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Characterization of Monoclonal Antibodies to Junin Virus Nucleocapsid Protein and Application to the Diagnosis of Hemorrhagic Fever Caused by South American Arenaviruses▽

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Junin virus (JUNV), Machupo virus, Guanarito virus, Sabia virus, and Chapare virus are members of New World arenavirus clade B and are the etiological agents of viral hemorrhagic fevers that occur in South America. In this study, we produced three monoclonal antibodies (MAbs) to the recombinant nucleocapsid protein of JUNV, designated C6-9, C11-12, and E4-2. The specificity of these MAbs was examined by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, and an epitope-mapping method. Using these MAbs, we developed antigen (Ag) capture ELISA systems. We showed that by using MAb C6-9, JUNV Ag was specifically detected. On the other hand, by using MAb C11-12 or E-4-2, the Ags of all human pathogenic South American arenaviruses were detected. The combined use of these Ag capture ELISA systems in the present study may be useful for the diagnosis of acute-phase viral hemorrhagic fever due to infection by a South American arenavirus.

The South American arenaviruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHPV) are members of New World arenavirus clade B. JUNV, MACV, GTOV, and SABV are the etiological agents of Argentine hemorrhagic fever (AHF), Bolivian hemorrhagic fever (BHF), Venezuelan hemorrhagic fever (VHF), and Brazilian hemorrhagic fever, respectively (4). CHPV was also recently shown to be associated with cases of hemorrhagic fever in Bolivia (5). AHF emerged in the 1950s, and since then, outbreaks have occurred annually without interruption (4). The mortality rate for AHF is estimated to be 15 to 30%, but early treatment with immune plasma reduces the rate to less than 1% (6). The region at risk has been progressively expanding into northern central Argentina, and almost 5 million people are currently considered to be at risk for AHF (6, 13). Phylogenetic analysis indicates that JUNV is more closely related to MACV than to SABV or CHPV, whereas SABV and CHPV are more closely related to each other than to other New World arenaviruses (5).

Arenaviruses are enveloped and contain a bisegmented RNA genome. The genome consists of two ambisense single-stranded RNA molecules, one designated L, which encodes the RNA-dependent RNA polymerase and a zinc-binding matrix protein, Z, and the other designated S, which encodes the major structural components of the virion, i.e., the nucleocapsid protein (NP) and the envelope glycoprotein precursor (15). The arenavirus NP is the most abundant protein among the viral structural proteins both in infected cells and in virions (2) and is commonly used as a target for detecting viral antigens (Ags) (20). Moreover, arenavirus NPs have been known to be the most conserved among the same virus species and, to some extent, among different arenavirus species (3, 8). Therefore, it seems likely that monoclonal antibodies (MAbs) raised against the NP of an arenavirus would also be useful for detecting other arenaviruses (20). Recently, an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was developed by using a recombinant NP (rNP) of JUNV, obtained from a recombinant baculovirus system, and was proposed to be useful for etiologic confirmation of AHF in seroepidemiological studies (20, 26). It is considered that an Ag capture ELISA using MAbs specific for viral Ags allows rapid diagnosis of the acute phase of viral hemorrhagic fever by detecting viral Ags in blood or tissue homogenates (20). In this study, we produced MAbs to the rNP of JUNV. These MAbs were characterized by ELISA, indirect immunofluorescence assay (IFA), and an epitope-mapping method. Ag capture ELISAs were developed by using these MAbs that are specific for JUNV and that are broadly applicable for the detection of human pathogenic New World arenaviruses.

MATERIALS AND METHODS

Cell culture. Hybridomas and their parental cell line, P3/Ag568, were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Invitrogen), and antibiotics (streptomycin and penicillin G; Invitrogen). Hypoxanthine-aminopterin-thymidine supplement (Invitrogen) was added to the me-

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dium for selection of hybridomas, as recommended by the supplier. BTI-TN-5B1-4 (High Five; Invitrogen) insect cells were maintained in TC100 (Invitrogen) supplemented with 10% FBS, 2% tryptose phosphate broth (Difco, Detroit, MI), and kanamycin (Invitrogen). HeLa cells were maintained in minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS and antibiotics (streptomycin and penicillin G; Invitrogen).

Recombinant baculoviruses. The baculoviruses Ac-JUNV-NP and Ac-His-Lassa virus (LASV)-NP, expressing the JUNV and His-LASV rNPs, respectively, were generated as described previously (20).

The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were obtained by chemical synthesis (Codon Devices, Cambridge, MA). The GenBank accession numbers of the nucleotide sequences of the MACV, GTOV, SABV, and CHPV NP genes are NC_005078, AF485258, NC_006317, and NC_010562, respectively. The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were digested with BamHI and subcloned into the BamHI restriction site of pAcYM1 (14), and the resulting plasmids were designated pAcYM1-MACV-NP, pAcYM1-GTOV-NP, pAcYM1-SABV-NP, and pAcYM1-CHPV-NP, respectively. High Five cells were transfected with mixtures of linearized BacPAK6 DNA (Clontech, Mountain View, CA) and the recombinant transfer vector according to the manufacturer's instructions and the procedures described by Kitts and Possee (10), and recombinant baculoviruses were obtained from them. The baculoviruses expressing the MACV, GTOV, SABV, and CHPV rNPs were designated Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, and Ac-CHPV-NP, respectively.

Expression and purification of rNPs. High Five cells infected with Ac-JUNV-NP, Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, Ac-CHPV-NP, or Ac-His-LASV-NP were incubated at 26°C for 72 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution. The High Five cells were lysed in PBS containing 1% NP-40 and 2 M urea. After the cell lysates were centrifuged at 15,000 $\times$ *g* for 10 min, the pellet fractions were collected and then solubilized in PBS containing 8 M urea. After the samples were centrifuged, the supernatant fractions were used as the purified Ags. The control Ag was produced from High Five cells infected with Ac-ΔP, which lacks the polyhedrin gene, in the same manner as for the negative control Ags. All Ags were aliquoted and kept at -80°C until use.

Establishment of MAbs. BALB/c mice were immunized three times with the purified JUNV rNP. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells by using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified JUNV rNP as an Ag in the presence of 2 M urea. MAbs were purified from the culture supernatant by using a MAbTrap GII antibody purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. The concentration of each purified MAb was also determined by use of a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Polyclonal antibodies. Polyclonal antibodies were induced in rabbits by immunization with the purified rNPs of JUNV, MACV, GTOV, SABV, and CHPV, respectively. Rabbit sera collected before immunization were used as controls.

IgG ELISA. The IgG ELISA was performed as previously described, except for Ag preparation (20–22). Briefly, ELISA plates (96 wells, Pro-Bind; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified JUNV, MACV, GTOV, SABV, CHPV, or His-LASV rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was covered with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (PBST-M), followed by incubation for 1 h for blocking at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then inoculated with MAbs (100 μl/well), which were diluted 1:1,000 with PBST-M. After a 1-h incubation period, the plates were washed three times with PBST and then the plates were inoculated with goat anti-mouse IgG antibody labeled with horseradish peroxidase (HRP; 1:1,000 dilution; Zymed Laboratories, Inc., South San Francisco, CA). After a further 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and the optical density at 405 nm (OD_{405}) was measured against a reference of 490 nm. The adjusted OD_{405} value was calculated by subtracting the OD_{405} value of the negative Ag-coated wells from that of the corresponding wells.

