Enzyme-Linked Immunosorbent Assay for Detection of Filovirus Species-Specific Antibodies

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Several enzyme-linked immunosorbent assays (ELISAs) for the detection of filovirus-specific antibodies have been developed. However, diagnostic methods to distinguish antibodies specific to the respective species of filoviruses, which provide the basis for serological classification, are not readily available. We established an ELISA using His-tagged secreted forms of the transmembrane glycoproteins (GPs) of five different Ebola virus (EBOV) species and one Marburg virus (MARV) strain as antigens for the detection of filovirus species-specific antibodies. The GP-based ELISA was evaluated by testing antisera collected from mice immunized with virus-like particles as well as from humans and nonhuman primates infected with EBOV or MARV. In our ELISA, little cross-reactivity of IgG antibodies was observed in most of the mouse antisera. Although sera and plasma from some patients and monkeys showed notable cross-reactivity with the GPs from multiple filovirus species, the highest reactions of IgG were uniformly detected against the GP antigen homologous to the virus species that infected individuals. We further confirmed that MARV-specific IgM antibodies were specifically detected in specimens collected from patients during the acute phase of infection. These results demonstrate the usefulness of our ELISA for diagnostics as well as ecological and serosurvey studies.

MATERIALS AND METHODS

Plasmids. Viral RNA extracted from the supernatant of Vero E6 cells infected with ZEBOV, SEBOV, CIEBOV, BEBOV, and MARV strain Angola was used for the cloning of the respective GP cDNAs lacking the transmembrane domain and cytoplasmic tail. The cDNAs of truncated EBOV and MARV GPs with a C-terminal histidine (His) tag (His-EBOV-GP and His-MARV-GP, respectively) were cloned into a pATX vector. Finally, the cDNA fragments of His-EBOV-GP and His-MARV-GP were inserted into the mammalian expression vector pcAGGS/MCS, which contains the chicken β-actin promoter (13). All clones were confirmed by sequencing prior to expression.

MAbs. Hybridoma cells producing EBOV GP-specific MAbs ZGP42/3.7 (IgG1) (24, 26), which recognizes a linear epitope on GP comprising the sequence D556-E558, and S139/1 monoclonal antibody (IgG2a), which binds to the hemagglutinin of influenza A viruses (37), was used as a negative control.

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FIG. 1. Phylogenetic analysis of filovirus GP amino acid sequences. The phylogenetic tree was constructed by using the neighbor-joining method. For the construction of this tree, we used 10 GP amino acid sequences, each comprising a whole GP amino acid sequence. Numbers at branch points indicate bootstrap values (1,000 replicates).

Sera and plasma. Five-week-old female BALB/c mice were immunized twice intraperitoneally with 100 μg virus-like particles (VLPs) (14, 21) in 3-week intervals, and the serum samples were collected 7 to 10 days after the second immunization. Convalescent-phase plasma samples were collected from cynomolgus macaques vaccinated and/or infected with EBOV as described previously (27). ZEBOV convalescent-phase human plasma (patients 2 to 7) and serum (patients 1 and 8) samples were obtained 51 to 135 days after the onset of ZEBOV infection during the 1995 outbreak in Kikwit, Democratic Republic of the Congo (25). SEBOV convalescent-phase patient serum samples (patients 9 and 10) were collected about 2 months after onset during the Ebola hemorrhagic fever outbreaks in Uganda in 2000 associated with SEBOV (2). These EBOV-infected human blood samples were kindly provided by T. G. Ksiazek (Centers for Disease Control and Prevention). MARV-infected human blood samples (patients 11 to 21) were collected within a few days after the onset of symptoms from infected patients from the 2004-2005 outbreak in Angola (29). Blood collections during outbreak investigations were approved under the special response protocol established between the World Health Organization and national authorities.

Expression and purification of His-EBOV-GP and His-MARV-GP. Human epithelial kidney 293T cells cultured in high-glucose DMEM containing 10% FCS and antibiotics were transfected with pCAGGS vectors expressing His-EBOV-GP (pChis-ZEBOV-GP, pChis-SEBOV-GP, pChis-CIEBOV-GP, pChis-ZEBOV-GP, or pChis-REBOV-GP) or His-MARV-GP (pChis-MARV-GP) using TransIT LT1 (Mirus). Forty-eight hours after transfection, the supernatants were collected, and the recombinant GPs were purified by using the Ni-nitrotriacetic acid (NTA) purification system (Invitrogen) according to the manufacturer's instructions. The majority of contaminant protein was removed with wash buffer containing 15 mM imidazole. Finally, bound proteins were collected with elution buffer containing 250 mM imidazole. To monitor inevitable nonspecific reactions (i.e., nonspecific antibodies) to FCS-derived impurities in each GP preparation, control antigens (FCS-derived proteins nonspecifically bound to the Ni beads) were prepared by using the Ni-NTA column under the same conditions. The eluted protein was concentrated by using Amicon Ultra 4 spin columns (Millipore) and dialyzed against phosphate-buffered saline (PBS) at 4°C overnight. Purified His-EBOV-GP and His-MARV-GP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Western blotting was performed by using ZGP42/3, AGP127, and anti-His MAbs (Covance).

