Antigen Capture Enzyme-Linked Immunosorbent Assay for Specific Detection of Reston Ebola Virus Nucleoprotein

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Antigen capture enzyme-linked immunosorbent assay (ELISA) is one of the most useful methods to detect Ebola virus rapidly. We previously developed an antigen capture ELISA using a monoclonal antibody (MAb), 3-3D, which reacted not only to the nucleoprotein (NP) of Zaire Ebola virus (EBO-Z) but also to the NPs of Sudan (EBO-S) and Reston Ebola (EBO-R) viruses. In this study, we developed antigen capture ELISAs using two novel MAbs, Res2-6C8 and Res2-1D8, specific to the NP of EBO-R. Res2-6C8 and Res2-1D8 recognized epitopes consisting of 4 and 8 amino acid residues, respectively, near the C-terminal region of the EBO-R NP. The antigen capture ELISAs using these two MAb detected the EBO-R NP in the tissues from EBO-R-infected cynomolgus macaques. The antigen capture ELISAs using Res2-6C8 and Res2-1D8 are useful for the rapid detection of the NP in EBO-R-infected cynomolgus macaques.

The family Filoviridae includes the genera Marburgvirus and Ebolavirus. The genus Ebolavirus has four species: Zaire Ebola virus (EBO-Z), Sudan Ebola virus (EBO-S), Côte-d’Ivoire Ebola virus (EBO-CI), and Reston Ebola virus (EBO-R). Ebolavirus has a negative-stranded RNA genome that encodes nucleoprotein (NP), P protein (VP35), matrix protein (VP40), glycoprotein (GP), an NP that influences the synthesis of viral mRNA (VP30), a protein associated with the membrane (VP24), and RNA-dependent RNA polymerase (L). Ebola virus reaches extremely high levels in Ebola virus infections (5, 7, 17, 31), the detection of Ebola antigens by antigen capture ELISA is suitable as a method of laboratory diagnosis (18, 29).

Monoclonal antibodies (MAbs) that discriminate Ebola virus species have been reported (28). However, an antigen capture ELISA that discriminates Ebola virus species has not been reported. In the present study, we developed antigen capture ELISAs using two novel MAb to the NP specific to EBO-R. The ELISAs will be a useful tool for rapid discrimination of EBO-R infection from those other Ebola virus species, especially in monkey quarantine or field studies.

MATERIALS AND METHODS

Cell culture. P3/Ag568 was used as the parental cell line for hybridomas. The cells were maintained in RPMI 1640 (Gibco BRL, Rockville, Md.) supplemented with 10% fetal bovine serum and antibiotics (streptomycin and penicillin; Gibco BRL). Hypoxanthine-aminopterin-thymidine supplement (HAT) (Gibco BRL) was added to the medium for the selection of hybridomas according to the manufacturer’s instructions. Hypoxanthine-thymidine supplement (HT) (Gibco BRL) was added to the medium to switch the medium from HAT to RPMI 1640.

Clinical specimens. Livers (monkey no. 2882 and 2887), spleens (monkey no. 2877, 2882, and 2885), and sera (monkey no. 2866 and 2862), which were collected from EBO-R-infected cynomolgus macaques during the EBO-R outbreak in the Philippines in 1996 (14, 25) and kept frozen at −80°C, were used in the study. EBO-R-NP antigens or genomic RNA were detected in these samples by antigen capture ELISA using 3-3D (29), immunohistochemistry (14), or RT-PCR using primers RES-N2 (5′-TGAGCTCCGAGAAAGACGTCGTT-3′) and RES-Nr2 (5′-ACCATCAGTGCTGTTCCACTAGTTC-3′). Five liver specimens and 79 serum specimens from EBO-R-noninfected cynomolgus macaques were used as the negative controls. Liver and spleen tissues were homogenized at approximately 10% (wt/vol) in phosphate-buffered saline (PBS) containing 0.05% Tween 20, 1% Triton X-100, and 5% nonfat milk (Triton-milk-PBS-T). After centrifugation at 15,800 × g for 10 min, the supernatants were used in the experiments. Sera were diluted with Triton-milk-PBS-T and used in the experiments.

Preparation of histidine-tagged Ebola virus rNPs (His-EBO-R-NP and His-EBO-Z-NP) for antigen capture ELISA. Recombinant NPs (rNP) of EBO-R (His-EBO-R-NP) and EBO-Z (His-EBO-Z-NP) were prepared to be the anti-
RESULTS

Development of two MAbs specific to the EBO-R NP. Thirty-two hybridoma clones secreting MAbs to EBO-R rNP were established, and the reactivities of these MAbs to His-EBO-R-NP were examined in the antigen capture ELISA format.
Two MAbs, Res2-6C8 and Res2-1D8, were reactive in the antigen capture ELISA format and were used in the present study. The isotypes of Res2-6C8 and Res2-1D8 were IgG2b and IgG1, respectively. Ebola virus species specificity of Res2-6C8 and Res2-1D8 was examined by IFA with HeLa cells that expressed the rNP of EBO-R or EBO-Z or by using Vero E6 cells infected with EBO-S. These two MAbs reacted to EBO-R NP (Fig. 1A and B), but not to EBO-Z NP (Fig. 1D and E) or EBO-S NP (Fig. 1G and H).

