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Enzyme-Linked Immunosorbent Assays for Detection of Antibodies to Ebola and Marburg Viruses Using Recombinant Nucleoproteins

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The full-length nucleoprotein (NP) of Ebola virus (EBO) was expressed as a His-tagged recombinant protein (His-EBO-NP) by a baculovirus system. Carboxy-terminal halves of NPs of EBO and Marburg virus (MBG) were expressed as glutathione *S*-transferase-tagged recombinant proteins in an *Escherichia coli* system. The antigenic regions on the NPs of EBO and MBG were determined by both Western blotting and enzyme-linked immunosorbent assay (ELISA) to be located on the C-terminal halves. The C-terminal 110 and 102 amino acids of the NPs of EBO and MBG, respectively, possess strong antigenicity. The full-length NP of EBO was strongly expressed in insect cells upon infection with the recombinant baculovirus, while expression of the full-length NP of MBG was weak. We developed an immunoglobulin G (IgG) ELISA using His-EBO-NP and the C-terminal halves of the NPs of EBO and MBG as antigens. We evaluated the IgG ELISA for the ability to detect IgG antibodies to EBO and MBG, using human sera collected from EBO and MBG patients. The IgG ELISA with the recombinant NPs showed high sensitivity and specificity in detecting EBO and MBG antibodies. The results indicate that ELISA systems prepared with the recombinant NPs of EBO and MBG are valuable tools for the diagnosis of EBO and MBG infections and for seroepidemiological field studies.

The two members of the family *Filoviridae*, Ebola and Marburg viruses (EBO and MBG, respectively), are responsible for severe forms of hemorrhagic fevers. The first recognized outbreaks of Ebola hemorrhagic fever occurred in Zaire and Sudan in 1976 (3, 7, 22, 23). After the discovery of EBO in 1976, several African countries were struck by outbreaks of Ebola hemorrhagic fevers caused by one of the three known human-pathogenic EBO subtypes: Zaire (EBO-Z), Sudan (EBO-S), or Côte d'Ivoire (EBO-CI) (13–15). Another outbreak of Ebola hemorrhagic fever caused by EBO (Reston subtype [EBO-R]) occurred among captured cynomolgus macaques in the Philippines in 1989 (5). EBO-R was carried from the Philippines to the United States by infected monkeys in 1989, 1990, and 1996, as well as to Italy in 1992 (13). MBG was first identified in the outbreaks of hemorrhagic disease in Germany and Yugoslavia in 1967 among technicians and animal handlers who worked with vervet monkeys (*Cercopithecus aethiops*) imported from Uganda or with tissues from these monkeys (19, 20). Since then, there have been sporadic cases of hemorrhagic fever due to MBG infection on three occasions (in 1975 in South Africa and Zimbabwe; in 1980 and 1987 in Kenya) (4). Recently, a relatively large outbreak of MBG infections has occurred in the Durba region of the Democratic Republic of the Congo since 1998 (1, 24).

As EBO-R was introduced from the Philippines to the United States and MBG was introduced from Uganda to Europe, there is always a possibility that the deadly hemorrhagic fever viruses could be introduced to areas previously free from outbreaks. Therefore, preparation of diagnostic materials for EBO and MBG infections is important even in countries without outbreaks of Ebola or Marburg hemorrhagic fevers. However, EBO and MBG must be handled in a biosafety level 4 (BSL-4) facility. This restriction makes it difficult to prepare diagnostic materials for EBO and MBG infections. To overcome this difficulty, we developed enzyme-linked immunosorbent assays (ELISA) using recombinant filovirus nucleoproteins (NPs) to detect immunoglobulin G (IgG) antibodies to EBO and MBG. We demonstrated that the ELISA had high sensitivity and specificity for detection of EBO and MBG antibodies. Thus, our ELISA systems are useful for diagnosis and epidemiological studies.