Antigens prepared from cell lysates and VLPs. Membrane lysates of 293T cells transfected with pCAGGS expressing full-length GP were prepared by using the Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce) according to the manufacturer's instructions. To generate VLPs, 293T cells were transfected with plasmids expressing major viral structural proteins, GP, NP, and VP40 (10, 33). After 48 h, supernatants were overlaid on 25% sucrose and ultraconcentrated at 28,000 × g at 4°C for 1.5 h. The VLPs were recovered from the pellet and disrupted with 0.05% Triton X-100 in the presence of 30 mM potassium chloride for the use of ELISA antigens. The GP amounts in the membrane lysates and VLPs were quantified by Western blotting using MAb ZGP42/3.7 or AGP127-8, and the GP concentrations of each preparation were calculated based on the standard band intensities provided by known concentrations of His-GP. Membrane lysates or supernatants of 293T cells transfected with empty pCAGGS vectors were used to prepare control antigens for ELISA using cell lysates or VLPs, respectively.

ELISA. ELISA plates (Nunc Maxisorp) were coated with the GP antigens (100 ng of GP/50 μl/well) or control antigens in PBS at 4°C overnight and then washed with PBS containing 0.05% Tween 20 (PBST). Unspecific binding of the antibodies was avoided by blocking with 3% skim milk (150 μl/well) for 2 h at room temperature. Monkey plasma samples were preincubated with 2% FCS to absorb antibodies to FCS components, since they were exposed to FCS by the injection of the vaccines or viruses diluted in DMEM containing FCS. After washing three times with PBST, 50 μl of appropriately diluted serum or plasma samples or the GP-specific MAB in PBST containing 1% skim milk was added and incubated for 1 h at room temperature. After washing three times with PBST, the bound antibodies were detected by using the following secondary antibodies conjugated with horseradish peroxidase diluted in 1% skim milk in PBST: goat anti-mouse IgG (Jackson Immunoresearch), goat anti-monkey IgG (Rockland), goat anti-human IgG (Jackson Immunoresearch), or donkey anti-human IgM (Jackson Immunoresearch). After incubation for 1 h at room temperature and three PBST washes, 50 μl of 3, 3', 5'-tetramethylbenzidine (Sigma) was added to each well, and the mixture was incubated for 15 min at room temperature. The reaction was stopped by adding 1 N sulfuric acid to the mixture, and the optical density (OD) at 450 nm was measured.

Phylogenetic analysis. Phylogenetic analysis was based on whole amino acid sequences of filovirus GPs. The sequences were analyzed by using GENETYX (Genetyx Corp., Japan) for Windows software, version 7. A phylogenetic tree was constructed by using the neighbor-joining bootstrap method (1,000 replicates) in MEGA 4.0 software (28). Amino acid sequences of ZEBOV strain Mayinga-76, ZEBOV strain Kikwit-95, SEBOV strain Boniface-76, SEBOV strain Malabo-79, CIEBOV strain Côte d’Ivoire-94, BEBOV, REBOV strain Reston-89, REBOV strain Sienna-Philippine-92, MARV strain Musoke-80, and MARV strain Angola/2005 used in phylogenetic analyses were obtained from GenBank under accession numbers Q05320, P87666, Q46614, Q466798, Q466810, ACI28624, Q466799, Q466853, P15253, and Q1P505, respectively.

Statistical analyses. OD values higher than 3 standard deviations above the averages of negative-control samples at a 1:100 dilution were considered positive. To test the specificity of each reaction, ELISA data (i.e., OD values) were analyzed by using one-way analysis of variance (ANOVA). The differences between OD values were compared by using the two-sided t test with the Bonferroni-Holm correction for multiple comparisons (4). All statistical analyses were performed with the computer program R (version 2.2.8).