IFA. The full-length cDNA of the JUNV NP obtained from Ac-JUNV-NP, which possessed a BamHI restriction site at both extremities, was cloned into the BamHI site of the pKS336 vector (23), and the resulting plasmid was designated pKS-JUNV-NP. Also, the chemically synthesized full-length cDNAs of the MACV, GTOV, SABV, and CHPV NPs were cloned into the BamHI site of the pKS336 vector and the resulting plasmids were designated pKS-MACV-NP, pKS-GTOV-NP, pKS-SABV-NP, and pKS-CHPV-NP, respectively. HeLa cells were then transfected with each of these expression plasmids by using a transfection reagent (FuGENE6; Roche Diagnostics) according to the manufacturer's instructions. The transfected cells were selected with 4 μg of blasticidin *S*-hydrochloride/ml in culture medium. The HeLa cell clones were obtained by analyzing the expression of each rNP by IFA with rabbit serum raised against the JUNV, MACV, GTOV, SABV, or CHPV rNP, as previously described (20).

Ag capture ELISA. Purified MAb C6-9, C11-12, or E4-2 was used to coat microwell immunoplates (Falcon; Becton Dickinson Labware) at 100 ng/well in 100 μl of PBS at 4°C overnight, followed by blocking with PBST-M for 1 h at room temperature. After the plates were washed with PBST, 100 μl of samples containing serially diluted rNP of JUNV, MACV, GTOV, SABV, CHPV, or LASV was added and the plates were incubated for 1 h at 37°C. The plates were then washed with PBST, and 100 μl of rabbit polyclonal antibody raised against the rNP of JUNV diluted 1:500 with PBST-M was added to each well. After 1 h of incubation at 37°C, the plates were washed with PBST, and HRP-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added. The plates were incubated for 1 h at room temperature. After another extensive washing with PBST, 100 μl of ABTS substrate solution (Roche Diagnostics) was added and the OD_{405} was measured with a reference wavelength of 490 nm after 30 min of incubation at room temperature. As a negative control, the OD of control Ag-inoculated wells was measured. The adjusted OD_{405} values were calculated by subtracting the OD_{405} value of the negative control well from the corresponding OD_{405} values. Means and standard deviations were calculated from the ODs of 12 negative control wells, and the cutoff value for the assay was defined as the mean plus 3 standard deviations.

Expression of truncated rNPs of JUNV. In order to determine the epitope on the JUNV rNP for the MAbs, a series of truncated JUNV rNPs were expressed as fusion proteins with glutathione *S*-transferase (GST). The DNA corresponding to each of the truncated NP fragments was amplified by PCR with specifically designed primer sets. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). The GST-tagged full-length rNP (GST-JUNV frNP) or truncated forms of the rNP (GST-JUNV trNPs) were expressed in *Escherichia coli* BL21 and then partially purified.

Western blotting. The MAbs were tested for reactivity to GST-JUNV frNP and a series of GST-JUNV trNPs by Western blotting as reported previously (9, 18, 24).

MAb epitope mapping. The epitopes for MAbs C6-9 and C11-12 were determined by epitope-blocking ELISA using synthetic peptides. The decapeptides were chemically synthesized by shifting one amino acid, with a consecutive overlap of nine amino acids to cover the JUNV NP (amino acids [aa] 5 to 26 for C6-9 and aa 543 to 564 for C11-12). ELISA plates were coated with purified JUNV rNP prepared by using a baculovirus expression system (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of PBS-M, followed by incubation for 1 h for blocking. MAb C6-9 or C-11-12 was mixed with each peptide (1 μg/well) and incubated for 1 h at 37°C, and then the mixture was added to each well of the plates. After a 1-h incubation period, the plates were washed three times with PBST, and then the plates were inoculated with goat anti-mouse IgG antibody labeled with HRP (1:1,000 dilution; Zymed). The following procedure was performed as described in the IgG ELISA section above.

For MAb E4-2, the epitope was determined by ELISA using GST-JUNV frNP and trNPs. ELISA plates were coated with purified GST-JUNV frNP or trNPs (approximately 100 ng/well) according to the method described in the IgG ELISA section above. MAb E4-2 or an anti-GST MAb was used for detection at a 1:2,000 or a 1:500 dilution, respectively. The adjusted OD_{405} was calculated by dividing the OD_{405} of MAb E4-2 by that of the anti-GST MAb from the corresponding wells.

RESULTS

Generation of MAbs. In order to obtain MAbs against the JUNV NP, BALB/c mice were immunized with the purified rNP of JUNV. The MAbs were purified and tested for reactivity to the rNP of JUNV by IgG ELISA. Three MAbs, designated MAb C6-9, MAb C11-12, and MAb E4-2, reacted with the rNP of JUNV by IgG ELISA even in the presence of 2 M urea.

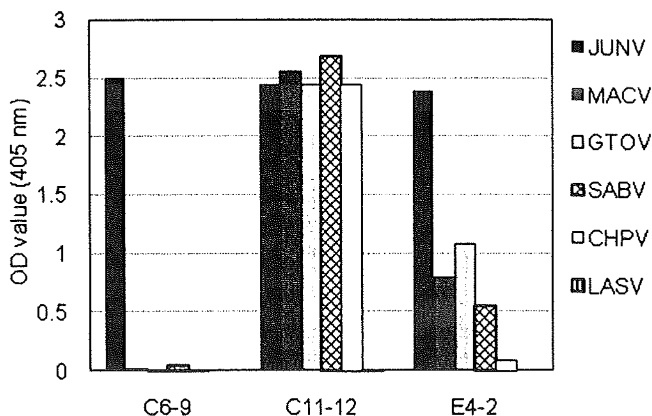

FIG. 1. Reactivity of each MAb with arenavirus rNP. Each purified rNP (100 ng/well) was used to coat microplates as described in the text, and the reactivities of each MAb to the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were measured. The MAbs are shown on the *x* axis. Results are expressed as the OD_{405}.

Reactivities of MAbs to rNPs of arenaviruses. The reactivities of MAbs to the rNPs of human pathogenic arenaviruses were examined by ELISA. MAb C6-9 reacted specifically with the rNP of JUNV but did not react with those of the other pathogenic South American arenaviruses (Fig. 1). On the other hand, MAb C11-12 reacted at the same level with the rNPs of all of the pathogenic South American arenaviruses, including JUNV, GTOV, MACV, SABV, and CHPV. MAb E4-2 reacted strongly with the rNP of JUNV, slightly more weakly with those of GTOV, MACV, and SABV, and very weakly with that of CHPV. However, MAb E4-2 reacted clearly with the rNP of CHPV when ELISA plate wells were coated with more-concentrated CHPV Ag (data not shown). None of the three MAbs reacted with the rNP of the human pathogenic Old World arenavirus LASV.

Reactivity was also examined by IFA. Consistent with the ELISA result, MAb C6-9 reacted only with HeLa cells expressing the rNP of JUNV and MAb C11-12 reacted with HeLa cells expressing the rNPs of all of the pathogenic South American arenaviruses (Table 1). On the other hand, MAb E4-2, which showed cross-reactivity to other arenaviruses by ELISA, reacted only with HeLa cells expressing the rNP of JUNV (Table 1). None of the three MAbs reacted with LASV NP-expressing HeLa cells (Table 1).

