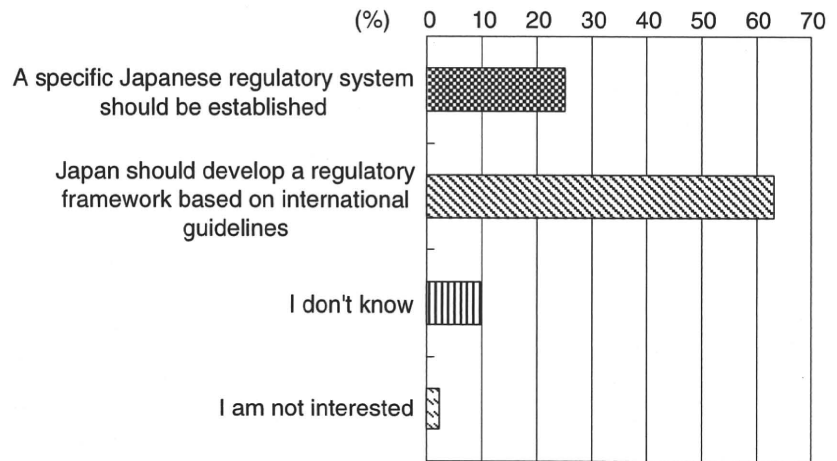**Fig. 7** What do you think about the regulatory framework regarding the medical applications of research and development into iPS cells and RM?

current situation, with increased interactions between scientists and journalists, works in a positive direction [13].

Requirement for Governance

It is important to recognize that the Japanese public thought that research and development of iPSCs and RM should be conducted under proper governance, in accordance with an international regulatory framework (Fig. 7). In addition, approximately half of the respondents thought that it was important to progress with research and development with international cooperation (Fig. 4). Thus the establishment of proper governance at the international level needs to be discussed, and discussions relating to the regulatory framework for research and development into PSCs, including iPSCs, are currently being conducted at the highest levels worldwide. For example, several international guidelines concerning general research and the clinical application of PSCs were presented by the International Society for Stem Cell Research (ISSCR) [14, 15]. However, the ISSCR is an academic society and their proposed

guidelines cannot be legally enforced in all countries. Moreover, the pace of regulatory developments differs between countries. In January of this year, the United States FDA approved safety evaluations regarding the clinical applications of ES cells [16]. International consensus guidelines are required to cover the safety aspects of such research and its medical applications.

In order to formulate such international guidelines, it is imperative to actively involve regulatory agencies to oversee safety testing, risk evaluation of tumorigenicity and clinical research procedures for cellular therapeutics. Current reports that have discussed the tumorigenicity risk of PSCs have emphasized the importance of appropriate preclinical studies and have pointed out the necessity for rapid discussions on evaluating safety standards and the effectiveness of PSCs in clinical applications [17, 18]. Yamanaka, one of the key researchers into iPSCs and RM, pointed out the risk of cancer due to iPSC implantation in long-term mouse experiments. He emphasized the importance of long-term safety and tumorigenicity risk evaluations [19]. Concerning this problem, a detailed description of regulatory systems will be

Fig. 8 Which of the following ideas about the use f iPS cells to make germ cells is closest to your opinion?
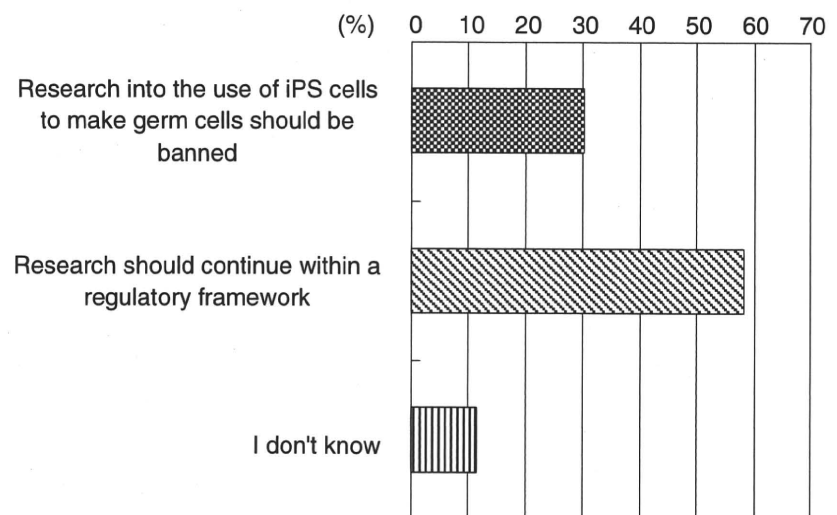
needed, considering the potential problems that can arise from imperfect articulation of regulatory guidelines concerning the clinical applications of RM and biologics derived from stem cells [20].