RESULTS

Expression and purification of recombinant EBOV and MARV GPs. The expression and secretion of His-EBOV-GP and His-MARV-GP in the supernatants of 293T cells transfected with a plasmid encoding His-GP were confirmed by immunoblotting using anti-GP and anti-His MAbs (data not shown). These recombinant GPs were purified as described in Materials and Methods. All purified His-GPs were detected by SDS-PAGE and immunoblotting using anti-GP and anti-His MAbs as prominent protein bands of the predicted size of the transmembrane anchor-minus EBOV and MARV GPs (Fig. 2). These purified GPs were used as antigens for the ELISA described in the following experiments.

Sensitivity of the GP-based ELISA. The sensitivity of the purified GP-based ELISA was tested by using anti-EBOV-GP MAB ZGP42/3.7 and anti-MARV-GP MAB AGP127-8. Serial 10-fold dilutions of the antibodies (10 −5 to 10 −2 μg/ml) were prepared, and the reactivity to each GP antigen was examined (Fig. 3a to c). The negative-control MAB, S139/1, did not bind to any His-GPs in the ELISA. At concentrations ranging from 0.1 μg/ml to 100 μg/ml, ZGP42/3.7 reacted with all His-EBOV-GPs but not His-MARV-GP, whereas AGP127-8 reacted specifically with His-MARV-GP but not any of the His-EBOV-GPs. The detection limit for specific antibodies using this assay was...
was approximately 0.01 to 0.1 μg/ml. On the other hand, ELISA using membrane lysates of GP-transfected cells or VLPs under similar conditions with the GP-based ELISA showed lower sensitivity, except for the Angola serum and VLP combination (Fig. 3d to i). This is most likely due to the interference by the residual detergent and/or irrelevant proteins in the lysates and VLP antigen preparations.

**Specificity of the GP-based ELISA.** Next, the species specificity of the ELISA was assessed by testing the antisera of mice immunized with VLP containing the respective EBOV and MARV GPs. We found that species-specific IgG antibodies were clearly detected in these mouse antisera (Fig. 4a to f). All the anti-EBOV IgG antibodies in the sera showed low reactivity to heterologous EBOV GPs, and no cross-reactivity to MARV GP was found (Fig. 4a to e). Similarly, anti-MARV VLP serum antibodies reacted to MARV GP but not to EBOV GPs (Fig. 4f). These results indicated that this purified GP-based ELISA sufficiently detected filovirus species-specific antibodies. On the other hand, the VLP-based ELISA was less sensitive and detected more appreciable cross-reactive antibodies in some of the mouse sera, likely specific to NP and VP40, than the purified GP-based ELISA (Fig. 4g to i).

**Analysis of clinical samples in the GP-based ELISA.** To further confirm the specificity of our ELISA, we used convalescent-phase plasma samples obtained from monkeys experimentally infected with ZEBOV or SEBOV (Fig. 5). The cutoff OD values (i.e., the mean plus 3 standard deviations of the five negative serum samples) were 0.23, 0.22, 0.29, 0.22, 0.17, 0.20, and 0.13 for His-ZEBOV-GP, His-SEBOV-GP, His-CIEBOV-GP, His-BEBOV-GP, His-REBOV-GP, His-MARV-GP, and control antigens, respectively. According to these thresholds, all infected monkey serum samples tested were EBOV antibody positive. We detected IgG antibodies in the ZEBOV-infected monkey plasma with higher reactivity against His-ZEBOV-GP than against any heterologous GP antigens. Although IgG antibodies in the SEBOV-infected monkey plasma showed binding to all His-EBOV-GPs, the highest reactivity was observed with the homologous antigen His-SEBOV-GP. Neither of these plasma antibodies reacted with MARV GP.

We then examined IgG antibody levels in serum or plasma derived from ZEBOV-, SEBOV-, and MARV-infected patients (Fig. 6a). The cutoff OD values obtained from the five negative-control sera for IgG antibodies were 0.20, 0.17, 0.24, 0.18, 0.14, 0.27, and 0.23 for His-ZEBOV-GP, His-SEBOV-GP, His-CIEBOV-GP, His-BEBOV-GP, His-REBOV-GP, His-MARV-GP, and control antigens, respectively. For most of the samples tested, IgG antibodies to homologous GP antigens were detected with the highest reactivity (Fig. 6a). All of the samples derived from ZEBOV-infected patients cross-reacted with His-CIEBOV-GP and His-BEBOV-GP antigens, whereas only one of the SEBOV-infected human samples (sample 9) showed cross-reactivity with His-MARV-GP. Overall, the level of cross-reactivity was consistent with the phylogenetic relationship among EBOV species (Fig. 1). On the other hand, for most of the samples from patients infected with MARV Angola, IgG antibodies to His-MARV-GP were specifically detected, except for specimen 17, which showed no IgG response to any GP. Interestingly, IgG antibodies detected in specimen 11 showed remarkable cross-reactivity with the heterologous antigens His-CIEBOV-GP and His-BEBOV-GP.