MATERIALS AND METHODS

Recombinant transfer vector. An entire cDNA clone of EBO-Z NP was supplied by the Special Pathogens Branch, Centers for Disease Control and Prevention (CDC), Atlanta, Ga. (18). An entire cDNA clone of MBG NP was provided by H.-D. Klenk, Phillips University, Marburg, Germany (2). The DNA of EBO NP was amplified by PCR from the source using primers EBO (Z) NP/F (5′-CAAGGATCCGAGTATGGATTCTCG-3′) and EBO (Z) NP/R (5′-ATGGATCCATGCTCATTCACTGATG-3′) (the *Bam*HI site is underlined). The amplification conditions were as reported previously (17). The amplified DNA of the 2.2-kbp fragment was subcloned into the *Bam*HI site of pQE31 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE31-EBO-NP. The inserted EBO NP DNA was sequenced and confirmed to be identical to the original sequence in order to exclude PCR errors. The DNA fragment of EBO NP with a histidine (His) tag was isolated from plasmid pQE31-EBO-NP by digestion of the plasmid with *Eco*RI and *Hin*d III. Then it was repaired for blunting using Klenow enzyme and was ligated into pAcYM1 (12). The resultant

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TABLE 1. Summary of the serum samples used in the experiments[a]

Sample no.	Agent	Reagent Type[b], species	Description
E1	EBO-CI	HMAF, mouse	Anti-EBO-CI mouse ascites fluid
E2	EBO-R	Plasma, monkey	Anti-EBO-R monkey plasma
E3	EBO-R	Plasma, monkey	Anti-EBO-R monkey plasma
E4	EBO-R	Plasma, monkey	Anti-EBO-R monkey plasma
E5	EBO-R	HMAF, mouse	Anti-EBO-R mouse ascites fluid
E6	EBO-R	Serum, rabbit	Anti-EBO-R rabbit serum
E7	EBO-S	Plasma, monkey	Anti-EBO-S monkey plasma
E8	EBO-S	HMAF, mouse	Anti-EBO-S mouse ascites fluid
E9	EBO-Z	Plasma, human	Human EBO plasma collected in 1976 outbreak
E10	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E11	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E12	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E13	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E14	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E15	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E16	EBO-Z	Plasma, human	Human EBO plasma collected in 1995 outbreak
E17	EBO-Z	Serum, human	Human EBO serum collected in 1976 outbreak
E18	EBO-Z	Serum, human	Human EBO serum collected in 1976 outbreak
E19	EBO-Z	Serum, human	Human EBO serum collected in 1976 outbreak
E20	EBO-Z	Serum, human	Human EBO serum collected in 1977 outbreak
E21	EBO-Z	Serum, human	Human EBO serum collected in 1995 outbreak
E22	EBO-Z	Serum, human	Human EBO serum collected in 1995 outbreak
E23	EBO-Z	Plasma, monkey	Anti-EBO-Z monkey plasma
E24	EBO-Z	Serum, rabbit	Anti-EBO-Z rabbit serum
E25	EBO-Z	Serum, rabbit	Anti-EBO-Z rabbit serum
E26	His-EBO-NP	Serum, rabbit	Anti-His-EBO-NP rabbit serum
M1	MBG	Serum, human	Human MBG serum
M2	MBG	Serum, human	Human MBG serum
M3	MBG	Serum, human	Human MBG serum
M4	MBG	Serum, monkey	Anti-MBG monkey serum
M5	MBG	HMAF, mouse	Anti-MBG mouse ascites fluid
M6	MBG	Serum, rabbit	Anti-MBG rabbit serum
M7	MBG	Serum, rabbit	Anti-MBG rabbit serum
M8	His-MBG-NP	Serum, rabbit	Anti-His-MBG-NP rabbit serum

[a] Serum samples were collected from EBO- or MBG-infected humans or from animals that were infected with EBO or MBG or that had been immunized with recombinant NPs of EBO or MBG.
[b] HMAF, hyperimmune mouse ascites fluid.

recombinant transfer vector with the correct orientation to the promoter was designated pAcYM1-His-EBO-NP.

The recombinant pAcYM1 transfer vector (pAcYM1-His-MBG-NP), which carries the DNA of the His tag and the entire MBG NP, was also constructed in the same way as pAcYM1-His-EBO-NP with some modifications. The MBG NP DNA, which was amplified from the source DNA using primers MBG-N (Bcl)/F (5′-TATTGATCAACACAGTTTGTTGGAGTTG-3′ [the *Bcl*I site is underlined]) and MBG-N (Hind)/R (5′-GCTAAGCTTATCTGGACTACAAGTTCA TCGC-3′ [the *Hin*dIII site is underlined]), was subcloned into the appropriate cloning site of pQE31 vector DNA (QIAGEN). Subsequent procedures were as described above.