Development of Ag capture ELISAs. Ag capture ELISAs were developed by using three MAbs as capture antibodies, and sensitivity and specificity were determined. The Ag capture ELISA with MAb C6-9 specifically detected the rNP of JUNV, whereas it could not detect the rNPs of the other South American arenaviruses. No less than 62.5 ng/well of the rNP of JUNV was detected by the Ag capture ELISA using MAb C6-9 (Fig. 2A). On the other hand, the Ag capture ELISAs using

TABLE 1. MAb reactivity with NPs of arenaviruses in IFA

MAb	Reactivity[a] with NP of:					
	JUNV	MACV	GTOV	SABV	CHPV	LASV
C6-9	+	−	−	−	−	−
C11-12	+	+	+	+	+	−
E4-2	+	−	−	−	−	−

[a] The symbols + and − indicate positive and negative reactions, respectively. The expression of each NP in HeLa cells was confirmed by IFA with a rabbit polyclonal antibody produced against each NP.

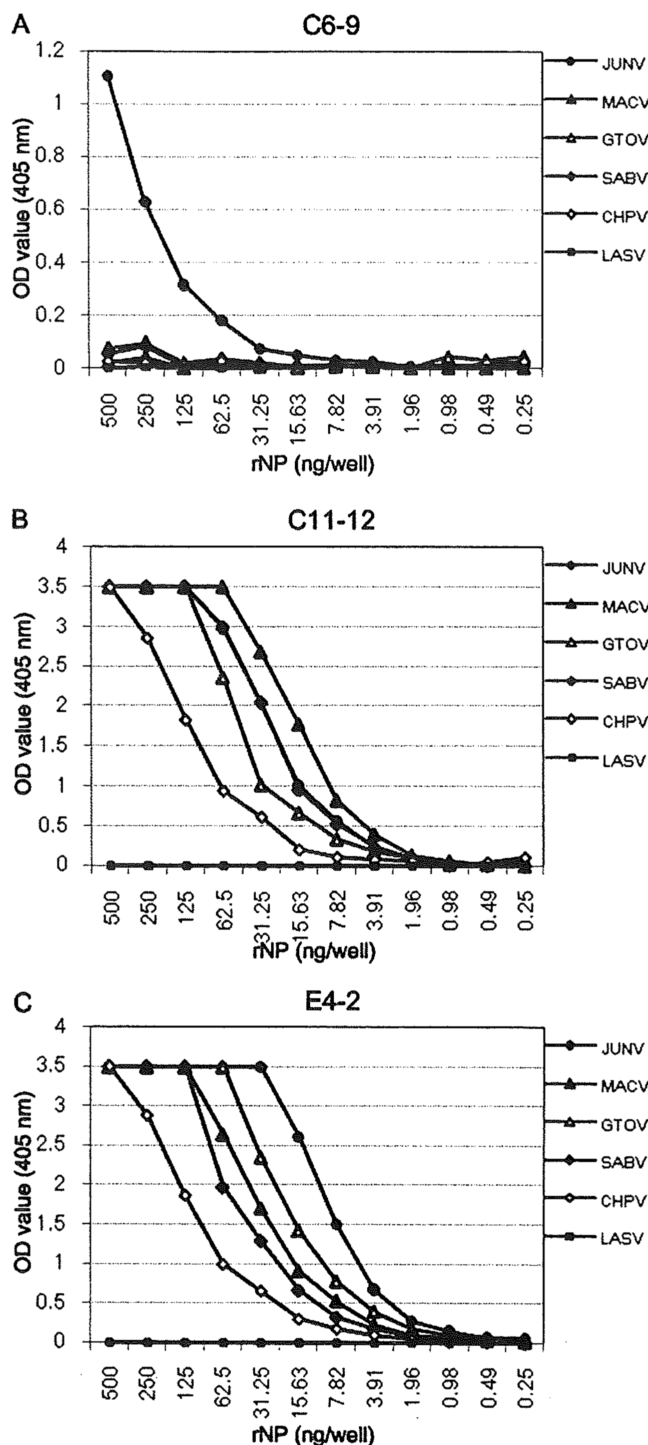

FIG. 2. Reactivity of each MAb in an Ag capture ELISA. Purified MAbs C6-9 (A), C11-12 (B), and E4-2 (C) were used to coat microplates as described in the text, and their abilities to capture the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were examined at various concentrations in the Ag capture format. Results are expressed as the OD_{405}.

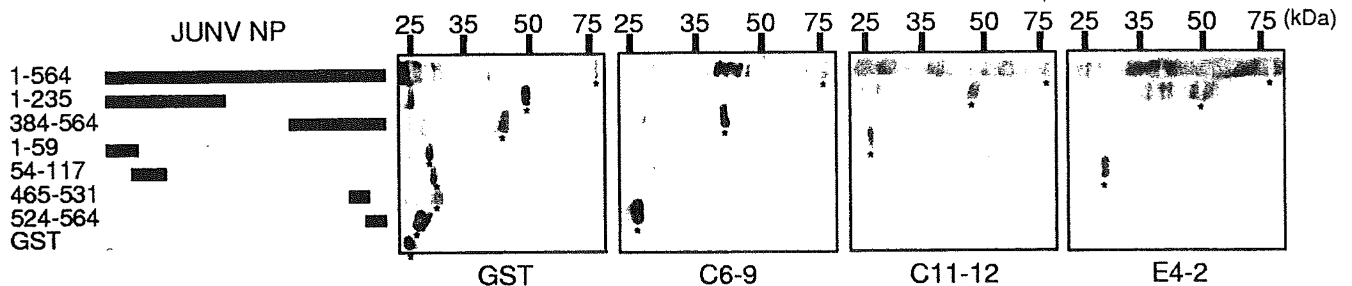

FIG. 3. Reactivities of MAbs C6-9, C11-12, and E4-2 with the GST-tagged JUNV NP by Western blotting. Schematic drawings of polypeptides of the JUNV NP and the amino acid positions of the polypeptides are shown on the left. The reactivities of MAbs and a control MAb against GST to these polypeptides by Western blotting are shown on the right. The asterisks indicate the polypeptides to which the MAbs reacted.

MAbs C11-12 and E4-2 were more sensitive at detecting the rNP of JUNV, with detection limits of 7.82 and 3.91 ng/well, respectively, and these Ag capture ELISAs also detected the rNPs of the other South American arenaviruses GTOV, MACV, SABV, and CHPV (Fig. 2B and C). In contrast, the LASV NP was not detected by any of the Ag capture ELISAs.

Determination of the epitope on the JUNV rNP recognized by the MAbs. In order to determine regions including epitopes on the JUNV rNP recognized by the MAbs, the reactivity of these MAbs was tested by Western blotting using the GST-JUNV frNP and a series of GST-JUNV trNPs as Ags. MAb C6-9 reacted with GST-JUNV frNP and trNPs at aa 384 to 564 and 524 to 564 (Fig. 3). MAb C11-12 reacted with GST-JUNV frNP and trNPs at aa 1 to 235 and 1 to 59 (Fig. 3). MAb E4-2 reacted with GST-JUNV frNP and trNPs at aa 1 to 235 and 54 to 117 (Fig. 3).

