Human Survivors of Disease Outbreaks Caused by Ebola or Marburg Virus Exhibit Cross-Reactive and Long-Lived Antibody Responses

Mohan Natesan, Stig M. Jensen, Sarah L. Keasey, Teddy Kamata, Ana I. Kuehne, Spencer W. Stonier, Julius Julian Lutwama, Leslie Lobel, John M. Dye, Robert G. Ulrich

Molecular and Translational Sciences and Virology Division, United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, USA; Department of Arbovirology, Emerging and Re-emerging Infection, Uganda Virus Research Institute, Entebbe, Uganda; Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Shea, Israel

A detailed understanding of serological immune responses to Ebola and Marburg virus infections will facilitate the development of effective diagnostic methods, therapeutics, and vaccines. We examined antibodies from Ebola or Marburg survivors 1 to 14 years after recovery from disease, by using a microarray that displayed recombinant nucleoprotein (NP), viral protein 40 (VP40), envelope glycoprotein (GP), and inactivated whole virions from six species of filoviruses. All three outbreak cohorts exhibited significant antibody responses to antigens from the original infecting species and a pattern of additional filoviruses that varied by outbreak. NP was the most cross-reactive antigen, while GP was the most specific. Antibodies from survivors of infections by Marburg marburgvirus (MARV) species were least cross-reactive, while those from survivors of infections by Sudan virus (SUDV) species exhibited the highest cross-reactivity. Based on results revealed by the protein microarray, persistent levels of antibodies to NP, VP40, and VP40 were maintained for up to 14 years after infection, and survival of infection caused by one species imparted cross-reactive antibody responses to other filoviruses.

Ebola and Marburg disease outbreaks occur as isolated events that are generally confined to Central Africa. Species of the Filoviridae Sudan virus (SUDV), Ebola virus (EBOV), Bundibugyo virus (BBDV), Tai Forest virus (TAFV) ebolavirus, and Marburg marburgvirus (MARV) are the cause of these severe human infections. In contrast to the historic trend, the recent epidemic caused by the Makona variant of Ebola virus (EBOV-Makona) began in the Western Africa country of Guinea and spread to several countries, including Liberia, Sierra Leone, and Nigeria, resulting in over 11,000 deaths (1). Several potential animal reservoirs or alternative hosts were reported (2–7), suggesting that human outbreaks may involve incidental exposures to infected animals. While the events that trigger cycles of human infections are not clearly understood, human-to-human disease transmission occurs through direct physical contact with infected body fluids (8, 9). The mortality rate can reach 90% without medical intervention, while supportive measures such as hydration and electrolyte correction substantially improve patient outcome (10, 11). Many questions regarding the relationship between human immunity and disease outbreaks remain unanswered. Further, the public health management of filoviral infections is hampered by the lack of effective vaccines and limited therapeutic options. Viral load was reported to be the most important marker of survival during the SUDV outbreak of 2000–2001 in the Gulu district of Uganda that resulted in 55 pediatric and 161 adult laboratory-confirmed cases (12). Although the physiological factors that influence viral load are not clear, humoral and cellular immunity contributes to resistance and recovery from infection (13, 14). For example, low antibody levels during the early phase of infection were hypothesized to increase fatal outcomes, whereas robust antibody responses were associated with survival (15, 16).

The use of plasma or gamma globulin from individuals who have recovered from infection may be an effective treatment for active cases of disease (17). Despite the promise of this approach, a recent clinical trial of convalescent plasma in Guinea did not find a significant survival benefit (18). However, the levels of antibodies against Ebola virus in the convalescent plasma used in the Guinea study were unfortunately unknown (18), suggesting the importance of using well-characterized plasma. As a further concern, it is not clear if antibodies collected from one disease outbreak will provide protection against infections caused by a different species or strain of filovirus. Antibody cross-reactivities (19) may also be useful for predicting efficacy against infections caused by other filoviruses for the case of vaccines currently under development that are based on antigens from a limited number of viral isolates (20, 21). Thus, methods that can be used to address the diversity and duration of antibody responses to infection will facilitate the development of effective therapeutics and vaccines.