Epitopes recognized by Res2-6C8 and Res2-1D8. The epitopes recognized by Res2-6C8 and Res2-1D8 were determined by IgG ELISAs using the truncated EBO-R rNPs (Fig. 2). Res2-6C8 and Res2-1D8 recognized the amino acid residues between aa 631 and 739, and the minimum epitopes were further determined. Res2-6C8 reacted to the truncated EBO-R rNPs corresponding to aa 636 to 739 and 631 to 639, but not to aa 637 to 739 and 631 to 638. On the other hand, Res2-1D8 reacted to the truncated EBO-R NP corresponding to aa 636 to 739 and 631 to 643, but not to aa 637 to 739 and 631 to 642. These results showed that the epitopes recognized by Res2-6C8 and Res2-1D8 were aa 636 to 639 (4 aa residues, DPDDL639) and aa 636 to 643 (8 aa residues, DPDDL643) of EBO-R NP, respectively. The amino acid sequences of the epitopes were aligned to that of EBO-R Pennsylvania isolate in 1989 to 1990, EBO-Z, and EBO-S. The 8 aa residues were identical to those of the EBO-R Pennsylvania isolate in 1989 to 1990 (Table 2). The amino acid sequences of EBO-Z and EBO-S NPs corresponding to the amino acids of EBO-R, DD636DPDDL643SK643, were Q636DPDDL643SK643, respectively (Table 2).

Development of the antigen capture ELISAs using these novel MAbs. The sensitivity of the antigen capture ELISAs prepared with these two MAbs was tested. As shown in Fig. 3A, Res2-6C8 and Res2-1D8 detected His-EBO-R-NP up to dilutions of 1:128,000 and 1:64,000 in the antigen capture ELISA, respectively. The ELISA prepared with 3-3D detected His-EBO-R-NP up to the dilution of 1:32,000. As expected, Res2-6C8 and Res2-1D8 did not detect His-EBO-Z-NP in the antigen capture ELISA (Fig. 3B).

Detection of EBO-R NP in the specimens from EBO-R-infected monkeys. Serum, liver, and spleen specimens from EBO-R-infected macaques were examined for the presence of EBO-R NP by antigen capture ELISAs using Res2-6C8, Res2-1D8, and 3-3D. Res2-6C8 and Res2-1D8 detected the EBO-R NP antigens in the sera, livers, and spleens (Table 3). Res2-6C8 detected the NP in all of the samples, while Res2-1D8 and 3-3D detected the NP in six of seven and five of seven samples.
respectively. The endpoint titers were higher in the ELISAs using Res2-6C8 and Res2-1D8 than in the ELISA using 3-3D.

**DISCUSSION**

We previously established an antigen capture ELISA using the MAb 3-3D, which reacted to the NPs of EBO-Z, EBO-S, and EBO-R (29). The minimum epitope recognized by MAb 3-3D was mapped on 26 aa residues between aa 648 and 673 at the C-terminal region of the EBO-Z NP (29), and the amino acid residues between aa 631 and 739 and aa 633 and 738 were required for the cross-reaction to EBO-R NP and EBO-S NP, respectively (29). In the present study, we developed two novel MAbs to EBO-R NP, Res2-6C8 and Res2-1D8, which detected EBO-R NP with high sensitivity and specificity in the antigen capture ELISA format. The minimum epitopes recognized by Res2-6C8 and Res2-1D8 were found to be 636DPDI639 and 636DPDIGQSK643, respectively (Fig. 2). The amino acid se-

**TABLE 2. Comparison of the epitopes of Res2-6C8 and Res2-1D8**

<table>
<thead>
<tr>
<th>Ebola virus species</th>
<th>Amino acids corresponding to epitope of:</th>
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<tr>
<td>EBO-R, 1996</td>
<td>636DPDI639</td>
</tr>
<tr>
<td>EBO-R, 1989/1990</td>
<td>636DPDI639</td>
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<tr>
<td>EBO-Z, 1976</td>
<td>636NQDS639</td>
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<tr>
<td>EBO-S, 1976</td>
<td>636EALP639</td>
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</table>


**FIG. 2. Reactivities of Res2-6C8 and Res2-1D8 to the truncated EBO-R rNPs in IgG ELISA.** Res2-6C8 recognizes the amino acid residues between aa 636 and 639 (sequence, DPDI). Res2-1D8 recognizes the amino acid residues between aa 636 and 643 (sequence, DPDIGQSK). The mean OD value ± SD in the IgG ELISA is shown.

