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Supplementary Materials for

Human polyclonal immunoglobulin G from transchromosomic bovines inhibits MERS-CoV in vivo

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This PDF file includes:

Materials and Methods

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/8/326/326ra21/DC1)

Tables S1A (Microsoft Excel format). Source data for Fig. 1B.
Table S1B (Microsoft Excel format). *P* value of Dunnett's test (V2, V3, V4, or V5 versus pre-V1D0) for Fig. 1B.
Table S1C (Microsoft Excel format). Specific titer activity of purified antibodies for Fig. 1C.
Table S1D (Microsoft Excel format). *P* value of Dunnett's test (SAB-300 or SAB-301 versus negative control) for Fig. 1C.
Table S2A (Microsoft Excel format). Source data for Fig. 2 (A and B).
Table S2B (Microsoft Excel format). *P* value of Dunnett's test for anti-spike antibody versus SAB-300 and SAB-301 for Fig. 2B.
Table S2C (Microsoft Excel format). Source data and *P* value of Dunnett's test for Fig. 2C.
Table S3A (Microsoft Excel format). Source data for Fig. 3 (A and B).
Table S3B (Microsoft Excel format). Source data for Fig. 3A.

SUPPLEMENTARY MATERIALS

Materials and Methods

Investigational new animal drug (INAD) and ethics statement

SAB Biotherapeutics has an Investigational New Animal Drug (INAD) file (#I-011204) with FDA Center for Veterinary Medicine (CVM) on the complete genetic engineering of the Tc bovine and the production of fully human antibody in the animals. SAB uses Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for accreditation of its animal care and use programs. The animal protocols contained in the study were approved by SAB Biotherapeutic Institutional Animal Care and Use Committee (IACUC). All murine protocols were approved by the University of Iowa International Animal Care and Use Committee.

MERS-CoV inactivated whole virion vaccine production

Vero CCL-81 cells (ATCC) were infected with MERS-CoV (Jordan N3/2012) strain at a multiplicity of infection of 0.01. Cells were maintained in DMEM (Lonza) supplemented with 5% fetal bovine serum (Sigma) until cytopathic effects (CPE) encompassed 70-80% of the monlayer. Media from MERS-CoV-infected cells were collected from Vero CCL-81 cells and MERS-CoV (4×10^9 pfu) was isolated and irradiated with a cobalt source until an absorbed dose of 6 kGy was achieved. The material was then tested for inactivation. Briefly, 800 µl (1 aliquot of virus stock) of irradiated material was inoculated onto 90% confluent T-25 flasks of Vero E6 cells (ATCC, CRL-1586) for blind passage and observed daily for production of CPE. After 5 days, the material was passaged again under the same conditions. After 5 more days, the media were harvested, and 8 serial 10-fold dilutions were made and plated onto a 96-well Operetta compatible plate (PerkinElmer) in quadruplicate. A separate plate containing MERS-CoV

infected cells was used as a positive control, and uninfected cells were used as negative controls. After incubating the Operetta plates for 72 hours, plates were fixed with 8% paraformaldehyde for 30 minutes, followed by 10% neutral buffered formalin overnight and to removal from the biosafety level-3 (BSL-3) laboratory.

Cells were washed 4 times with 1X phosphate-buffered saline (PBS) for 5 minutes each on a rocking platform. Antigen retrieval was performed by incubation in 0.1% sodium borohydrate in 0.1X PBS for 15minutes. Cells were rinsed briefly twice with PBS, then washed 4 times for 5 minutes each with 1X PBS. Cells were permeabilized (0.25% Triton X-100 in 1X PBS), rinsed briefly and washed with PBS 4 times for 5 minutes and blocked with 10%v/v normal goat serum diluted in 1X PBS for 60 minutes at room temperature (RT). Primary antibody anti-MERS-CoV spike protein (rabbit polyclonal antibody to MERS-CoV spike protein, catalog #40070-RP02 Sino Biological) was used at a 1:1,000 dilution (diluent: 1.5% v/v normal goat serum diluted in 1X PBS), and samples were incubated at 4℃ overnight. Cells were rinsed twice with 1X PBS and washed twice for 5 minutes in blocking buffer, then 4 times for 5 minutes each with 1X PBS.

Secondary antibody (Molecular Probes™ goat anti-rabbit AlexaFluor 594 or Alexa 488, Thermo Fisher Scientific) was used at 1:2,500 dilution and incubated for 30 minutes at RT (samples kept covered in foil to prevent photobleaching). Cells were rinsed briefly twice with 1X PBS and washed 4 times for 5 minutes with 1X PBS. Nuclei were stained (for cell counting by the Operetta) by adding a 1:2500 dilution of Hoechst 33342 dye diluted in 1X PBS and incubated at RT for 15 minutes. Cells were washed twice for 5 minutes with 1X PBS and evaluated by microscopy. Real-time polymerase chain reaction (PCR) analysis of irradiated, passaged material demonstrated that MERS-CoV mRNA was below the limit of detection of the assay.