In the study reported here, we examined antibody responses from survivors of separate disease outbreaks in Uganda caused by MARV, BBDV, and SUDV infections by utilizing microarrays comprising whole virus and key protein antigens from the six species of filoviruses. Our results serve to elucidate the antigenic relationships among proteins and viruses from the perspective of human responses to infection.

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Editor: R. L. Hodinka, University of South Carolina School of Medicine Greenville Address correspondence to Mohan Natesan, mohan.natesan.ctf@gmail.com, or Robert G. Ulrich, robert.g.ulrich.civ@mail.mil.

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MATERIALS AND METHODS

Disease and control serum. Peripheral blood serum from 61 survivors of SUDV strain Gulu (SUDV-Gulu) (37 cases), BDBV-Bundibugyo (20 cases), and MARV-Kabale (9 cases) infection outbreaks, along with serum from regional control subjects who had no documented history of filovirus infection, was examined. The MARV-Kabale sera were collected a year after infection, the BDBV-Bundibugyo sera 7 years after infection, and the SUDV-Gulu sera 12 to 14 years after infection. Additional control sera were obtained from volunteers with no history of infection (U.S. origin). Consent forms and personal health questionnaires were obtained from all subjects. Institutional approvals for the study were obtained from the Uganda Virus Research Institute in Entebbe, Uganda; the Ugandan National Council for Science and Technology; and the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).

Protein and virus microarrays. Recombinant proteins from EBOV, SUDV, MARV, BDBV, TAFV, and species of Reston ebolavirus (RESTV; a cause of asymptomatic human infections [22]) were cloned, expressed as His-tagged proteins, and purified (80% to 95% homogeneity), as previously described (23). The envelope glycoprotein (GP) ectodomain missing the transmembrane (GPA[TM]) from BDBV, RESTV, TAFV (Ivory Coast), SUDV (Boniface), EBOV (Mayinga), and MARV (Musoke) were also produced in mammalian cells. The following high-titer virus strains (inactivated by gamma irradiation) were generously provided by David Norwood (Diagnóstics Systems Division, USAMRIID): EBOV (Mayinga), SUDV (Sudan-Boniface), RESTV (Reston-H28), BDBV (Bundibugyo), and MARV (Musoke, Ravn, C67, and Angola). TAFV (Ivory Coast) inactivated by gamma irradiation was obtained from BEI Resources, Manassas, VA. To optimize assay signals, the virus concentrations were adjusted by evaporation (Savant; Thermo Scientific, CA), and data were processed by previously described methods (23). The microarrays were probed (1 h, 22°C) with serum diluted 1:150 in probe buffer (1 × Tris-buffered saline) and washed three times, and antibody binding was detected by incubation (1 h, 22°C) with Alexa Fluor 647-conjugated secondary antibody (Invitrogen, Carlsbad, CA) diluted 1:2,000 in probe buffer. The microarray-processed slides were rinsed with purified water and dried.

Data acquisition and analysis. Processed slides were scanned by the use of a laser scanner (GenePix 4400A; Molecular Devices, Sunnyvale, CA), and data were processed by previously described methods (23). Data were quantile normalized using the preprocess core package of the R statistical software program, version 3.2.1 (http://www.R-project.org). A two-class unpaired test (significance analysis of microarrays [SAM]) was applied (24) for comparison of significance determinations from the experimental groups, using 1,000 permutations, a q value of <0.001, and a permutation algorithm to estimate the false-discovery rate. The GEN-E program (Broad Institute, Cambridge, MA) was used to generate heat maps from normalized and log2-transformed data for hierarchical clustering of antibody interactions on the basis of average-linkage Euclidean distance determinations. Analysis of variance (ANOVA) performed with Dunnett’s tests was used to compare the antibody responses to GP proteins of BDBV, SUDV and MARV infection survivors. The statistical analyses were conducted using the SAS software system, version 9.4 (SAS Institute, Inc., Cary, NC).