To further determine exact epitope positions on the rNP of JUNV, we performed epitope-blocking ELISAs with a series of overlapping synthetic peptides. As shown in Fig. 4A, peptides containing PPSLLFLP (aa 551 to 558) blocked the reaction of MAb C6-9 with the purified rNP of JUNV. Similarly, peptides containing WTQSLR (aa 12 to 17) blocked the reaction of MAb C11-12 with the purified rNP of JUNV (Fig. 4B).

Because the epitope recognized by MAb E4-2 could not be determined by epitope-blocking ELISA, it was analyzed more in detail by using a series of GST-JUNV trNPs by ELISA (Fig. 4C). The reactivity of MAb E4-2 was normalized by dividing the OD_{405} value of MAb E4-2 by that of an anti-GST MAb. MAb E4-2 reacted with the GST-JUNV trNP containing the polypeptide KEVDRLMS (aa 72 to 79). The ELISA result was consistent with that of Western blotting (data not shown). The epitopes recognized by the MAbs are summarized in Fig. 5.

DISCUSSION

Detection of a viral Ag and/or the viral genome is crucial for rapid diagnosis of patients with hemorrhagic fever caused by South American arenaviruses, especially for patients in the acute phase. The application of reverse transcriptase PCR (RT-PCR) and TaqMan PCR for detection of the JUNV, MACV, and GTOV genomes has been reported (1, 11, 12, 27). Serological diagnosis is also useful for the diagnosis of AHF, especially in patients in the convalescent phase (7, 17, 19, 20, 26).

An Ag capture ELISA using a cocktail of MAbs against JUNV (25) was applied in an epidemiological study of rodents in Argentina (16). MAbs reactive with the NP of JUNV have been shown to cross-react with those of MACV and other nonpathogenic arenaviruses (25). In the present study, by using MAbs raised against the rNP of JUNV, we developed Ag capture ELISAs specific for JUNV and broadly reactive to human pathogenic New World arenaviruses.

The three MAbs to JUNV NP (designated C6-9, C11-12, and E4-2) reacted with the rNP of JUNV prepared using a baculovirus expression system by IgG ELISA and with rNP expressed in mammalian cells by IFA (Fig. 1 and Table 1). All Ag capture ELISAs using MAbs E4-2, C11-12, and C6-9 detected the rNP of JUNV (Fig. 2), suggesting that these ELISAs are useful tools for the diagnosis of AHF.

Interestingly, an Ag capture ELISA using MAb E4-2 detected the Ags of all of the pathogenic South American arenaviruses tested, in addition to that of JUNV (Fig. 2). IgG ELISA showed that the reactivity of MAb E4-2 with the rNP of JUNV was stronger than that with the rNPs of other South American arenaviruses (Fig. 1). The minimal length of the epitope required to be recognized by MAb E4-2 was 8 aa with the sequence KEVDRLMS (Fig. 4 and 5). However, the GST-JUNV trNP at aa 1 to 80 was more reactive than that at aa 1 to 79, which includes minimal epitope sequences, but was still less reactive than those at aa 72 to 564, 67 to 564, and 1 to 564 (Fig. 4). Even though we could not express GST-JUNV trNPs at aa 1 to 81 or more in *E. coli* because of their toxicity, it is possible that some additional amino acids at the C terminus of the minimal epitope are required for complete reaction with MAb E4-2. Actually, comparison of the amino acid sequences of NPs at positions 72 to 83 among South American arenaviruses showed that the amino acid differences with respect to JUNV were 1 aa for GTOV, 2 aa for MACV, 3 aa for SABV, and 5 aa for CHPV (Fig. 5), and these differences correlated well with the levels of reactivity of MAb E4-2 to the rNPs of the viruses (Fig. 1).

The Ag capture ELISA using MAb C11-12 also detected the Ags of all of the other pathogenic South American arenaviruses (Fig. 2). MAb C11-12 reacted with the rNPs of all of the pathogenic South American arenaviruses by IgG ELISA and IFA (Fig. 1 and Table 1). These results suggest that MAb C11-12 would be useful for detecting the Ags of all South American arenaviruses by Ag capture ELISA and IFA. Furthermore, the amino acid sequence (WTQSLR) of the epitope recognized by MAb C11-12 was located at the N terminus of the JUNV NP and was conserved among all of the pathogenic