**FIG. 3. Reactivity of MAbs Res2-6C8 (□), Res2-1D8 (△), and 3-3D (○) to the rNPs of EBO-R and EBO-Z in the antigen capture ELISA.** His-EBO-R-NP (A) and His-EBO-Z-NP (B) were used as antigens. The mean ± SD of four assays is shown.
Antigen sequence of EBO-R NP, 636DPDIGQSK643, was different from those of EBO-Z and EBO-S NP (Table 2). This is consistent with the result in which the two MAbs did not react to the NP of EBO-Z and EBO-S in the IFA (Fig. 1D, E, G, and H). The amino acid sequences of the epitopes recognized by the two MAbs were identical to those of EBO-R Pennsylvania isolate in 1989 to 1990 (Table 2) (11, 13). Considering the genetic stability among EBO-R isolates (11, 13), it is expected that antigen capture ELISA systems using Res2-6C8 and Res2-1D8 detect either EBO-R strain.

We determined the cutoff value in the antigen capture ELISA based on the mean OD value + 3 SDs of 79 sera and 5 liver specimens from EBO-R-uninfected cynomolgus macaques. Since the mean OD value + 3 SDs was lower than 0.2 in any specimen, the cutoff value was determined to be 0.2 in the present study. The sensitivity of the antigen capture ELISA using either Res2-6C8 or Res2-1D8 for His-EBO-R-NP was similar to that using 3-3D (Fig. 3A). On the other hand, His-EBO-Z-NP was not detected in the antigen capture ELISAs using Res2-6C8 and Res2-1D8 (Fig. 3B), as expected from the results in the IFA and the epitope sequences, demonstrating that the antigen capture ELISAs using Res2-6C8 and Res2-1D8 were specific to EBO-R.

Authentic EBO-R NP in the serum, liver, and spleen specimens from the macaques naturally infected with EBO-R was detected by the antigen capture ELISAs using the Res2-6C8, Res2-1D8, and 3-3D MAbs (Table 3). The sensitivity of the ELISA in detecting the authentic EBO-R NP was higher with Res2-6C8 and Res2-1D8 than with 3-3D. Since the clinical specimens used in this study were stored for 6 years, it is possible that the EBO-R NP was somewhat degraded and was detected with higher sensitivity by Res2-6C8, which recognizes 4 aa of the NP, than by 3-3D, which recognizes 109 aa. The results, nevertheless, indicated that both Res2-6C8 and Res2-1D8 were highly sensitive in detecting EBO-R NP in clinical specimens.

An outbreak of EBO-CI occurred among a troop of chimpanzees in the Tai National Park, Ivory Coast, in 1994 (20). It was also reported that Ebola hemorrhagic fever patients had been infected with Ebola virus while butchering dead chimpanzees in the Tai National Park, Ivory Coast, in 1994 (20). Fatal infection with EBO-Z, EBO-S, EBO-CI, and EBO-R was also demonstrated in cynomolgus macaques (7). EBO-R caused outbreaks among cynomolgus macaques in the Philippines (2–4, 12, 16, 25, 30, 38) and is most likely to cause epizootics among Asian macaques. Since EBO-R has never caused symptomatic infection in humans (3, 4, 25, 38), rapid differentiation of the species of Ebola virus is crucial, especially when samples from nonhuman primates are tested. Although RT-PCR has higher sensitivity to Ebola virus than the antigen capture ELISA, the sequencing of the PCR products is essential for confirming Ebola virus infection (21–23, 30, 34, 35). The viral load in the blood and other organs has been shown to reach extremely high levels in Ebola virus-infected animals (5, 7, 17, 31). Thus, the newly developed antigen capture ELISAs using Res2-6C8 and Res2-1D8 might be a promising tool for the diagnosis of EBO-R infection, especially in monkey quarantine and field studies.

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REFERENCES


TABLE 3. Detection of EBO-R NP from clinical specimens

<table>
<thead>
<tr>
<th>Sample</th>
<th>Monkey no.</th>
<th>Titer by antigen capture ELISA*</th>
<th>Result by:</th>
<th>RT-PCR†</th>
<th>IHC‡</th>
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<tr>
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<td>Res2-6C8</td>
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<tr>
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<td>320</td>
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<td>40</td>
<td>&lt;10</td>
<td>+</td>
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</tbody>
</table>

* Antigen titer in the specimens. The antigen titer in the ELISA was determined as the reciprocal of the highest dilution showing a positive reaction. The titer was determined by a single test. The cutoff OD value was 0.2.
† RT-PCR with primers RES-Nf2 and RES-Nr2.
‡ IHC, immunohistochemistry for the detection of Ebola virus NP (14).
§ ND, not done.