Sera and plasma. The sera and plasma used in the study are summarized in Table 1. Of 26 anti-EBO serum samples, 14 were collected from EBO-infected patients in the Democratic Republic of the Congo (formerly called Zaire) in 1976 (7) and 1995 (14, 15). Serum samples were also collected from animals infected with EBO-Z, EBO-CI, EBO-S, or EBO-R and from rabbits immunized with the purified recombinant NP of EBO-Z (His-EBO-NP).

Of eight MBG antibody-positive serum samples, three were collected from MBG-infected patients and the others were collected from animals infected with MBG or from rabbits immunized with the purified NP of MBG (His-MBG-NP).

Forty-eight serum samples collected from people without EBO or MBG infections in West African countries were used as the control sera.

Generation of recombinant baculoviruses. *High five* (Tn5) insect cells were transfected with mixtures of purified *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) DNA and the recombinant pAcYM1 vector (pAcYM1-His-EBO-NP or pAcYM1-His-MBG-NP) by the procedures described by Kitts et al. (8), with the modifications of Matsuura et al. (12), resulting in the production of recombinant baculoviruses. The baculoviruses, which express His-EBO-NP and His-MBG-NP, were designated Ac-His-EBO-NP and Ac-His-MBG-NP, respectively. A baculovirus (Ac-ΔP) which lacks polyhedrin expression was used as a negative-control virus.

Expression of whole NPs of EBO and MBG. The recombinant baculoviruses were grown in Tn5 cells as reported previously (12). Tn5 insect cells, which were infected with Ac-His-EBO-NP or with Ac-His-MBG-NP, were incubated at 26°C for 72 h. Then the cells were washed twice with phosphate-buffered saline (PBS) and were lysed in cold 1% NP-40–PBS solution. The cell lysate was centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant fraction was collected as a source of recombinant His-EBO-NP for purification. The His-EBO-NP was purified with a Ni^{2+}-resin purification system (QIAGEN GmbH) according to the manufacturer's instructions. The expression of recombinant His-EBO-NP and His-MBG-NP was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11) gels (10% polyacrylamide) stained with Coomassie blue and was confirmed by Western blot analysis using anti-His antibody (RGS-His Antibody; QIAGEN GmbH) (17).

Expression of truncated NPs. It was found by a preliminary experiment that the C-terminal halves of EBO NP and MBG NP possessed strong antigenic activities. So the C-terminal halves of EBO NP and MBG NP were each divided into four fragments as shown in Fig. 1. The DNA corresponding to each truncated NP fragment was amplified with the primer sets designed. Each amplified DNA was subcloned into the cloning site of plasmid pGEX-2T (Amersham Pharmacia Biotech, Tokyo, Japan). Each insert was sequenced and was confirmed to be identical to the original sequence. The glutathione *S*-transferase (GST)-tagged truncated NPs of EBO and MBG, and GST alone, were expressed in an *Escherichia coli* (BL21 strain) system and were purified using glutathione Sepharose 4B (Amersham Pharmacia Biotech).

The recombinant GST-tagged EBO and MBG NPs were designated GST-EBO-NP/C-half and GST-EBO-NP5 to -NP8, and GST-MBG-NP/C-half and GST-MBG-NP5 to -NP8, respectively, as shown in Fig. 1.