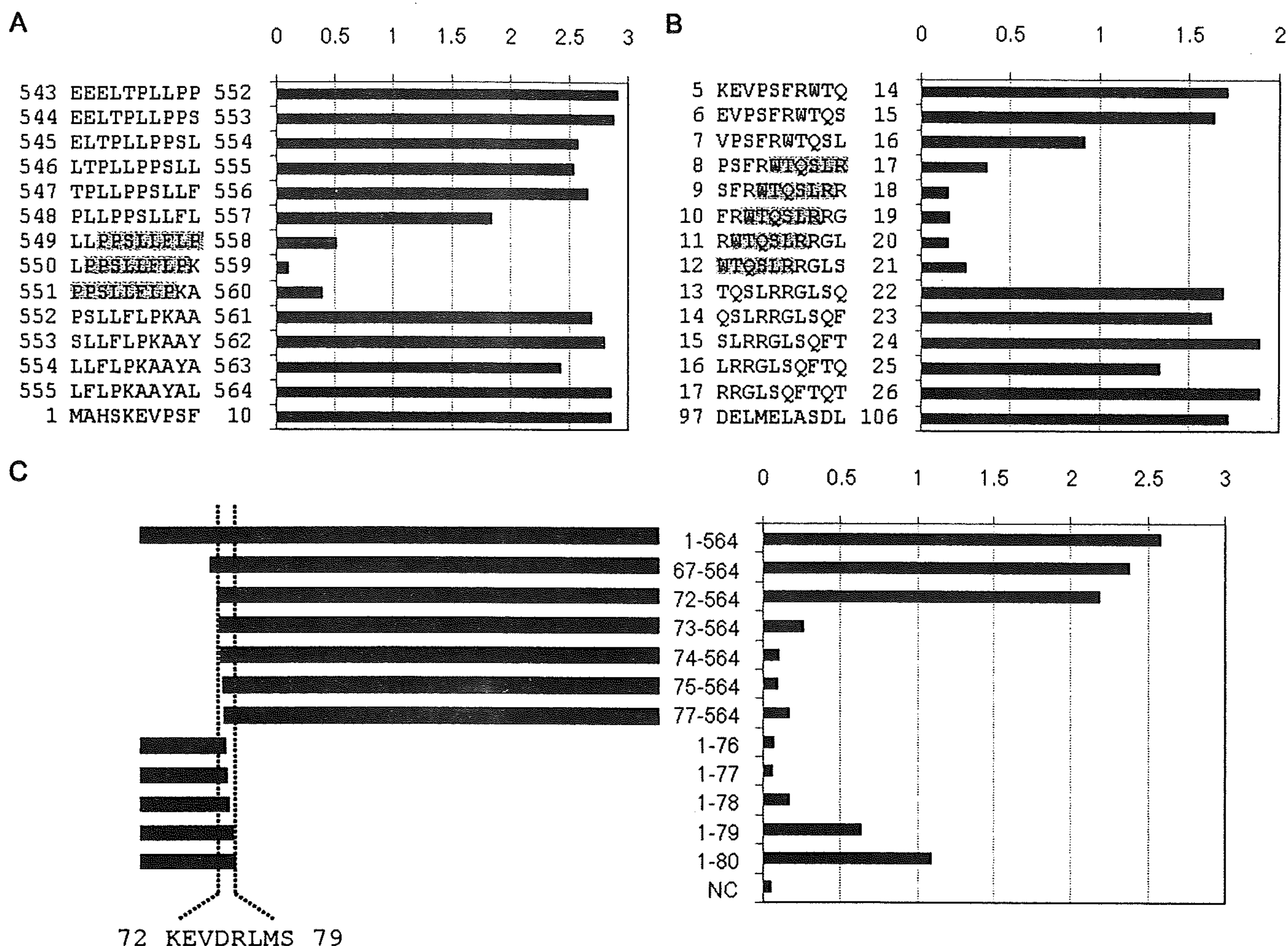

FIG. 4. Determination of the epitope on JUNV NP recognized by MAbs C6-9 (A), C11-12 (B), and E4-2 (C). (A, B) The ability of synthetic decapeptides to block the reactivity of MAbs to the JUNV rNP was examined by ELISA. The amino acid sequences and positions of synthetic peptides used in the assay are shown on the *y* axis. The synthetic peptides at aa 1 to 10 and 97 to 106 were used as negative control peptides for MAbs C6-9 and C11-12, respectively. Results indicate the OD_{405}. MAb C6-9 was confirmed to react with the 8 aa residues (PPSLLFLP) at positions 551 to 558, as represented by the shaded box (A). Similarly, MAb C11-12 was confirmed to react with the 6 aa residues (WTQSLR) at positions 12 to 17 (B). (C) The reactivity of MAb E4-2 with GST-tagged partial polypeptides of the JUNV NP was examined by ELISA. Schematic drawings of polypeptides of the JUNV NP are shown on the left, and the amino acid positions of the polypeptides are indicated on the *y* axis. NC represents the GST protein without any JUNV NP sequences. The reactivity of MAb E4-2 to each partial JUNV NP is indicated by the adjusted OD_{405}, which was calculated by dividing the OD_{405} of MAb E4-2 by that of the anti-GST MAb to the corresponding Ag. MAb E4-2 was confirmed to react with the 8 aa residues (KEVDRLMS) at positions 72 to 79, as indicated at the bottom.

South American arenavirus isolates so far deposited in GenBank (Fig. 5). However, slight differences in the sensitivity of detection of the NPs of the South American arenaviruses by Ag capture ELISA were observed. This may be due to the reactivity of the detector antibody, anti-JUNV NP rabbit serum, which was raised against the purified rNP of JUNV. Since the N-terminal region of the NPs recognized by MAbs C11-12 and E4-2 (aa 1 to 80) was relatively conserved among the NPs of South American arenaviruses, Ag capture ELISAs using MAbs C11-12 and E4-2 are considered to be useful for detecting most South American arenavirus isolates. Therefore, these Ag capture ELISAs may be applicable not only for the diagnosis of AHF but also for the diagnosis of BHF, VHF, and Brazilian hemorrhagic fever and may also be applicable for newly emerging viral hemorrhagic fevers caused by CHPV, although further study is needed.

On the other hand, the Ag capture ELISA using MAb C6-9 only detected JUNV Ag (Fig. 2). Furthermore, MAb C6-9 only reacted with the rNP of JUNV by IgG ELISA and IFA (Fig. 1 and Table 1). The amino acid sequence (PPSLLFLP) of the epitope recognized by MAb C6-9 was conserved among JUNV isolates so far deposited in GenBank (data not shown) but differed from those of other South American arenavirus isolates (Fig. 5). Since only the proline at position 552 in the epitope sequence is different in MACV, this proline is likely to be critical in the reaction of MAb C6-9. Therefore, the Ag capture ELISA using MAb C6-9 may detect most, if not all, JUNV isolates. Considering that the symptoms due to JUNV infection in humans are indistinguishable from those due to other South American arenaviruses, the Ag capture ELISA using MAb C6-9 may be a useful diagnostic tool, especially for AHF.

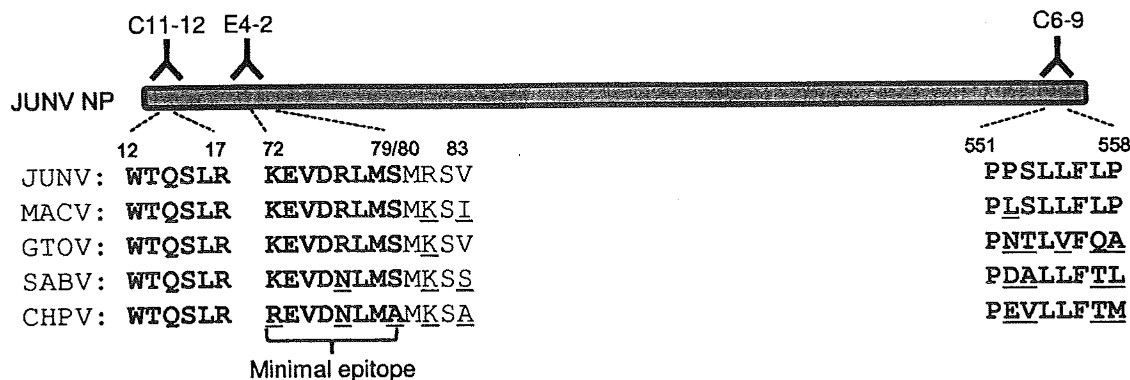

FIG. 5. Schematic representation of epitopes of the JUNV NP recognized by MAbs. The amino acid sequences of the epitopes of the JUNV NP recognized by MAbs C6-9, C11-12, and E4-2 are in boldface, and the amino acid positions are shown above the sequence. The amino acid sequences of the epitopes are compared to those of MACV, GTOV, SABV, and CHPV. The amino acid residues different from those of the JUNV NP are underlined. Because the amino acid sequence of the corresponding region is conserved among the strains of each virus species, a single sequence represents each virus species. The GenBank accession numbers for the S genes of JUNV are NC_005081, DQ272266, AY746353, AY619641, AY358023, D10072, U70802, U70803, and U70804. Those for the S genes of MACV are NC_005078, AY924208, AY924207, AY924206, AY924205, AY924204, AY924203, AY924202, AY571959, AY624355, AY619645, AY571904, AF485260, and AY129248. Those for GTOV are NC_005077, AY497548, AF485258, and AY129247. Those for SABV and CHPV are NC_006317 and NC_010562, respectively.

While the efficacy of newly developed Ag capture ELISAs in the diagnosis of viral hemorrhagic fever caused by South American arenaviruses was not evaluated by using serum samples from patients, it is generally accepted that an Ag capture ELISA is useful for the detection of viral Ags in blood and/or organ tissue specimens from patients in the acute phase.

The amino acid sequences of the epitope regions recognized by MAbs E4-2 and C6-9 were different from those of the corresponding region of LASV. On the other hand, the amino acid sequence (WTQSLR) of the epitope recognized by MAb C11-12 is the same in JUNV and LASV, even though the MAb failed to react to the LASV NP. However, aa 8 and 11 are proline and arginine in the NPs of South American arenaviruses while they are lysine/arginine and leucine in the LASV NP, respectively. Thus, it is possible that some amino acid sequence differences around the minimal epitope region affect the reaction with MAb C11-12.