In addition to the safety aspects, ethical aspects are also important. In this study, subjects were asked about the issue of making germ cell from iPSCs. More than half of the respondents thought that research should progress within a carefully established regulatory framework (Fig. 8). In Japan, a MEXT sub-committee approved the production of germ cells from PSCs for basic research in December 2008, though the fertilization of germ cells derived from PSCs is banned [21]. In the present study, we were unable to investigate in detail the public opinion on research concerned with making germ cells from iPSCs. In addition, this investigation was conducted over a different time period from that when the political decision on moderating research into making germ cells from iPSCs was made. Further research into the changes in public opinion and social, political, and scientific changes is needed.

Thus, in order to address the public requirement for the development of proper regulatory frameworks, it will be necessary to establish an international consensus on regulatory frameworks that has domestic legal authority to govern stem cell research and its application in each country. An internationally consistent regulatory system will promote future worldwide research and development into iPSCs and RM

Conclusion

The results of this study show that the Japanese public was familiar with the terms iPSCs and RM, and that they generally accepted the necessity of research into iPSCs. At the same time, they decided to adopt a "wait and see" approach, and thought that research and development into iPSCs and RM should be conducted under the proper regulations, in accordance with an international regulatory framework. This seems to demonstrate the prudence of the Japanese public. In order to address the public requirements, it will be necessary to discuss an international consensus on regulatory frameworks that have an impact on domestic stem cell research and its application in each country. It is also important to consider the methods of information flow concerning iPSCs and RM, and further research into this issue will be conducted.

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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.

Ebola virus (EBOV) and Marburg virus (MARV) belong to the family *Filoviridae* and cause severe hemorrhagic fever in primates (20). While MARV consists of a single species, Lake Victoria marburgvirus, four distinct EBOV species are known: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire ebolavirus* (CIEBOV), and *Reston ebolavirus* (REBOV). The phylogenetically distinct *Bundibugyo ebolavirus* (BEBOV) was recently identified in Uganda and was proposed to be a new species of EBOV (Fig. 1) (31).

EBOV and MARV are filamentous, enveloped, single-stranded, negative-sense RNA viruses. The virus genome encodes seven structural proteins, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L). EBOV also expresses at least one secreted nonstructural glycoprotein (sGP) (20). GP is responsible for receptor binding and fusion of the viral envelope with host cell membranes (11, 22, 35) and has an important role in the pathogenesis of filovirus infection (3, 23, 36). GP is the main target of neutralizing antibodies, and most of the known ZEBOV-specific monoclonal antibodies (MAbs) show little cross-reactivity to other filovirus species (24, 27, 34).

Serological diagnostic methods based on enzyme-linked im-

munosorbent assays (ELISAs) using the recombinant EBOV and MARV NP antigens have been developed to detect filovirus-specific antibodies (5, 17). Using a ZEBOV NP antigen, NP-specific antibodies were broadly detected in animals infected with ZEBOV, SEBOV, CIEBOV, or REBOV (17), indicating strong cross-reactivity among EBOV species. It is predicted, however, that the antibody response to GP is more species specific due to the larger genetic variability with this protein, which is supposed to be the main target of the host humoral immune response. Therefore, in this study we developed a filovirus species-specific ELISA using recombinant GP antigens to serologically distinguish filovirus species.