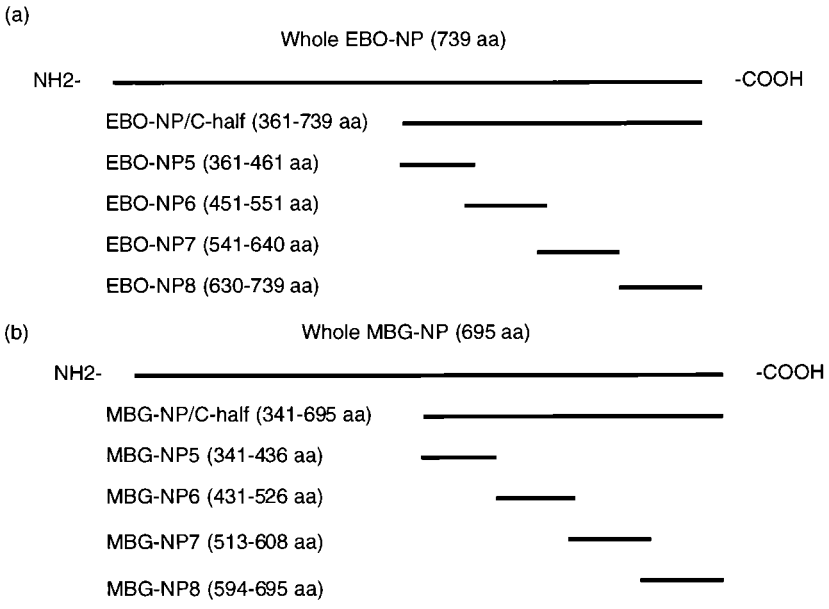

FIG. 1. Whole and truncated NPs of EBO (a) and MBG (b). Truncated EBO and MBG NPs were expressed as fusion proteins with GST on the N-terminal side.

Western blotting. The recombinant NP fragments (His-EBO-NP, GST-EBO-NP/C-half, and GST-EBO-NP5 to -NP8; His-MBG-NP, GST-MBG-NP/C-half, and GST-MBG-NP5 to -NP8; GST) were tested for reactivity to the serum samples in Table 1 by Western blotting (17).

ELISA. ELISA was performed as described previously (10) except for the antigen preparation. For the IgG ELISA to detect antibodies to EBO and MBG in human sera, ELISA plates were coated with a predetermined optimal quantity of recombinant NP antigens (usually 25 to 50 ng/well) at 4°C overnight. The plates were inoculated with test samples (100 μl/well), which were diluted fourfold from 1:100 to 1:6,400 with 0.05% Tween 20–PBS containing 5% skim milk (T-PBS-M). The adjusted optical density (OD) measured at 410 nm was calculated by subtracting the OD of the noncoated well from that of the corresponding His-EBO-NP antigen-coated well. In experiments where GST-tagged recombinant NP fragments were used, the adjusted OD was calculated by subtracting the OD of the GST-coated well from that of the corresponding well. The means and standard deviations (SD) of the adjusted ODs were calculated from those of the 48 control sera. The cutoff value for the assay was set at the mean plus 3 SD.

Antigenic index. We defined the term "antigenic index" to compare the degrees of antigenic activity among the truncated and GST-tagged NP fragments. First, ELISA plates were coated with the GST-tagged truncated NPs (GST-EBO-NP/C-half, EBO-NP5 to -NP8, GST-MBG-NP/C-half, MBG-NP5 to -NP8, and GST) at 4°C overnight to express the equal antigenic activities of GST in ELISA using anti-GST goat serum (1:1,000; Amersham Pharmacia Biotech). Then ELISA was performed against the GST-tagged NP fragments of EBO and MBG n each test sample. The plates were inoculated with each test sample (100 μl/ml) diluted with T-PBS-M at twofold dilutions of 1:500 through 1:64,000 (see Table 2).

Examples of the calculation of the antigenic index are shown in Table 2. The antigenic index was calculated as the maximum dilution at which the OD was over the threshold divided by the dilution level at the threshold. The threshold was defined as the OD of the GST-coated well at a given dilution (Table 2). Thus, the higher the antigenic index, the stronger the antigenic activity. In every experiment, anti-GST goat serum (1:1,000; Amersham Pharmacia Biotech) was tested to confirm the equality of antigenic activity of GST among the GST-tagged NP fragments on the ELISA plates.

RESULTS

Expression and purification of recombinant NPs of EBO and MBG. Expression of His-EBO-NP and His-MBG-NP in

TABLE 2. Definition of the antigenic index

Dilution level of test serum	OD of ELISA well coated with:					
	GST-EBO- or GST-MBG-					GST
	NP/C-half	NP5	NP6	NP7	NP8	
500	>1.999	1.130	>1.999	0.893	>1.999	0.324[a]
1,000	>1.999	0.415	0.915	0.775	>1.999	0.223
2,000	>1.999	0.482	0.624	0.826	>1.999	0.25
4,000	>1.999	0.407	0.379	0.330	1.814	0.236
8,000	1.386	0.255	0.495	0.204	1.284	0.222
16,000	0.926	0.243	0.403	0.253	0.763	0.184
32,000	0.458	0.146	0.207	0.265	0.470	0.271
64,000	0.442	0.22	0.138	0.315	0.294	0.267
Calculation[b]	>64,000/500	4,000/500	16,000/500	4,000/500	32,000/500	500/500
Antigenic index	>128	8	32	8	64	1

[a] The threshold was defined as the OD of the GST-coated well at a 1:500 dilution, 0.324 in this case. For each antigen, the OD at the maximum dilution level at which the OD is higher than the threshold is underlined.