Analysis of antigen diversity. CLUSTAL W2 (25) was used to generate three multiple-sequence alignments for NP, VP40, and GP mucin-like domain amino acid sequences. Each multiple-sequence alignment had a different gap opening penalty (5, 10, or 25), with Blosums62 used as the protein weight matrix and all other options left as defaults. T-Coffee Combine (26, 27) was then used to generate a single alignment that had the best agreement of all three multiple-sequence alignments for each protein. Shannon entropy was used as a measure of amino acid variability (BioEdit Sequence Alignment Editor v7.1.3.0 [28]). Each alignment was filtered using Gblocks (29, 30) with strict settings of no gap positions within the final blocks, strict flanking positions, and no small final blocks.

For phylogenetic reconstruction, Gblocks identified a 406-residue conserved region at the N terminus of NP (BDBV, TAFV, RESTV, SUDV, and EBOV residues 20 to 425; MARV residues 2 to 407) and a 266-residue conserved region of VP40 that spanned the majority of the protein’s length (BDBV, TAFV, RESTV, SUDV, and EBOV residues 46 to 312; MARV residues 34 to 300). An unappaged, highly variable region of 33 residues at the N-terminal portion of the GP moiety was selected for use in phylogenetic reconstruction (BDBV, TAFV, and RESTV residues 2 to 34; EBOV and MARV residues 1 to 33). Molecular phylogenies were generated by the maximum likelihood method implemented in the PhyML program (v3.0 aLRT) (31). The Blosums62 substitution model was selected together with 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (αNP = 0.654; αVP40 = 0.996; αGPmucin = 15.371). Reliability for internal branches was assessed using a bootstrap method with 1,000 replicates. Comparison of dendrograms was completed using Compare2Trees software (32).

RESULTS

Antigen complexity. With respect to potential targets of antibody responses, filovirus genomes encode seven antigens consisting of the nucleoprotein (NP), envelope glycoprotein (GP), viral protein 24 (VP24), VP30, VP35, and VP40, and RNA polymerase. For qualitative assessment of survivor sera, the conserved antigens NP and VP40 may capture serological responses to the broadest number of infections; the more variable GP may be useful for detecting antibodies that discriminate between viral variants, while VP40 is the most abundant protein in the virus particle (33). Our previous observations (23) indicated that antibody responses of infected rhesus macaques to EBOV or MARV were most specific for the GP of the infecting virus whereas antibody recognition of NP was more promiscuous. We further examined the potential impact of antigen variability on human antibody recognition. The NP antigen is highly conserved among Ebola virus species, with >60% sequence identity among isolates, but exhibits only ~30% amino acid sequence identity with MARV NP (see Fig. S1 in the supplemental material). Thirty percent of NP residues (216 residues of 739 total) were completely conserved among the six species examined, with an average variability per residue (H/res) of 0.638 (data not shown), based on calculations of Shannon entropy per residue of the NP multiple-sequence alignment. NP sequences exhibit a relatively large amount of rate variation (αNP = 0.654), with many sites evolving very slowly and others evolving at a high rate, indicating the potential effect of selective pressures that contribute to sites of high variability (34). While VP40 also exhibits a high degree of conservation among Ebola virus species, with >75% sequence identity evident among isolates, it exhibits less conservation with MARV VP40 (~25% sequence identity) than Ebola virus NP does with MARV NP (see Fig. S1). Amino acid sites of VP40 exhibit less variation and similar substitution rates (αVP40 = 0.996). In contrast, the mucin-like domain of GP exhibited minimal sequence identity (sequence identity = 5% to 26% [see Fig. S1]) among all filovirus isolates examined and no conserved residues were observed. The average variability per residue of the GP mucin-like domain was 50% greater than that of NP sequences (H/res = 1.07), with residues exhibiting similar substitution rates (αGPmucin = 15.371). Comparing NP, the most conserved protein, with GP, the most divergent protein, the overall similarity of the protein sequence dendrograms (see Fig. S2 in the supplemental material) was 77.8%, with 100% conserved edges among the trees for BDBV, TAFV, and EBOV and the SUDV, RESTV, and MARV...
The heat map in Fig. 2 provides an overview of antibody responses to individual proteins, using hierarchically clustered data obtained from survivor and control sera. Two major data clusters corresponding to infected and negative-control sera were immediately apparent. While they formed two clusters, the control sera exhibited minimal binding to filoviral proteins. As shown by analysis of the total antibody response to proteins, sera from MARV, BDBV, and SUDV infection survivors clustered as distinct groups within the study cohorts (Fig. 2). Sera from two of the MARV infection survivors clustered independently of the other MARV sera due to high reactivity to MARV (mammalian) GPαTM combined with a lower level of antibody recognition of VP40. Further, sera from each outbreak presented high levels of antibody recognition for antigens from the infecting species of filovirus (Fig. 3; see also Table S4 in the supplemental material). In total, 5 viral proteins were recognized by sera from infections caused by MARV, 9 by sera from infections caused by BDBV, and 16 by sera from infections caused by SUDV (see Table S4). Moreover, these results indicated that substantial levels of specific antibody were detectable at sampling times more than a decade after primary SUDV infection and 7 years after BDBV-Bundibugyo infection.

