendix, Table S1 and Table S2). Accordingly, hAH1.3 inhibited with comparable potencies rCedV-HeV-GFP (50% inhibitory concentration [IC₅₀]: 25.47 ng/mL) and rCedV-HeV-g2-GFP (IC₅₀: 22.07 ng/mL) (Fig. 4E). Although the structure suggests that the effect of individual NiV G substitutions within the hAH1.3 epitope relative to HeV G (I385T, H386K, K388Q, and S483T) is expected to be moderate, the additive effect of all four mutations likely explains the lack of hAH1.3 cross-reactivity with NiV (Fig. 4 C and F and SI Appendix, Fig. S1).

Structure-Guided Formulation of a Tetravalent mAb Mixture. Superimposition of the m102.4-, HENV-32-, nAH1.3-, and hAH1.3-bound G structures indicated that the four mAbs target entirely distinct, nonoverlapping antigenic sites on HNV G (Fig. 5A). Using BLI, we confirmed that all four mAbs can simultaneously recognize the HeV or HeV-g2 G head domains, as observed using rCedV-HeV-GFP (black) or rCedV-HeV-g2-GFP (blue). The limit of detection for the neutralization assay was 50 fluorescent foci. The hAH1.3 IC₅₀ values were not statistically different (Student’s t test) between rCedV-HeV-GFP and rCedV-HeV-g2-GFP (P = 0.736). Moreover, we observed a Pearson correlation of 0.9974 for the hAH1.3 IC₅₀ values between rCedV-HeV-GFP and rCedV-HeV-g2-GFP with P values smaller than 0.0001. Error bars: SD. (D) Molecular surface representation of the HeV G head showing the hAH1.3 footprint colored by residue conservation between HeV G and HeV-g2 G. Semi-Conservative sub, semi-conservative substitution. (E) hAH1.3-mediated neutralization of rCedV-HeV-GFP (black) or rCedV-HeV-g2-GFP (blue). The limit of detection for the neutralization assay was 50 fluorescent foci. The hAH1.3 IC₅₀ values were not statistically different (Student’s t test) between rCedV-HeV-GFP and rCedV-HeV-g2-GFP (P = 0.736). Moreover, we observed a Pearson correlation of 0.9974 for the hAH1.3 IC₅₀ values between rCedV-HeV-GFP and rCedV-HeV-g2-GFP with P values smaller than 0.0001. Error bars: SD. (D) Molecular surface representation of the HeV G head showing the hAH1.3 epitope. Conservative sub, conservative substitution. Semi-Conservative sub, semi-conservative substitution. Semi conservative sub, semi-conservative substitution.

Discussion

Since its discovery in 1994, 65 spillovers of HeV to domestic horses have been detected in Australia with sporadic human...
exposure and disease. A new HEV variant, termed HEV-g2, was recently described from two independent discoveries: one from a retrospectively diagnosed horse succumbing to henipavirus disease in 2015, which enabled a second fatal equine case to be diagnosed in October 2021, and the other from a long-term effort to monitor and survey the flying fox population in different states of Australia (26). The HEV-g2 genome differs from that of the prototypic HEV sufficiently to have failed detection by real-time RT-PCR–based disease surveillance in horses and viral reservoir surveillance of flying foxes. The identification of HEV-g2 extends the known HNV genetic diversity and also highlights the broad geographical range of potential HEV spillover.

We show here that HEV-g2 retains the same receptor tropism as prototypic HEV and utilizes EB2 and EB3 as bona fide entry receptors. Moreover, a panel of nine mAbs targeting several epitopes on the HEV F or G glycoprotein cross-reacts and retains potent neutralizing activities against HEV-g2. We describe a mAb, designated hAH1.3, cross-reacting with HEV and HEV-g2 G by targeting an antigenic site which is conserved among HEV variants but not NiV. As the epitopes of the hAH1.3, m102.4, HENV-32, and nAH1.3 neutralizing mAbs are distinct, we formulated a tetravalent mAb mixture potently neutralizing HEV and HEV-g2 which is expected to have a much higher barrier for the emergence of escape mutants.