[b] The antigenic index of each GST-EBO-NP and GST-MBG-NP fragment was calculated as the maximum dilution level at which the OD was over the threshold divided by the dilution level at the threshold.

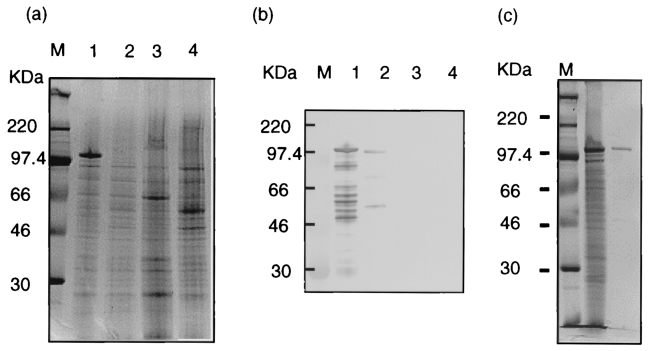

FIG. 2. (a) SDS-PAGE analysis of expression of recombinant His-EBO-NP and His-MBG-NP in the supernatant fractions from 1% NP-40 lysates of Tn5 cells infected with Ac-His-EBO-NP (lane 1), Ac-His-MBG-NP (lane 2), or Ac-ΔP (lane 3), or mock infected (lane 4). M, marker. (b) Demonstration of expressed His-tagged EBO NP and MBG NP by Western blotting using the monoclonal antibody to His tag (RGS-His Antibody; QIAGEN) in Tn5 cells infected with the recombinant baculovirus Ac-His-EBO-NP (lane 1), Ac-His-MBG-NP (lane 2), or Ac- ΔP (lane 3), or mock infected (lane 4). (c) SDS-PAGE analysis of purified His-EBO-NP. Left lane, marker (M); center lane, supernatant fraction from 1% NP-40 lysate of Ac-His-EBO-NP-infected Tn5 cells; right lane, purified His-EBO-NP.

insect cells upon infection with Ac-His-EBO-NP and Ac-His-MBG-NP, respectively, was demonstrated by SDS-PAGE analysis (Fig. 2a and c). Expression of His-EBO-NP was high, whereas expression of His-MBG-NP was relatively low. Although the expression of His-MBG-NP was weak, it was demonstrated by Western blotting (Fig. 2b). GST-EBO-NP/C-half, GST-EBO-NP5 to -NP8, GST-MBG-NP/C-half, and GST-MBG-NP5 to -NP8 were strongly expressed in *E. coli* and could be purified (data not shown).

Antigenicities of truncated EBO and MBG NPs determined by Western blotting. All the sera reacted to His-EBO-NP in Western blotting (Table 3). Serum sample E19 reacted to GST and was excluded for the next evaluation. The remaining 25 EBO serum samples reacted to GST-EBO-NP/C-half, and 24 of these (all but E9) reacted to GST-EBO-NP8. Nineteen, 19, and 6 of 25 tested sera reacted to GST-EBO-NP5, -NP6, and -NP7, respectively. These results suggest that whole NP of EBO (His-EBO-NP), GST-EBO-NP/C-half, and GST-EBO-NP8 are promising candidates for antigens in an EBO antibody detection system.

All the eight MBG sera reacted to whole MBG NP, GST-MBG-NP/C-half and GST-MBG-NP8 (Table 4). Six and five of the eight sera reacted to GST-MBG-NP5 and GST-MBG-NP6, respectively, but only one serum reacted to GST-MBG-NP7.