In general, RT-PCR is more sensitive in detecting viruses in patients' specimens than is an Ag capture ELISA. Recently, a real-time RT-PCR has been established for the detection of all of the pathogenic South American arenaviruses, but it has not yet been applied for clinical specimens, so the possibility that it does not detect novel virus strains or species cannot be ruled out (27). Furthermore, arenaviruses are known to have high genetic variability, and false-negative results are occasionally obtained with some particular primer sets for RT-PCR. On the other hand, the Ag capture ELISAs established in the present study recognized highly conserved epitopes, suggesting that Ag capture ELISA may be useful for the diagnosis of suspected patients.

In conclusion, we developed Ag capture ELISA systems using newly produced MAbs against the JUNV NP and showed that JUNV Ag was detected specifically by the Ag capture ELISA using MAb C6-9. On the other hand, the Ags of all human pathogenic South American arenaviruses could be detected by an Ag capture ELISA using MAb C11-12 or E-4-2. The combined use of these Ag capture ELISAs in the present study may be useful for the diagnosis and differentiation of viral hemorrhagic fevers caused by South American arenavirus infections.

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Argentine Hemorrhagic Fever Diagnostic Test Based on Recombinant Junín Virus N Protein

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Junín arenavirus is the etiologic agent of Argentine hemorrhagic fever. Due to its morbidity and high mortality rate in untreated cases, this endemic disease is of mandatory report in Argentina. Secure and accurate diagnostic methods are needed for the epidemiological surveillance of the disease. Current assays rely on antigens prepared from lysates of virus infected mammalian cells. The bio-safety issue related to the manipulation of large quantities of virus restricts such antigen production to laboratories with the appropriate containment facilities. In this report, we describe the development of an enzyme linked immunosorbent assay for the etiologic confirmation of the disease, based on recombinant antigens expressed in insect cells. Eight different variables of the assay were optimized with the Taguchi approach for experimental design (L18 design, seven three-level factors and one two-level factor). The area under the receiver operating characteristics (ROC) curve was 0.966, showing the high accuracy of the test discriminating positive from negative samples. Taking into account the biosafety benefits, the high yields of antigen in cell culture, and the general performance of the assay, it is expected that it will be a useful alternative to the current ELISA for the detection of antibodies in sera from convalescent patients. ***J. Med. Virol. 80:2127–2133, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: JUNV; ELISA; AHF; arenavirus; Taguchi approach; baculovirus; nucleoprotein

INTRODUCTION

The family *Arenaviridae* comprises 22 recognized members and new arenaviruses have been characterized recently [Salvato et al., 2005; Cajimat et al., 2007; Lecompte et al., 2007; Delgado et al., 2008; Palacios et al., 2008]. Some of them cause severe hemorrhagic fevers in humans [Salvato et al., 2005]. These are enveloped, bisegmented RNA viruses. The genome consists of two single-stranded RNA molecules, designated L (7.2 kb) and S (3.4 kb). The S segment encodes the major structural components of the virion: the major nucleocapsid protein (N) and the envelope glycoprotein precursor (GPC). The L segment encodes the RNA-dependent RNA polymerase (L) and a zinc-binding matrix protein (Z) [Meyer et al., 2002].

Junín virus (JUNV) is the etiological agent of Argentine hemorrhagic fever (AHF). The clinical symptoms of AHF include hematological, neurological, cardiovascular, renal and immunological alterations. The mortality rate for AHF may be as high as 30%, but early treatment with immune plasma reduces fatal cases to less than 1% [Enria et al., 2008]. The natural reservoir of the virus is the rodent *Calomys musculinus* (Sigmodontinae, Muridae). The human population at risk is composed mainly of field workers, who are believed to become infected through cuts or skin abrasions or via airborne dust contaminated with urine, saliva or blood from infected rodents [Maiztegui et al., 1986].

Since the emergence of the disease in the 1950s, annual outbreaks have occurred without interruption. The region at risk has been progressively expanding into

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North-central Argentina and almost 5 million of humans are today considered to be at risk for AHF [Maiztegui et al., 1986; Enria et al., 2008].

AHF is a disease of mandatory reporting in Argentina. ELISA is the routine assay for the etiologic confirmation of clinical diagnosis for convalescent patients and for the surveillance of the zoonosis [Riera et al., 1997; Morales et al., 2002]. Nevertheless, the use of the live virus for the production of antigens represents a potential risk and restricts its production to facilities with biosafety levels between 2 and 3 in Argentina, which depends on the strain employed (attenuated or not).

Here, we describe the expression of JUNV nucleocapsid protein (N) using a recombinant baculovirus and the development of an enzyme-linked immunosorbent assay based on the recombinant antigen.

MATERIALS AND METHODS

Insect Cells and Recombinant Baculoviruses

IPLB-Sf21AE (Sf21), Sf9 and BTI-TN-5B1-4 (High Five™, Invitrogen, Carlsbad, CA) insect cells were propagated at 28°C in TC100 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum and 0.1% gentamicine.

The gene encoding the nucleoprotein of JUNV (MC2 strain) was reconstructed from two clones of a cDNA library. Briefly, three fragments were generated by restriction endonuclease digestion and cloned in two steps into the pBS-KS$^+$ vector (Stratagene, Cedar Creek, TX), from which the insert was cut with *Bam*HI and *Sac*II, blunt ended with nuclease S1 and ligated to *Bam*HI linkers. After digestion with that enzyme, the N gene was cloned into the transfer vectors pAcUW2B and pAcRP25. Clones with the correct polarity (assessed by restriction analysis) were selected and co-transfected with a polyhedrin negative (polh$^-$) AcMNPV or wild-type AcMNPV (C6 strain) DNA in Sf21 cells. Recombinant baculoviruses were selected by visual screening of polh$^+$ or polh$^-$, according to the parental DNA employed. Individual plaques were cloned by three successive plaque purifications. Two clones were sequenced and selected to represent the two kinds of recombinants: AcJUN-N+, which expresses high levels of JUNV N in addition to polyhedrin, and AcJUN-N, which only over-expresses N.

Time Course of Expression

The analysis of the kinetics of expression was performed essentially as described by O'Reilly et al. [1992].

Partial Purification of Antigen

High Five™ cells were infected at an moi of 10 PFU/cell with the recombinant baculovirus, either Ac-JUN-N, Ac-LCM-N [Matsuura et al., 1986] or Ac-LacZ. The cells were harvested at 72 h p.i., washed with PBS and resuspended in 2 M urea and 1% Triton-X100 in PBS. The solutions also contained a cocktail of protease inhibitors (100 µM leupeptin, 1 µM pepstatin A, 1 mM PMSF and 10 mM EDTA). After incubation for 2 hr (with shaking), cells were centrifuged at 17,000*g* for 5 min. Leaving most of the recombinant protein in the pellet. It was finally solubilized in 10 M urea, quantified by the Bradford method and diluted to a concentration of 50 µg/ml (10 M urea in PBS).