Fig. 5. G-targeted tetravalent mAb mixture and vaccine-elicited antibodies broadly neutralize HEV-g2. (A) hAH1.3 (blue), m102.3/m.102.4 (green), nAH1.3 (pink), and HENV-32 (red) mAbs recognize nonoverlapping epitopes on the HEV G head (gray). The HEV G head is rendered in the same orientation as in Fig. 4A, where the head–head dimerization interface faces to the right and the receptor-binding interface faces to the top. (B) BLI analysis of binding of m102.4, HENV-32, nAH1.3, and HAH1.3 IgG to the immobilized HEV G head showing the absence of competition among mAbs. The red trace shows the HEV head-I53-50A fusion–loaded anti–penta-His biosensor sequentially dipped into a solution containing 100 nm m102.4, followed by 100 nm m102.4 + 100 nm HENV-32, followed by 100 nm m102.4 + 100 nm HENV-32 + 100 nm nAH1.3, followed by 100 nm m102.4 + 100 nm HENV-32 + 100 nm nAH1.3 + 100 nm hAH1.3, and buffer alone. Controls with only 100 nm m102.4 IgG (orange), HENV-32 IgG (green), nAH1.3 IgG (cyan), and hAH1.3 IgG (blue) are shown for comparison. Negative sensor refers to an uncoated anti–penta-His biosensor dipped into 100 nm m102.4 + 100 nm HENV-32 + 100 nm nAH1.3 + 100 nm hAH1.3, and negative I53-50A refers to the HEV G head-I53-50A fusion–loaded anti–penta-His biosensor dipped into buffer alone. (C) Neutralization of rCedV-HEV-GFP (black) or rCedV-HEV-g2-GFP (blue) by the tetravalent m102.4/HENV-32/nAH1.3/hAH1.3 mAb mixture (1:1:1:1 molar ratio). (D) Study design for vaccination of rhesus macaques. Two animals were immunized three times (4 wk apart) with 200 μg of an alum-adjuvanted equimolar mixture of the purified NiV-B and NiV-M sG tetramers. Sera were collected on day 42 and day 84 postimmunization. (E) Day 84 NHP 171269 serum neutralizing activity against rCedV-HEV-GFP (black) and rCedV-HEV-g2-GFP (blue). (F) Day 84 NHP 180227 serum neutralizing activity against rCedV-HEV-GFP (black) and rCedV-HEV-g2-GFP (blue). The limit of detection for the neutralization assay was 50 fluorescent foci. Error bars: SD.
relative to individual constituting mAbs (2). Our data support the clinical development of multivalent mAb mixtures for post-exposure therapy as such countermeasures are expected to retain broad neutralizing activity against known and unknown NiV and HeV variants. NiV sG subunit vaccine-elicited neutralizing antibody responses are equivalently potent against HeV and HeV-g2, indicating that this newly emerged HeV-g2 variant does not erode cross-neutralizing polyclonal antibody responses more than HeV. We expect these findings to hold true in horses vaccinated with Equivac HeV and in humans, as the only HNV vaccine in clinical development is based on HeV sG which elicits better cross-protective titers than NiV sG against heterotypic challenge in animals (38). Collectively, our data provide a comprehensive assessment of available countermeasures against this newly described HeV-g2 variant and delineate multiple strategies for pandemic preparedness.

**Materials and Methods**

** Constructs.** The soluble ectodomain constructs of HeV F (whole genome from GenBank accession no. NC_001906.3; National Center for Biotechnology Information protein sequence NP_047111.2) and HeV-g2 F (GenBank accession no. UCY33669.1) were codon-optimized for a mammalian cell expression system, synthesized, and cloned into a pCDNA3.1+ vector by GenScript. These constructs include residues 1 to 487 followed by a GCNt trimerization system, synthesized, and cloned into a pCDNA3.1+ vector by GenScript. These constructs include residues 176 to 604 for HeV G and residues 176 to 602 for NiV G head domains as well as the corresponding human EB2 (GenBank accession no. P52799), human EB3 (GenBank accession no. NP_001397), and P. alecto EB3 (GenBank accession no. NP_001397) sequences. For NiV G head-domain constructs, residues 176 to 604 for HeV G were fused to the C terminus of the 16-GS His tag and to the C terminus of a GSGGGS linker at the very C terminus, where I53-50A has been fused to the I53-50A component with a His tag at the very C terminus, where I53-50A has been fused to the I53-50A component with a 16-GS linker in between and a GSGGGS linker at the C terminus.

The soluble Fc fusion constructs of HeV F (whole genome from GenBank accession no. AAC31913.2) and HeV-g2 F (GenBank accession no. UCY33670.1) were codon-optimized for a mammalian cell expression system, synthesized, and cloned into a pCDNA3.1+ vector by GenScript. These constructs include residues 176 to 604 for HeV G and residues 176 to 602 for NiV G head domains as well as the corresponding human EB2 (GenBank accession no. P52799), human EB3 (GenBank accession no. NP_001397), and P. alecto EB3 (GenBank accession no. NP_001397) sequences. For NiV G head-domain constructs, residues 176 to 604 for HeV G were fused to the C terminus of the 16-GS His tag and to the C terminus of a GSGGGS linker at the very C terminus, where I53-50A has been fused to the I53-50A component with a 16-GS linker in between and a GSGGGS linker at the C terminus.

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**Fabs.** The soluble Fab fragments were produced by transient transfection using the ExpiFectamine 293 Transfection Kit (Thermo Fisher) according to the manufacturer’s protocol. The Fab constructs were purifed with Protein A from a selected stable m102.4-producing hybridoma media with a protein A affinity column (31). Soluble m102.4 Fab was produced by fragmenting mouse m102.4 IgG using Pierce Mouse IgG1 Fab and Fab’2 Preparation Kits according to the manufacturer’s protocol. The HeV-G-m102.4 complex was obtained by mixing the two proteins in a 1:1.5 molar ratio and passed through a Superdex 200 column (Thermo Fisher). The fractions containing both proteins were collected and concentrated to 10 mg/mL in a buffer containing 20 mM Hepes (pH 7.2) and 10 mM NaCl.