TABLE 3. Reactivities of the truncated NP fragments of EBO to EBO antibody-positive samples determined by Western blotting and ELISA

Sample	Reactivity[a]/antigenic index of:						
	His-EBO-NP	GST-EBO-					GST
		NP/C-half	NP5	NP6	NP7	NP8	
E1	+	+/16	+/8	−/4	−/2	+/8	−/1
E2	+	+/8	−/1	−/1	+/4	+/8	−/1
E3	+	+/4	−/1	+/4	−/1	+/8	−/1
E4	+	+/8	−/1	+/8	−/2	+/16	−/1
E5	+	+/64	+/64	+/32	−/4	+/32	−/1
E6	+	+/64	+/8	−/1	−/8	+/64	−/1
E7	+	+/NT[b]	−/NT	+/NT	−/NT	+/NT	−/NT
E8	+	+/>128	+/32	+/32	+/32	+/>128	−/1
E9	+	+/8	+/1	+/2	−/1	−/16	−/1
E10	+	+/>128	+/8	+/16	−/8	+/>128	−/1
E11	+	+/>128	+/4	+/16	+/8	+/>128	−/1
E12	+	+/>64	+/2	+/2	−/2	+/64	−/1
E13	+	+/>128	+/16	+/16	−/16	+/>128	−/1
E14	+	+/>128	+/2	+/8	−/16	+/>128	−/1
E15	+	+/32	−/4	+/4	+/4	+/64	−/1
E16	+	+/16	+/2	+/2	−/2	+/16	−/1
E17	+	+/8	+/2	+/1	−/1	+/2	−/1
E18	+	+/8	+/8	−/1	−/2	+/8	−/1
E19	+	+/32	+/2	+/16	+/1	+/2	+/1
E20	+	+/32	+/4	+/1	−/1	+/8	−/1
E21	+	+/>128	+/8	+/16	−/8	+/>128	−/1
E22	+	+/>128	+/2	−/2	−/1	+/64	−/1
E23	+	+/32	−/4	−/2	−/2	+/16	−/1
E24	+	+/>128	+/>128	+/>128	+/64	+/>128	−/1
E25	+	+/>128	+/32	+/64	−/16	+/>128	−/1
E26	+	+/>32	+/>32	+/16	+/>32	+/>32	−/1
No. of WB[c] positives	26	26	20	20	7	25	1
Avg $\log_2$ antigenic index[d]		5.2	2.4	2.6	2.1	4.9	0

[a] By Western blotting.
[b] NT, not tested.
[c] WB, Western blotting.
[d] In the calculation of $\log_2$ antigenic indices of >32, >64, and >128, the values of the antigenic indices were regarded as 32, 64, and 128, respectively.

TABLE 4. Reactivities of recombinant MBG NP fragments to MBG antibody-positive samples determined by Western blotting and ELISA

Sample	Reactivity[a]/antigenic index of:						
	His-MBG-NP	GST-MBG-					GST
		NP/C-half	NP5	NP6	NP7	NP8	
M1	+	+/>64	+/2	−/8	−/8	+/>64	−/1
M2	+	+/>64	−/2	+/8	−/2	+/>64	−/1
M3	+	+/NT[b]	+/NT	+/NT	−/NT	+/NT	−/1
M4	+	+/>16	−/2	−/2	−/2	+/>16	−/1
M5	+	+/16	+/4	+/8	−/2	+/8	−/1
M6	+	+/>128	+/64	+/>128	+/64	+/>128	−/1
M7	+	+/16	+/2	−/2	−/1	+/8	−/1
M8	+	+/>128	+/4	+/32	−/1	+/>128	−/1
No. of WB[c] positives	8	8	6	5	1	8	0
Average $\log_2$ antigenic index[d]		5.4	2.0	3.3	1.7	5.1	0

[a] By Western blotting.
[b] NT, not tested.
[c] WB, Western blotting.
[d] In the calculation of $\log_2$ antigenic indices of >16, >64, and >128, the values of the antigenic indices were regarded as 16, 64, and 128, respectively.

Antigenicities of the truncated NP fragments determined by ELISA. All the EBO sera reacted to GST-EBO-NP/C-half in ELISA, with antigenic indices of 4 to >128 (Table 3). Furthermore, the antigenic indices of GST-EBO-NP8 to all EBO sera were equal to or greater than 2. All the EBO sera reacted to GST-EBO-NP8 in ELISA. Although the E9 serum did not react to GST-EBO-NP8 in Western blotting, it reacted to GST-EBO-NP8 in ELISA with an antigenic index of 16. The average of the $\log_2$ antigenic indices for each fragment is shown in Table 3. Based on these results, we conclude that GST-EBO-NP/C-half and GST-EBO-NP8 possess the highest antigenicities followed by GST-EBO-NP6, GST-EBO-NP5, and GST-EBO-NP7.