MATERIALS AND METHODS

Plasmids. Viral RNA extracted from the supernatant of Vero E6 cells infected with ZEBOV, SEBOV, CIEBOV, BEBOV, REBOV, or 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 MAb ZGP42/3.7 (IgG1) (24, 26), which recognizes a linear epitope on GP comprising the sequence GEWAFWENKKN, and MARV GP-specific MAb AGP127-8 (IgG1) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) and RPMI medium (Sigma), respectively, supplemented with fetal calf serum (FCS) and antibiotics. Mouse ascites were obtained by a standard procedure, and MAbs were purified from ascites fluid using protein A-agarose columns (Bio-Rad). The 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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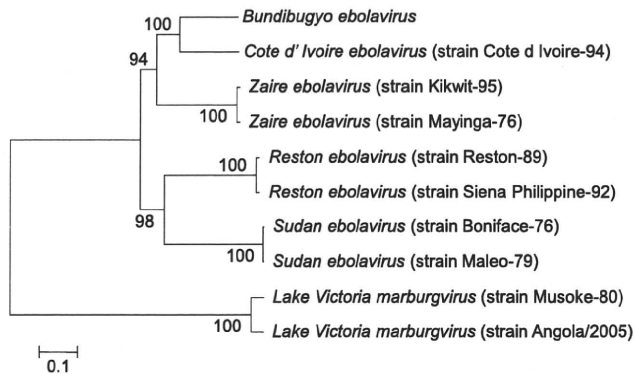
FIG. 1. Phylogenetic analysis of filovirus GP amino acid sequences. The phylogenetic tree was constructed by using the neighbor-joining method. For the construction of this tree, we used 10 GP amino acid sequences, each comprising a whole GP amino acid sequence. Numbers at branch points indicate bootstrap 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 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 admitted 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-BEBOV-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-nitrilotriacetic 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 ZGP42/3.7, AGP127-8, 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 ultracentrifuged 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′,5,5′-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 Maleo-79, CIEBOV strain Côte d'Ivoire-94, BEBOV, REBOV strain Reston-89, REBOV strain Siena Philippine-92, MARV strain Musoke-80, and MARV strain Angola/2005 used in phylogenetic analyses were obtained from GenBank under accession numbers Q05320, P87666, Q66814, Q66798, Q66810, ACI28624, Q66799, Q89853, P35253, and Q1PD50, 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
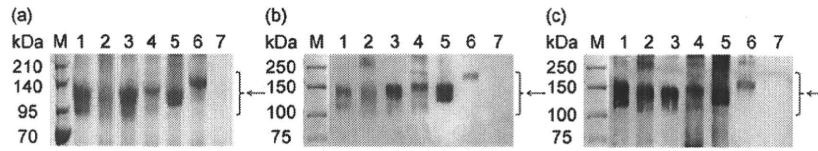
FIG. 2. Identification and characterization of purified His-GPs. (a) His-EBOV-GP and His-MARV-GP were analyzed by 8% SDS-PAGE and stained with Coomassie brilliant blue. (b and c) Immunoblotting of purified His-GPs was performed by using MAbs to EBOV (ZGP42/3.7) and MARV GPs (AGP127-8) (b) and His tags (c). Arrows indicate the locations of the His-GPs. The protein bands represent His-ZEBOV-GP (lane 1), His-SEBOV-GP (lane 2), His-CIEBOV-GP (lane 3), His-BEBOV-GP (lane 4), His-REBOV-GP (lane 5), and His-MARV-GP (lane 6). Lane 7 shows FCS-derived proteins used as a control antigen (see Materials and Methods).