Having established that sera from outbreak survivors presented high levels of antibodies to protein antigens from the infecting species, we examined antibody cross-reactivity in greater detail (Fig. 4). Serum antibodies from MARV infection survivors showed the least cross-reactivity with other filoviral antigens in comparison to those from BDBV and SUDV infection survivors. In comparison to the MARV infection survivors, the BDBV infection survivor group exhibited the highest antibody cross-reactivity, with significant levels observed for proteins from the following heterologous viruses: EBOV (NP and VP40), SUDV (NP), TAFV (VP40), and RESTV (NP). Lastly (see Table S4 in the supplemental material), the SUDV infection survivors presented significant levels of antibodies that were directed against EBOV (NP and VP40), BDBV (NP and VP40), MARV (GP mucin and NP), and RESTV (GP mucin, GPαTM, NP, and VP40), as well as against TAFV (NP and VP40). As shown by analysis of all sera from the infection survivors, the order of antibody cross-reactivity among the three protein antigens examined was VP40 > NP > GP, consistent with VP40 presenting the highest level of sequence conservation and GP mucin the lowest (data not shown).

**Human antibody interactions with viruses.** We used inactivated viruses to ascertain the antibody response to the composite assembly of filovirus antigens. General cross-reactivity of survivor sera was observed in the whole-virus microarray (Fig. 4D), although the antibody responses to the infecting species were higher. More-detailed assessments of antibody interactions with filoviruses are presented in Fig. 5 and Table S5 in the supplemental material. Sera from the MARV infection survivors exhibited antibody interactions with all of the filoviruses, while only the antibody levels against MARV isolates were significant. In addition to detection of significant antibodies against the infecting species, the sera from the BDBV infection survivors also recognized EBOV, RESTV, and SUDV isolates, while the sera from the SUDV infection survivors exhibited significant antibody interactions with EBOV, MARV, and RESTV. We concluded from these results that the individual viral proteins allowed detection of a greater level of antibody cross-reactivity among filoviral species than the whole-virus preparations.

**Determination of antibody responses.** Antibody responses to filovirus proteins were determined by analysis of variance (ANOVA) with Dunnett’s test (** P < 0.01; *** P < 0.001; ****, P < 0.0001; NS, not significant).

**Human antibody interactions with viral proteins.** To address the extent of antibody cross-reactivity to different species of filoviruses following recovery from infection, we examined serological responses of 37 survivors from the 2000 SUDV-Gulu outbreak, 20 from the 2007 BDBV-Bundibugyo outbreak, and 4 from the MARV-Kabale/Ibanda outbreak, collected 1 to 14 years after infection (see Table S1 in the supplemental material). Antibody interactions were measured by means of a multiplexed microarray platform, comprising GPαTM proteins (11 total) expressed in insect and mammalian cells, GP mucin, NP, and VP40 from six species expressed in *Escherichia coli* (18 total), and nine isolates of whole inactivated filoviruses (see Table S2 and S3). We first examined antibody responses to the GP ectodomains (GPαTM) in comparison to the GP mucin-like domain. For this purpose, we used GP expressed in both insect and mammalian cell backgrounds to minimize the impact of any variability in glycosylation and in nonglycosylated mucin-like domains that were produced in *E. coli*. We focused on antibody responses from each disease cohort and the infecting virus from each outbreak (Fig. 1).