Similar results were obtained using MBG NP fragments (Table 4). All the MBG sera reacted to GST-MBG-NP/C-half and -NP8 with, antigenic indices from 8 to >128. According to the average $\log_2$ antigenic indices, GST-MBG-NP/C-half and GST-MBG-NP8 possess the highest antigenicities among the truncated MBG NP fragments.

IgG ELISA for EBO antibody detection using His-EBO-NP, GST-EBO-NP/C-half, and GST-EBO-NP8. The mean and SD of ODs of the 48 control sera at a 1:400 dilution in ELISA using His-EBO-NP as an antigen were −0.004 and 0.120, respectively, resulting in a mean plus 3 SD of 0.356. According to this threshold, 13 of 14 EBO patients' serum samples were EBO antibody positive, giving an IgG ELISA sensitivity of 93% (Fig. 3a). Furthermore, 50 of 51 control sera, including 3 MBG sera, were judged negative, resulting in 98% specificity.

The mean and SD of ODs of the control sera in ELISA using GST-EBO-NP/C-half were 0.136 and 0.169, respectively (mean plus 3 SD, 0.644). According to this threshold, 13 of 14 EBO patients' sera were judged EBO antibody positive, and 50 of 51 control sera (including 3 MBG sera) were judged EBO antibody negative, resulting in 93% sensitivity and 98% specificity (Fig. 3b). If the threshold is set at 0.475, the value of the mean plus 2 SD, all the EBO patients' sera were positive and 49 of the 51 control sera were negative, resulting in 100% sensitivity and 96% specificity.

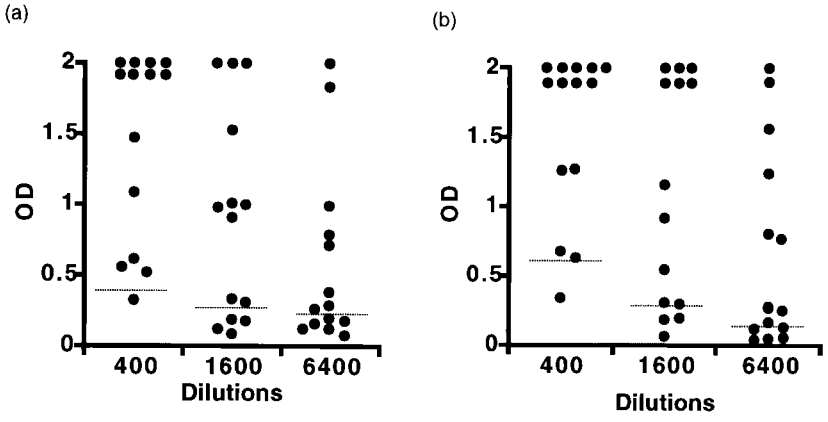

FIG. 3. Results of an IgG ELISA using His-EBO-NP (a) and GST-EBO-NP/C-half (b) for EBO antibody detection. The ODs of the 14 EBO sera at dilutions of 1:400, 1:1,600, and 1:6,400 are shown here. Lines indicate the threshold at each dilution level. The threshold was set at the mean plus 3 SD, which was calculated from the ODs of the 48 control sera.

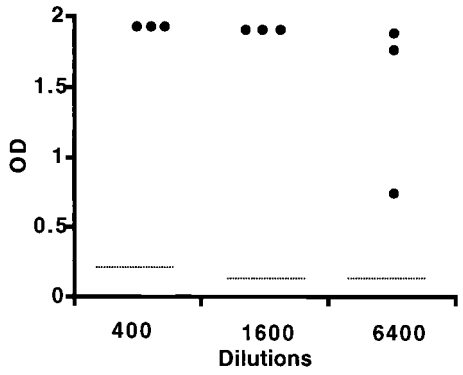

FIG. 4. IgG ELISA using GST-MBG-NP/C-half for MBG antibody detection. The ODs of the three MBG sera at dilutions of 1:400, 1:1,600, and 1:6,400 are shown here. Lines indicate the threshold at each dilution level. The threshold was set at the mean plus 3 SD, which was calculated from the ODs of the 48 control sera.

The mean and SD of ODs of the control sera at a 1:400 dilution in ELISA using GST-EBO-NP8 were 0.266 and 0.406, respectively. Because of high average and SD values, it was difficult to evaluate the efficacy of ELISA using GST-EBO-NP8.