**Generation of Fab Fragments from IgGs.** The 1FS, 12B2, S83, and nAH1.3 Fab constructs were obtained by fragmentation of mouse IgGs using Pierce Mouse IgG1 Fab and Fab’2 Preparation Kits according to the manufacturer’s protocol.
Assays were performed on an Octet RED (Fortebio) instrument at 25 °C with shaking at 1,000 rpm. All proteins were diluted in kinetics buffer (KB) (Sartorius) to the desired concentration and loaded on appropriate biosensors at 10 to 20 μg/mL. All sensors were hydrated in water for 10 min prior to each experiment. Fab murine IgGs (1F5, 12B2, and 5B3) were loaded on anti-mouse Fc capture (AMC) biosensors. G human IgGs (m102.4 and HENV-32) and ephrin-Fc fusion proteins were loaded on anti-human Fc capture (AHC) biosensors, and G murine IgGs (nAH1.3 and hAH1.3) were loaded on AMC biosensors. HeV-g2 G head-I53-50A was loaded on anti-penta-His (HISK1) biosensors. All experiments were performed in technical replicates.

For kinetic measurements of Fab binding to F proteins, trimeric HeV F or HeV-g2 F immobilized on Ni-NTA biosensors was dipped into solutions containing a threefold dilution series of Fab fragments at 300 to 1.23 nM.

For kinetic measurements of G mAb or ephrin-Fc binding to G, mAbs or ephrin-Fc fusion proteins immobilized on AHC biosensors were dipped into various concentrations of HeV/HeV-g2 G head domain in KB in a threefold dilution series either from 30 to 0.124 nM or 3 μM to 12.4 nM.

All kinetics parameters and statistics were estimated from ForteBio software using a 1:1 binding model. Mean kon and koff values were determined with a global fit applied to all data.

For evaluation of hAH1.3 cross-reactivity, HeV, HeV-g2, or NIV G head domains immobilized on Ni-NTA biosensors were dipped into solutions containing 100 nM hAH1.3 IgG.

For competition binding assays using hAH1.3 and EB2, the HeV G head domain immobilized on Ni-NTA biosensors was dipped into solutions containing 100 nM hAH1.3 IgG and then 250 nM EB2 + 100 nM hAH1.3 IgG, or 100 nM hAH1.3 IgG only or 250 nM EB2 only.

For the tetravalent mAb mixture binding assays (m102.4, HENV-32, nAH1.3, and hAH1.3), HeV G head-I53-50A-loaded biosensors were dipped into 100 nM m102.4 in 10x KB, 100 nM m102.4 + 100 nM HENV-32, 100 nM m102.4 + 100 nM HENV-32 + 100 nM nAH1.3, 100 nM m102.4 + 100 nM HENV-32 + 100 nM hAH1.3, and KB alone sequentially.

ELISA. Ninety-six-well MaxiSorp plates (Thermo Fisher) were coated overnight at 4 °C with 2 μg/mL nAH1.3, hAH1.3, m102.4, HENV-32, HENV-103, or HENV-117 IgG in 20 mM Tris and 150 mM NaCl (pH 8). Plates were slapped dry, washed three times in Tris-buffered saline Tween (TBST), and blocked with SuperBlock (Thermo Fisher) for 1 h at 37 °C. Plates were slapped dry and washed four times in TBST; 1:2 serial dilutions of either HeV or HeV-g2 G head domain (with initial concentration at 10 μM) were made in 50 μL TBST and incubated at 37 °C for 1 h. Plates were slapped dry and washed four times in TBST followed by addition of 50 μL 1:5000 6xHis tag monoclonal antibody (HIS.HB) horseradish peroxidase (HRP) (Thermo Fisher, MA-21315-HRP) for 1 h at 37 °C. Plates were slapped dry and washed four times in TBST followed by addition of 50 μL TMB Microwell Peroxidase (SeraCare). The reaction was quenched after 4 min with 1 N HCl and the A450 of each well was read using a BioTek plate reader. Data were plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL, X is log(concentration) to determine half-maximal effective concentration (EC50) values from curve fits. All experiments were performed in technical triplicates.
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Competing interest statement: C.C.B. is a US federal employee and coinventor on US and foreign patents pertaining to soluble forms of Nipah virus and Hendra virus G glycoproteins. C.C.B. and M.A. are coinventors on US and foreign patents pertaining to Cedar Virus and methods of use and recombinant Cedar virus chimeras, whose assignee is the United States as represented by the Henry M. Jackson Foundation for the Advancement of Military Medicine.