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 anti-

bodies 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

◆Zaire ■Sudan ▲Cote d'Ivoire ◇Bundibugyo □Reston △Angola ×Control antigens
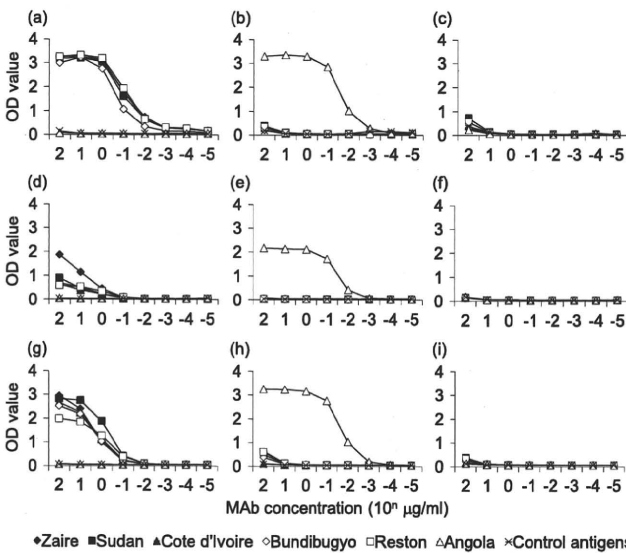
FIG. 3. Sensitivity of ELISAs. His-GPs (a, b, and c), GP-expressing cell lysates (d, e, and f), and VLP (g, h, and i) were used as antigens. The GP amounts were standardized by Western blotting as described in Materials and Methods. Serial 10-fold dilutions of MAbs to EBOV (a, d, and g) and MARV (b, e, and h) were prepared and tested. S139/1 (specific to influenza virus hemagglutinin) was used as a negative-control antibody (c, f, and i).
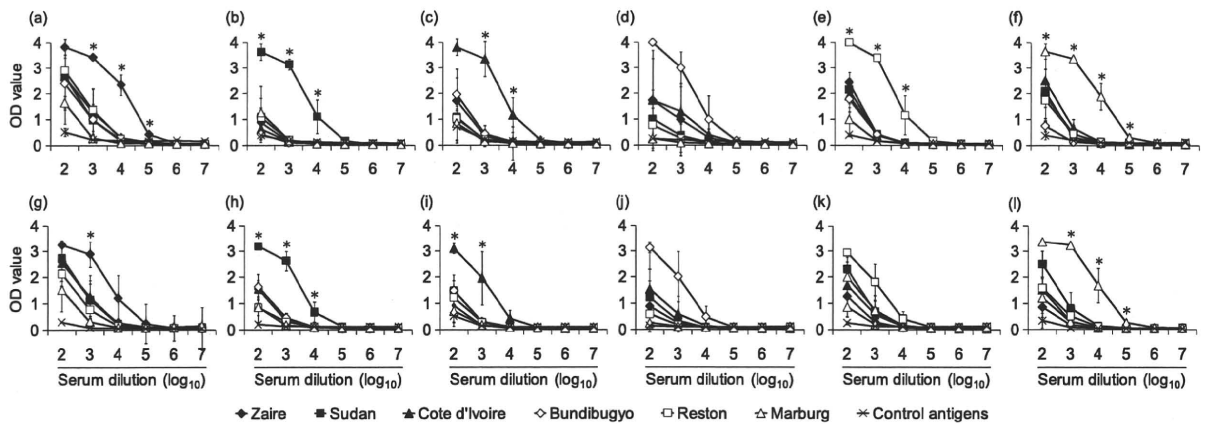
FIG. 4. IgG antibodies detected in mouse antisera. Serial 10-fold dilutions of anti-ZEBOV (a and g), anti-SEBOV (b and h), anti-CIEBOV (c and i), anti-BEBOV (d and j), anti-REBOV (e and k), and anti-MARV (f and l) sera obtained from mice immunized with EBOV and MARV VLPs were tested for IgG antibodies reacting with His-GPs (a, b, c, d, e, and f) and VLPs (g, h, i, j, k, and l). Averages and standard deviations for three mice of each group are shown. Asterisks indicate statistically significant differences in OD values between the homologous antigen and all other antigens (P < 0.05).

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
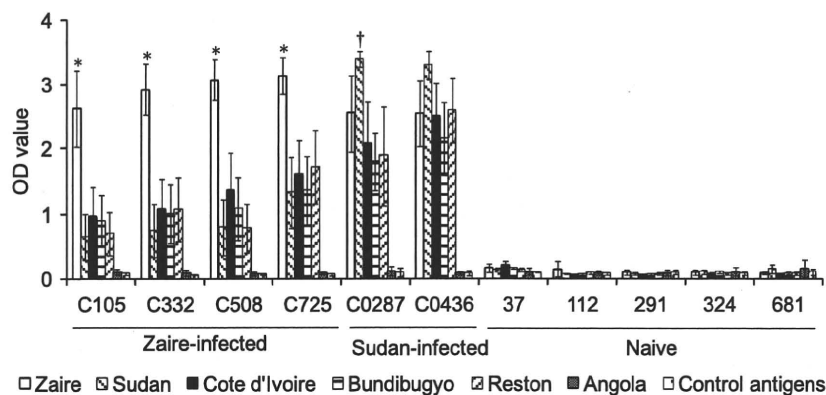