Antibodies from MARV infection survivors recognized GP mucin and GPαTM produced in insect or mammalian cells equivalently, whereas antibody recognition of the GP mucin for SUDV infection survivors was greater (Fig. 1) than antibody recognition of either GPαTM. For BDBV infection survivors, microarray display of GP mucin and mammalian-cell-produced GPαTM resulted in equivalent levels of antibody binding, while microarray display of the GPαTM produced by insect cells resulted in the greatest amount of antibody binding. Further, the results suggest that substantial amounts of antibodies were directed toward the nonglycosylated polypeptide of the mucin-like domain.
DISCUSSION

The broad protein-level and virus-level cross-reactivities that we observed for antibodies from humans years after their recovery from infections caused by Ebola and Marburg viruses indicate that these serological immune responses are long lived and extend beyond the original filovirus exposure. Protein- and virus-specific antibodies were studied by using a multiplexed microarray that contained conserved (VP40 and NP) and highly variable (GP) antigens from six species of filoviruses, together with nine isolates of whole inactivated filoviruses. The sera examined were collected from survivors and controls. Hierarchical clustering performed by the Euclidean distance average linkage method was used for visualization. Normalized and log2-transformed data were applied for creating the heat map. The proteins are listed in the rows (*, insect cell expression; **, mammalian cell expression), the cells represent individual serum samples, and the survivor and control groups are listed on the bottom. The purple bars show healthy controls from Uganda, the blue bars U.S. healthy controls, and the green bars BDBV, MARV, and SUDV infection survivor groups.

FIG 2 Recognition of filoviral proteins by antibodies from Ebola and Marburg survivors. The heat map displays IgG reactivity associated with survivors and controls. Hierarchical clustering performed by the Euclidean distance average linkage method was used for visualization. Normalized and log2-transformed data were applied for creating the heat map. The proteins are listed in the rows (*, insect cell expression; **, mammalian cell expression), the cells represent individual serum samples, and the survivor and control groups are listed on the bottom. The purple bars show healthy controls from Uganda, the blue bars U.S. healthy controls, and the green bars BDBV, MARV, and SUDV infection survivor groups.

FIG 3 IgG responses of Ebola and Marburg survivors to autologous GP mucin, NP, and VP40 recombinant proteins. Panel A shows reactivity of BDBV infection survivors, panel B reactivity of SUDV-Gulu survivors, and panel C reactivity of MARV infection survivors. The filled symbols denote survivors and open symbols controls. Each symbol corresponds to an individual serum sample, and the black line represents geometric mean of all samples in each group. *, statistical significance was measured by SAM.
The highest levels of human antibody interactions were directed toward antigens from the same infecting species, with antibody levels being the most pronounced for NP, followed by VP40 and GP. The relative levels of antibody responses to viral antigens did not represent results based solely on stoichiometry of virion components, because VP40 is the most abundant protein in the virus particle (33). Antibody cross-reactivity was observed for all heterologous VP40 and NP proteins, while the relationship between antibody responses to these antigens and recovery from infection is unknown. Antibody epitopes were previously identified in the C-terminal region of NP (35, 36), a protein that is essential for replication of the viral genome and nucleocapsid assembly. Each virion contains about 3,200 NP molecules (37), and the most conserved region of NP forms a condensed helix that may have a crucial role in virus replication (37).

The longevity of cellular and antibody memory immune responses to infections by SUDV was previously reported (13, 38, 39). In another study of EBOV-Zaire survivors, specific IgG levels increased over days 5 to 30 after onset of symptoms and then declined slowly over several years, while remaining detectable >11 years after infection (38, 39). Among the results reported here from analyses performed with recombinant antigens and inactivated viruses from six species of filoviruses, we observed antibodies that were specific for NP that persisted for up to 14 years after SUDV infection and antibodies against VP40 and GP whose levels were significantly elevated 7 years after the outbreak of BDBV infections. Nonhuman primates are often used to model certain aspects of the human response to filoviral infections, and yet no studies of long-term immunity have been reported. We previously examined convalescent (30 days from infection) serum antibody responses of MARV- or EBOV-challenged rhesus macaques that were vaccinated with virus-like particles of GP, NP, and VP40 from MARV or EBOV (23). Similarly to the results seen with sera collected years after human infections, rhesus antibody cross-reactivities were observed among NP and VP40 of Ebola virus species, while GP recognition by rhesus antibodies was much more specific than that seen in the human results. In addition to the possible influence of vaccination, which could drive higher GP specificity for the rhesus macaque, the reported results hint that primate disease models are not identical to naturally occurring human infections.