Plasma collection and hIgG production

Prior to vaccination 1 (V1), plasma was collected from each Tc bovine that served as the negative control. Up to 2.1% of body weight of hyperimmune plasma per animal was collected from immunized Tc bovines on days 10 and 14 after each vaccination starting fromV2–V5. Plasma was collected using an automated plasmapheresis system (Baxter Healthcare, Autopheresis C Model 200). Plasma samples were stored frozen at -80℃ until purifications were performed. The frozen Tc plasma bags were thawed at RT overnight, and equal volumes of plasma from each time point and each Tc bovine within a group were pooled. Samples were then pH adjusted to 4.80 with dropwise addition of 20% acetic acid, fractionated by caprylic acid at a caprylic acid/total protein ratio of 1.0 for 30 minutes at RT, and then clarified by centrifugation at 10,000 × *g* for 20 minutes at RT.

The supernatant containing IgG was then neutralized to a pH of 7.50 with 1M of Tris, filtered by a 0.22 μm filter, and affinity purified by an anti-human IgG light chain specific column, KappaSelect (GE Healthcare Life Sciences). Residual bovine IgG in the KappaSelect-purified IgG sample was then removed by passing through an anti-bovine IgG heavy chain specific affinity column, Capto HC15 (GE Healthcare Life Sciences). Fully human IgG recovered from the column was then formulated by a Millipore Labscale tangential flow filtration system. The final purified fully human IgG was in a buffer at a pH of 5.5 consisting of 10 mM of glutamic acid monosodium salt, 262 mM of d-sorbitol, and 0.05 mg/mL of Tween 80. The purified hIgG was sterile filtered with 0.22 μm filter at a concentration of 10 mg/ml. Analysis of purified IgG product by high-performance liquid chromatography size-exclusion chromatography did not indicate IgG aggregates or IgG dimers.

MERS-CoV spike-protein-specific ELISA

Determination of MERS-CoV spike protein-specific human IgG antibody titers was performed in Maxisorp Immuno 96-well ELISA plates (Thermo Scientific) coated overnight at 4℃ with 100 µl/well of 2 µg/ml recombinant MERS-CoV spike protein (Al-Hasa strain) in PBS. Plates were washed with PBST (PBS with 0.05% Tween 20) and blocked at RT for 1 hour with 1% bovine serum albumin in PBS. After washing with PBST, serum samples or purified fully hIgG SAB-300 and SAB-301 were serially diluted in PBST, added to the plates, and incubated for 1 hour at RT. Following washing with PBST, diluted goat anti-human IgG-Fc conjugated with horseradish peroxidase (HRP) (Bethyl) was added to plates and incubated for 1 hour at RT. After final washing with PBST, the bound anti-spike antibodies were detected colorimetrically by using the 3, 3,' 5, 5'-tetramethylbenzidine (TMB) substrate kit (Kirkegaard & Perry Laboratories). Absorbance was read in a microplate reader at 450 nm. The titer (units/ml) is defined as the highest dilution of serum sample resulting in an optical density at 450 nm (OD_{450}) reading that was 2.5-fold higher than blank. The specific titer activity (units/mg) is defined as the highest dilution of 1 mg of SAB-300 or SAB-301 resulting in an OD450 reading that was 2.5-fold higher than blank.

Human IgG subclass proportion by ELISA

For detection of human IgG1, IgG2, IgG3, or IgG4 in SAB-300 and SAB-301, human IgG subclass ELISAs were performed in Maxisorp Immuno 96-well ELISA plates in accordance with the anti-human IgG subclass antibody manufacturer's instructions. Briefly, mouse anti-human IgG1 Fc, mouse anti-human IgG2 Fc, mouse anti-human IgG3-hinge, or mouse anti-human IgG4 Fc (Hybridoma Reagent Laboratory) was used as a capture antibody, respectively. Mouse anti-human IgG HRP (Southern Biotech) for IgG1 and IgG2 detection, mouse anti-human IgG HRP (Invitrogen) for IgG3 detection, and mouse anti-human IgG HRP (Jackson ImmunoResearch) for

IgG4 detection were used. Human IgG subclass standard serum (Bethyl) was used as the reference. Human IVIG (Grifols) was used as a positive control.

MERS-CoV exposure studies in transduced adenovirus/hDPP4 mice

Transduced adenovirus/hDPP4 BALB/c mice were infected with EMC/2012 strain of MERS-CoV as described (*41*). BALB/c mice (n = 3 per group) were injected intraperitoneally with 100 or 500 μg (5–25 mg/kg) of negative control or test hIgG (SAB-300; SAB-301). Doses were selected based on lower and upper doses of other Tc bovine hIgGs with similar neutralizing titers against other viral pathogens (*35, 36*). The equivalent human doses in a human are approximately 0.5 to 2.5 mg/kg based upon the body surface area normalization method (*49*). Mice were infected intranasally with MERS-CoV (1×10^5 pfu) in a total volume of 50 μl 12 hours before or 24 and 48 hours after administration of the hIgG. To obtain MERS-CoV titers, lungs were removed into PBS and homogenized using a manual homogenizer. Virus in homogenate was detected by titration on Vero 81 cells. Viral titers are expressed as $\log_{10}$ pfu/g tissue.