FIG. 5. IgG antibodies detected in experimentally infected monkey plasma by ELISA using His-GPs. Monkeys C105, C332, C508, and C725 were infected with ZEBOV, whereas monkeys C0287 and C0436 were infected with SEBOV. Infected monkey sera were diluted at 1:1,000. Naïve monkey sera were diluted at 1:100. Each bar represents the average and standard deviation of data from three independent experiments. Asterisks indicate statistically significant differences in OD values between the Zaire antigen and all other antigens (P < 0.05). The dagger shows statistically different reactions between His-SEBOV-GP and all the other antigens (P < 0.05) except His-ZEBOV-GP.
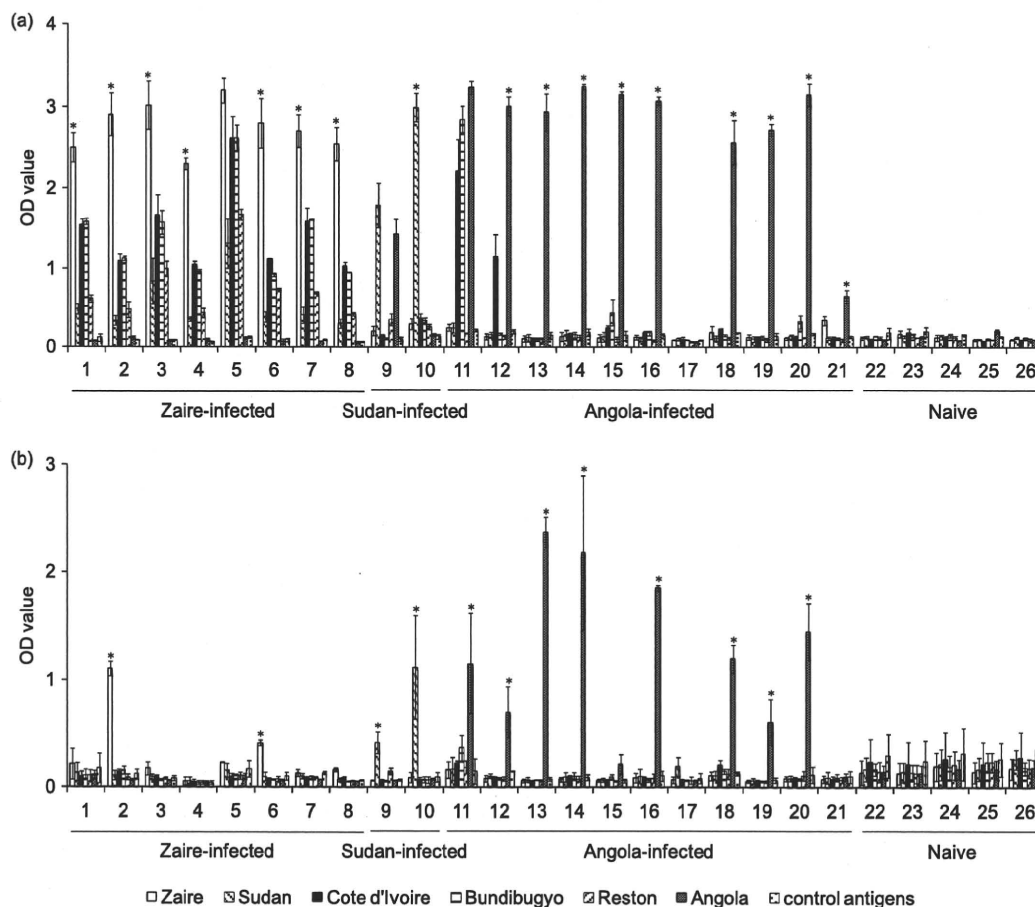
FIG. 6. IgG and IgM antibodies detected in human samples. OD values for specific IgG (a) and IgM (b) antibodies in the patient sera are shown. Sera from 21 individuals were analyzed at 1:1,000 dilutions. Naïve human sera (1:100 dilution) were used as a negative control. Each bar represents the average and standard deviation of data from independent experiments. Asterisks indicate statistically significant differences in OD values between the homologous antigen and all other antigens (P < 0.05).

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 individu-

als, 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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