We next evaluated whether GP-specific IgM antibodies could be detected in the patient serum or plasma samples using the GP-based ELISA (Fig. 6b). The cutoff values for IgM ELISA were 0.23, 0.32, 0.31, 0.28, 0.30, 0.22, and 0.36 for His-ZEBOV-GP, His-SEBOV-GP, His-CIEBOV-GP, His-BEBOV-GP, His-REBOV-GP, His-MARV-GP, and control antigens, respectively.
antigens, respectively. ZEBOV- or SEBOV-specific IgM antibodies were detected only in patients 2, 6, 9, and 10. In contrast, MARV-specific IgM antibodies were detected in 8 out of the 11 specimens derived from MARV Angola-infected patients. No obvious IgM cross-reactivity to heterologous GP antigens was found in these samples.

**DISCUSSION**

In this study, we established a GP-based ELISA to detect filovirus species-specific antibodies. To date, lysates from Vero E6 cells infected with live EBOV and MARV or recombinant EBOV and MARV NPs have been used as antigens in ELISAs for the detection of filovirus-specific antibodies (5, 7, 17). Since the NPs of EBOV and MARV contain similar amino acid sequences (18), common antibody epitopes seem to be present (12). Indeed, cross-reactivity among all EBOV species was to be expected (16, 17). Therefore, NP antigens may be useful for the detection of genus-specific antibodies but not for the detection of species-specific humoral responses (7, 16, 17).

The heterogeneity of EBOV and MARV GPs has been demonstrated at the genetic level through sequence analyses (17, 19). An ELISA using recombinant ZEBOV GP expressed in a baculovirus-insect cell expression system was reported previously (16), but it is known that the protein glycosylation pathways in insect cells differ from those in mammalian cells (6). This may significantly affect the antigenic properties of filovirus GPs, since large amounts of both N- and O-linked carbohydrate chains are present in GP molecules. To overcome this difficulty, we used mammalian 293T cells for the expression of GP antigens and verified the sensitivity and specificity of GP-based ELISAs. Our results were consistent with a previous study suggesting that anti-EBOV GP antibodies were highly species specific and showed little cross-reactivity to GPs of other EBOV species (27). These findings indicated that most antibodies induced against filovirus GPs recognized...
epitopes in the variable regions of the protein. Expectedly, the serological classification mirrors the phylogenetic relationship of the different GPs (Fig. 1). Interestingly, serological characterization of anti-BEBOV antibodies clearly supports the molecular investigations (31) suggesting that BEBOV represents a new species within the EBOV genus.

IgG antibodies in some of the serum and plasma samples collected from infected monkeys and humans showed appreciable cross-reactivity to heterologous antigens, whereas antibodies in the mouse sera produced by immunization with VLPs specifically reacted to the homologous antigens. This result led us to the conjecture that VLP immunization and live-virus infection induce a distinct antibody repertoire or that the antibody repertoire of mice differs from that of primates. Interestingly, the plasma of patient 11 infected with MARV Angola contained IgG, but not IgM, antibodies cross-reactive to His-CIEBOV-GP and His-BEBOV-GP. It might be possible that prior to infection with MARV Angola, this patient was infected with CIEBOV, BEBOV, or another unknown filovirus whose GP has epitopes shared among CIEBOV and BEBOV. In the plasma of patient 17, neither IgG nor IgM antibodies were readily detected. An explanation for this observation might be differences of immunological conditions in individuals, or alternatively, the blood samples have been collected before a detectable antibody response was induced.

Notably, our GP-based ELISA detected MARV Angola-specific IgM antibodies in most of the plasma samples collected during the acute or subacute phase of infection, although it was reported previously that the detection of antibodies is of only limited use for acute-case diagnosis due to a lack of a detectable antibody response (8). The present study suggests that if proper antigen and sensitive assays are available, IgM antibodies can be useful for the diagnosis of acute EBOV and MARV infections and support the use of antigen capture ELISA and reverse transcription-PCR, the most commonly used technologies.

Despite the more recent discovery of REBOV in domestic pigs in the Philippines (1) and the discovery of fruit bat species as potential reservoirs for EBOV and MARV (9, 15, 30, 32), the search for the reservoirs and potential amplifying hosts remains ongoing. Advanced diagnostic technologies are welcome here, and our new GP-based species-specific antibody detection ELISA may be a useful tool for future ecological and seroepidemiological studies in areas of Central Africa and parts of Asia where the disease is endemic.
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