IgG ELISA for MBG antibody detection using GST-MBG-NP/C-half. The mean and SD of ODs of the control sera at a 1:400 dilution in ELISA using GST-MBG-NP/C-half were −0.005 and 0.045, respectively. The threshold was set at 0.135 (the value of the mean plus 3 SD). According to this threshold, three MBG-positive sera (M1, M2, and M3) were judged MBG antibody positive and 62 control sera, including 14 EBO patients' sera, were judged MBG antibody negative (Fig. 4).

DISCUSSION

So far, ELISA and indirect immunofluorescent methods using cells infected with EBO have been developed and used for detection of EBO-specific antibody (6, 10, 21). These methods require preparation of antigens by handling live EBO and MBG. In order to make it possible to detect antibodies to EBO and MBG without handling the live viruses, we tried to develop an IgG ELISA using recombinant EBO NP and MBG NP.

We first attempted to understand the antigenicities of the recombinant EBO and MBG NPs. We revealed that EBO-NP8 and MBG-NP8, representing the C-terminal portion of NP, had the highest antigenicities by both Western blotting and ELISA. Furthermore, the C-terminal halves of the EBO and MBG NPs showed high levels of antigenicity, suggesting that these regions can be used as antigens for detection of EBO or MBG antibody. This report is the first demonstration of the antigenic regions within the NPs of filoviruses.

We evaluated the efficacy of an ELISA prepared with purified His-EBO-NP, GST-EBO-NP/C-half, or GST-EBO-NP8 as the antigen. The ODs of EBO sera, which were collected from EBO patients, by ELISA using His-EBO-NP and GST-EBO-NP/C-half at dilutions of 1:400, 1:1,600, and 1:6400 are shown in Fig. 3. We eventually set the threshold at the mean plus 3 SD of ODs of control sera tested at a 1:400 dilution. With this threshold, the ELISA system using these two recombinant EBO NPs has high sensitivity and specificity. One EBO patient's serum was judged negative in ELISA both with His-EBO-NP and with GST-EBO-NP/C-half. We included this serum as a positive-control serum because it was collected from an EBO-infected patient. Although data are not shown here, IgG antibody to EBO was detected in this serum by an indirect immunofluorescent method using recombinant EBO NP-expressing HeLa cells (submitted for publication).

Because the expression of His-MBG-NP was low in insect cells (Fig. 2), we evaluated GST-MBG-NP/C-half as an antigen for ELISA instead. Unfortunately, the sensitivity of IgG ELISA using GST-MBG-NP/C-half could not be evaluated in this study because the number of MBG antibody-positive sera was so small. Further study is needed to evaluate accurately the sensitivity of this ELISA system. However, all the MBG patients' sera tested MBG antibody positive. Furthermore, the specificity of the IgG ELISA was 100%. These results suggest that the ELISA may be useful not only for diagnosis of surviving MBG-infected patients but also for epidemiological field studies.

To determine the antigenic regions on the EBO NP, 26 sera, which contained IgG antibody to EBO-Z, EBO-S, EBO-CI, or EBO-R, were used. The IgG antibody induced by infection with EBO-S, EBO-CI, or EBO-R reacted to the recombinant EBO NP, His-EBO-NP, which was derived from EBO-Z, indicating that the recombinant EBO NP from EBO-Z can be used to detect IgG antibody not only to EBO-Z but also to other EBO subtypes.

Recently, Prehaud et al. reported the usefulness of the recombinant EBO NP and glycoprotein (Gabon 94 strain) for IgG and IgM antibody detection (16). They studied the efficacy of the recombinant EBO NP and glycoprotein expressed in an *E. coli* system using seven sera collected from EBO-infected patients.

Ksiazek et al. reported the limitation of EBO IgG detection for the diagnosis of EBO infection (9). Some patients with EBO infections died before the EBO IgG response. For the accurate diagnosis of acute EBO and MBG infections, some methods of detecting virus are necessary: antigen ELISA, reverse transcription-PCR, or isolation of infectious agents. IgM antibodies can also be useful for inferring recent onset of the immune response. Thus, other diagnostic systems, such as a filovirus antigen capture ELISA and an IgM capture ELISA using recombinant NPs of filoviruses, are under construction in our laboratory.

In conclusion, we have developed ELISA systems to detect IgG antibodies against filoviruses using recombinant NPs. These ELISA systems can be used for anti-filovirus antibody detection in a facility without a BSL-4 laboratory.

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