Fluorescence reduction neutralizing-50% assay ($FRNA_{50}$)

$FRNA_{50}$ were performed to determine neutralizing activity of anti-MERS-CoV antibody samples. Vero E6 cells (8×10^4 cells/well) were plated on 96-well Operetta (PerkinElmer) compatible plates. Serum samples were heat-inactivated at 56°C for 1 hour followed by serial dilutions from 1:40 to 1:327,680 in Dulbecco's modified Eagle's Medium (Lonza). In parallel, MERS-CoV virus stock was diluted to 0.96 pfu/μL using serum-free Dulbecco's modified Eagle's Medium (Lonza). Virus was mixed with serum dilutions and incubated at 37°C and 5% CO_2 for 1 hour. At the end of 1 hour, 100 μL of virus +serum mixture was added to the 96-well plates and incubated for 24 hours at 37°C and 5% CO_2. Negative control (no virus and no serum)

samples and positive control (virus + antigen affinity purified rabbit polyclonal antibody to MERS-CoV spike protein #40069-RP02, Sino Biological) samples were used. Cells were fixed by adding 20% neutral-buffered formalin and processed by labeling with a primary MERS-CoV anti-spike antibody. A secondary antibody goat anti-rabbit IgG (heavy and light chain) conjugated to Alexa Fluor 594 (A-11037, Thermo Fisher Scientific) detected the primary antibody and was visualized on an Operetta High Content Imager (Perkin Elmer). Samples were evaluated to determine the dilution factor required for neutralization, then IgG present in a given volume was normalized to the mass of IgG to remove dilution associated biases of the samples. The quantity of IgG (μg) that reduces the relative fluorescent intensity by50% was reported as the $FRNA_{50}$.

Tissue culture infectious dose-50% assay ($TCID_{50}$)

The $TCID_{50}$ assay was performed as previously described (*48*). Briefly, in a two-fold dilution series, SAB-300, SAB-301, and negative control hIgG were diluted 1:20 in Vero E6 cell growth media and further diluted down to 1:40960. MERS-CoV-Jordan-N3/2012 strain was added at a final concentration of 3,950 $TCID_{50}$/ml and incubated for 30 minutes at RT. Mock-infected Tc bovine sera and MERS-CoV-Jordan strain alone in Vero E6 cell media served as negative and positive controls, respectively. The inhibitory capacity of each SAB-300, SAB-301 and negative control hIgG dilution was assessed by a $TCID_{50}$ assay, and the dilution at which MERS-CoV-Jordan strain was inhibited by 50% was recorded as the percent of virus titer compared to media alone control.

Antibody dependent enhancement assay

To test for ADE of MERS-CoV infection, 1×10^4 Raji or Vero E6 cells per well were seeded into 96-well plates (Corning) and cultured overnight. MERS-CoV-Jordan strain at a $TCID_{50}$ of

10,000 was added to SAB-300 or SAB-301, incubated at 37°C for 1 hour, and then added to Raji or Vero E6 cells for 2 hours at 37℃. Cells were washed once in PBS, and fresh normal growth media RPMI-1640 plus 10% fetal bovine serum (Sigma Aldrich) was added. Cells were incubated for a further 48 hours at 37℃. At 48 hours post-infection, supernatant was collected and frozen at -80°C until infectivity was assessed by $TCID_{50}$ assay as previously described [27].

RNA was extracted from Raji cell pellets using the PureLink® RNA mini kit (Life Technologies) according to the manufacturers' instructions. Levels of MERS-CoV RNA were assessed using the Taqman® Fast virus one-step master mix (Applied Biosystems) according to the manufacturers' instructions using a triplex of primers obtained from Life Technologies. These primers targeted a region of the genome upstream of the envelope gene (UpE) and the leader sequence of the nucleocapsid messenger RNA (leader primer), and transferrin receptor protein 1 served as an endogenous control (Thermo Fisher). PCR reactions in Microamp® fast optical reaction plates (Applied Biosystems) were read on a 7500 fast DX real-time PCR instrument (Applied Biosystems), and data were analyzed using the Δ cycle threshold (Ct) method (*50*).

Cells and viruses

Raji cells (ATCC #CCL-86), Vero E6 cells (ATCC #CRL-1586) and Vero CCL81 cells (ATCC #CCL81.1) were grown per ATCC recommendations. The Jordan-N3/2012 strain (GenBank KC776174.1) of MERS-CoV was provided by Dr. Gabriel Defang (Naval Medical Research Unit-3, Cairo, Egypt) (*40*). The clinical sample from which the MERS-CoV Jordan-N3/2012 strain was isolated was provided by Dr. Tarek Al-Sanouri and Dr. Aktham Haddadin (Jordan Ministry of Health). The EMC/2012 strain of MERS-CoV (passage 8) was provided by Drs. Bart

161 Haagmans and Ron Fouchier (Erasmus Medical Center) (*51*). Recombinant adenoviral vectors
162 expressing-hDPP4 were prepared by the University of Iowa Gene Transfer Vector Core (*41*).