Western Blot

Proteins were resolved by SDS–PAGE (10% acrylamide) [Sambrook et al., 1989]. Transfer to PVDF membrane (Hybond™-P; Amersham Biosciences, Little Chalfont, UK) was carried out during 30 min at 15 V using a semi-dry Trans-blot® SD device (BioRad, Hercules, CA). The membrane was then blocked with 5% fat-free powdered milk and 0.1% tween 20 in PBS (PMT) for 30 min at room temperature. The incubation with the monoclonal antibody anti JUNV N, QB06-AE05 [Sanchez et al., 1989] (diluted 1:500 in PMT), was performed for 2 hr at room temperature. The membrane was washed with 0.1% tween 20 in PBS, and incubated for 1 hr room temperature with a 1:1,000 dilution of the HRP (horseradish peroxidase) conjugated goat anti-mouse antibody (P0447, Dako, Glostrup, Denmark) and finally washed three times (15 min each). Chemiluminescence was generated by adding 1.25 mM luminol (Sigma, St. Louis, MO), 200 mM *p*-coumaric acid (Sigma) and 2.7 mM H_2O_2 in 0.1 M Tris–HCl, pH 8.5. Images were obtained with the EC3™ 500 Imaging System (UVP, Upland, CA).

Serum Samples

Thirty-six serum samples from AHF patients were provided by Dr. Silvana Levis and Dr. Delia Enría from the Instituto Nacional de Enfermedades Virales Humanas (INEVH), Pergamino, Argentina. Positivity was established in that institute either by ELISA or neutralization test. One hundred twenty normal sera from the non-endemic area were employed as negative controls.

Taguchi Approach for Optimizing ELISA

The optimization of the ELISA was performed using the Taguchi approach for experimental design, essentially as previously described [Taguchi, 1993; Jeney et al., 1999]. We employed an L-18 array without modifications, which permits the study of seven 3-level factors and a 2-level factor (factor: variable; level: value assigned to the variable). The factors evaluated are shown in Table I. The 36 experiments (18 for background analysis, without antigen coating) were performed by triplicate and randomly distributed in the inner 60 wells of three plates (assigned according to the incubation times). The background was processed with the "the smaller the better" transformation:

$$\frac{S}{N} = -10\log_{10}\left[\frac{b_1^2 + b_2^2 + b_3^2}{3}\right]$$

S/N stands for the signal to noise ratios characteristic of the Taguchi approach, although, no noise factors were

TABLE I. Assignment of Factors and Levels for the ELISA Optimization with the Taguchi Approach

Factor	Description	Level 1	Level 2	Level 3
T	Tween 20 concentration in PMT	0.05%	0.1%	—
N	N concentration (coating)	0.2 μg/well	0.5 μg/well	1 μg/well
U	Urea concentration (coating)	0	4 M	8 M
A	Anti human IgG serum (dil.)	1/500	1/1,000	1/2,000
S	Streptavidin/HRP (dil.)	1/500	1/1,000	1/2,000
I	Time of incubations	30 min	1 hr	2 hr
D	Time of development	8 min	12 min	16 min
ie	Internal error control	—	—	—

included in this study; b_{1-3} are the triplicates of the background values.

For the positive samples values (a_{1-3} are the triplicates of the signal values), the transformation "the bigger the better" was applied:

$$\frac{S}{N} = -10\log_{10}\left[\frac{1/a_1^2 + 1/a_2^2 + 1/a_3^2}{3}\right]$$

To facilitate the rapid interpretation of data and based on the transformation "the larger the better," we evaluated a new calculation of the S/N ratio:

$$\frac{S}{N} = -10\,\mathrm{Log}_{10}\left[\frac{\left(\frac{1}{a_1^2}+\frac{1}{a_2^2}+\frac{1}{a_3^2}\right)}{\left(\frac{1}{b_1^2}+\frac{1}{b_2^2}+\frac{1}{b_3^2}\right)}\right]$$

IgG ELISA

The ELISA optimization was performed following the above mentioned design. All the solutions were prepared with PBS. Flat bottom microplates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at room temperature with 0.5 μg per well of recombinant JUNV N antigen (100 μl, 4 M urea). Plates were blocked with 5% fat-free milk powder and 0.1% tween 20 (PMT), for 2 hr at 37°C (blocking solution was discarded and the plates frozen until use). Microplates were incubated with the serum samples diluted 1/400 in PMT for 2 hr at 37°C and washed five times with 0.1% tween 20 in PBS. Thereafter, incubated with the rabbit anti-human IgGs antiserum (E0482, DAKO) diluted 1/2,000 with PMT, washed, and followed with HRP conjugated streptavidin (P0397, DAKO) 1/1,000 in PMT. After the last round of washes, 100 μl of TMB (1-Step Turbo TMB-ELISA, Pierce, Rockford, IL) were added to each well. Plates were kept in the dark for 45 min (16 min for 1/10 dilutions of sera) and the reaction was stopped with 100 μl 2 M HCl. The absorbance was read at a wavelength of 450 nm (Tecan Absorbance Microplate Reader, Männedorf, Switzerland). Wells coated with LacZ produced and purified in the same way as N were used for background subtraction.

The assay variability was calculated with a positive control sample of moderate reactivity. The intra-assay variability (CV × 100) was calculated as the mean of three different assays. The inter-assay variation was calculated over three independent assays, using the mean of duplicates.

RESULTS AND DISCUSSION

Recombinant nucleocapsid proteins of Old World arenaviruses have been proposed as antigens for diagnostic tests with human and animal sera [Barber et al., 1987, 1990; Lloyd et al., 1989; Lukashevich et al., 1993; Homberger et al., 1995; Ter Meulen et al., 1998; Gunther et al., 2001; Saijo et al., 2007]. Nevertheless, this is the first ELISA developed for the diagnosis of a New World arenavirus hemorrhagic fever using a recombinant viral protein. We chose to express JUNV N in the baculovirus system because high levels of expression are usually reached [O'Reilly et al., 1992].

The nucleocapsid ORF of MC2 JUNV strain [Ghiringhelli et al., 1991] was reconstructed from a cDNA library and cloned into two different transfer vectors (pAcUW2B-JUN-N and pAcRP25-JUN-N). They were used to generate the recombinant baculoviruses: AcJUN-N+ (polh$^+$) and AcJUN-N (polh$^-$).

The high level of N expression in insect cells infected with the recombinant baculovirus was demonstrated by SDS–PAGE, as can be seen in Figure 1. In panel B, the

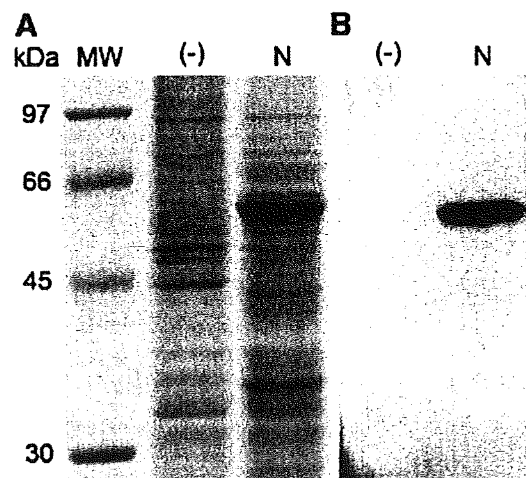

Fig. 1. Expression of N in insect cells. SDS–PAGE (A) and western blot (B) analysis of High Five™ cells infected with the recombinant baculovirus over-expressing N (72 h p.i., moi 10); uninfected cells were run as control (−). The monoclonal antibody employed in the immunoblot specifically binds N of JUNV [Sanchez et al., 1989].

identity of N was tested by western blot with a monoclonal antibody [Sanchez et al., 1989].