A further discussion of our results with respect to antibody responses to GP is also warranted. As the virus matures within the infected cell, GP is processed by cellular cathepsin L and B to remove the heavily glycosylated mucin-like domain and glycan cap (40), resulting in a 19-kDa GP1 complex with the GP2 (24-kDa) transmembrane subunit. Ebola GP is expressed following RNA editing, while the unedited transcript encodes a soluble GP that is cleaved by furin and released from infected cells (41). The conserved Ebola secretory glycoprotein (sGP) (42) is represented in the microarray by GPΔTM, whereas MARV does not have a sGP. Substantial amounts of antibodies to GP are directed toward the polypeptide of the heavily glycosylated mucin-like domain, which is missing from sGP. We base this conclusion on the observation that nonglycosylated mucin-like domains bound polyclonal antibodies to levels that were similar to those seen with the full-length ectodomains of glycosylated GP. This observation is consistent with a previous study that found that mutation of two N-linked sites on GP1 of EBOV enhanced immunogenicity, pos-
sibly by unmasking epitopes (43), while removal of the entire mucin-like domain reduced protective immunity in mice.

It is likely that serological immune responses to infection also represent a summation of the antibodies directed toward the different macromolecular forms of GP. For example, trimeric GP complexes on the virion surface serve as receptors for entry into host cells and as targets for neutralizing antibodies (44, 45), while, in addition to virion-bound GP, all filoviruses except Marburg viruses express a soluble GP that is released from infected cells (46). Our results do not distinguish between antibodies that interact with the different macromolecular complexes of GP, and studies that can associate host responses with each isoform may be important for development of vaccines and therapeutics. For further consideration, protein coding sequences differ slightly among isolates within a filovirus species and also within replicating populations of any isolate, as driven by factors such as the low fidelity of the RNA polymerase (47).

An important unsolved issue is that of determining if previous infection with one filovirus species confers immune protection against exposures to new strain variants or other species. There are no firm correlates of protection for filoviral infections. Approximately half of the sera from SUDV-Gulu outbreak survivors neutralized SUDV-Gulu in plaque reduction neutralization tests (PRNT), as previously reported (49). In contrast, less than 50% of sera from MARV infection survivors neutralized MARV isolates in PRNT (unpublished observations). In agreement with a previous report by Macneil and coworkers (19), our results indicate that there is a substantial amount of antibody cross-reactivity across isolates and species of filoviruses. Serological surveys based on enzyme-linked immunosorbent assay (ELISA) methods have recorded high antibody prevalence rates among populations that have not had documented cases of filovirus infections, as well as high overall seroprevalence rates across communities with prior infections (50, 51). Long-term monitoring of these previously characterized human populations will be necessary to determine the rate of the incidence of infection compared to that seen with naive communities. In addition, human or animal hosts are required to maintain an infectious reservoir to seed cycles of filovirus disease outbreaks. However, insufficient country- or region-wide surveillance data are available to determine the frequency of prior exposures to Ebola or Marburg viruses, and estimates of infection rates based on clinic observations alone are prone to errors due to unknown rates of asymptomatic cases (52, 53) and skewed levels of access to health care facilities. Antibody levels are generally elevated in cases of asymptomatic infections (54), suggesting that a systematic expansion of serological surveillance efforts based on high-throughput methods that are accurate and sensitive may help to track patterns of disease spread and aid in predicting the most vulnerable populations. An advantage to the microarray assay that was developed for this study is that concur-

![Microarray analysis of IgG reactivity of Ebola virus and Marburg virus infection survivors to whole virus.](http://cvi.asm.org/)}
rent detection of antibody responses to multiple antigens from each species might increase the accuracy of results, while the inclusion of probes for multiple viral species facilitates a broader, high-throughput analysis of infection history.

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