With the aim of determining the best conditions for the expression of N, time course experiments (24, 48, 72, and 96 h p.i.) were performed varying the multiplicities of infection (moi 0.1, 1, and 10), the insect cell line (Sf9 or High Five™), and the recombinant baculovirus employed (polh^{+} or polh^{-}). The highest yields of N were observed at 72 hr in High Five™ cells infected at an moi = 10 PFU/cell. AcJUN-N, which does not express polyhedrin, was selected to continue the setup of the ELISA. We speculate that the highly expressed polyhedrin in recombinant AcJUN-N+ could compete with N for the adsorption to the well surface. Nevertheless, AcJUN-N+ could be used for the production of antigen in larvae at a higher scale, with a significant reduction of costs.

Due to its high insolubility, N was partially purified by differential solubilization in urea solution. Most of the cellular proteins were solubilized in 2 M urea while N remained insoluble. It was then solubilized in 10 M urea (data not shown).

Optimization of IgG-ELISA

In a multifactorial assay such as the ELISA, the numerous variables tend to generate almost a different protocol for every ELISA developed. In order to simplify the optimization of the assay, we used the Taguchi approach [Taguchi, 1993]. In a full factorial study, the analysis of the same number of factors and levels would have required 4,374 experiments (wells) and the same number for the background study.

The L18 array permitted the study of seven 3-level factors and one 2-level factor. Including the background analysis, 36 experiments were run randomly distributed in the wells, but restricted by factor I (incubation time). The results are depicted in Table II. The conditions with the best ratios were chosen, even when the incidences of some factors were negligible. This was not the case for the anti-human IgG antibody, where the maximum dilution was adopted (less reagent used with a minimum loss in assay performance as judged by the incidence of that factor). The resulting protocol was described in Materials and Methods Section.

Since the main objective in the optimization of the assay is to obtain the highest ratio between positive sample and background signal, a new formula was developed to rapidly test the results of the optimization (see Materials and Methods Section). The results in this case were in concordance with those obtained separately (positive and background) according to the incidence of each factor as established by the ANOVA (Table II). This showed the suitability of this formula to rapidly interpret the data in ELISA optimization.

The implementation of the Taguchi method permitted a rapid optimization of the assay (2 days) with a statistic background (ANOVA for the incidence of each factor) and a minimum number of experiments (108 wells, including triplicates and background analysis). Curiously, the Taguchi method, to our knowledge, has been adopted for ELISA optimization only once before [see Jeney et al., 1999].

The resulting assay showed an intra and inter-assay variability of 4.9% (CV × 100) and 11.6%, respectively.

Figure 2 shows reactivity of positive serum for AHF and LCM against the recombinant N from Ac-JUN-N and LCMV. Cross-reactivity exists between both arenaviruses as has been also shown in hyperimmune rabbit sera [Saijo et al., 2007]. The serum chosen as positive for AHF in this figure showed the highest level of cross-reactivity (right panel). This probably reflects the fact that both arenaviruses coexist in the same area [Ambrosio et al., 2006]. Nevertheless, the mean JUNV/LCMV reactivity ratio (at 1/400 dilution) for the 36 positive sera was 6.1 (data not shown). Also, sera from a vaccinated person (healthy) was included to show that this IgG ELISA can detect antibodies against the vaccine Candid# 1.

Evaluation of Diagnostic Suitability

In order to determine the accuracy of the assay, that is, the ability to distinguish between positive and

TABLE II. Taguchi Totals, Optimum Conditions, and ANOVA Results

	Positive				Background				Ratio positive/background			
	Taguchi totals				Taguchi totals				Taguchi totals			
	Level			%F (ANOVA)	Level			%F (ANOVA)	Level			%F (ANOVA)
	1	2	3		1	2	3		1	2	3	
T	48.105	**48.619**	—		−43.315	**−41.586**	—		4.941	**7.091**	—	
N	47.136	48.591	**49.359**		−42.654	**−41.763**	−42.933		4.558	**7.056**	6.435	
U	**48.742**	48.582	47.762		−46.336	−40.617	**−40.396**	**31.07**	2.502	**8.069**	7.478	**31.16**
A	**50.317**	48.987	45.782	**7.54**	−44.256	−42.602	**−40.492**	**9.75**	6.217	**6.492**	5.340	
S	**55.046**	47.145	42.896	**52.76**	−46.309	−41.771	**−39.270**	**34.88**	**8.770**	5.519	3.760	**21.54**
I	43.803	47.660	**53.624**	**33.97**	−41.184	**−41.002**	−45.165	**15.17**	2.663	6.730	**8.656**	**31.19**
D	46.863	48.369	**49.854**		**−41.661**	−42.472	−43.217		5.336	5.968	**6.745**	
Ie	48.237	48.073	48.777		−42.349	−41.410	−43.591		6.076	6.675	5.298	

Optimum conditions are highlighted in bold. *F* percentage (%*F*) derives from de ANOVA and indicates the percentage of each factor from the total *F* ratios. Only significant values are shown.

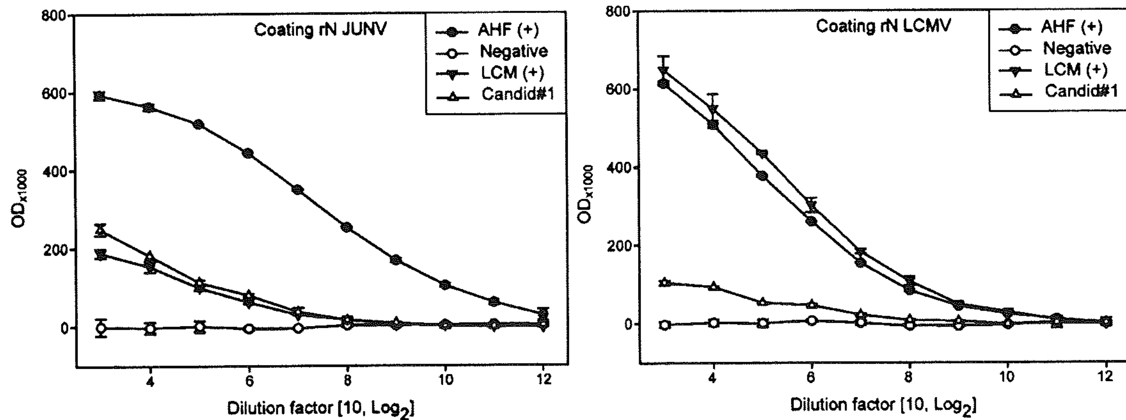

Fig. 2. Reactivity of AHF and LCM patients to recombinant N. Reactivity of serially diluted sera in the recombinant IgG ELISA. **Left panel**: plate coated with N from Ac-JUN-NP; **Right panel**: plates coated with N from a recombinant baculovirus expressing LCMV-NP [Matsuura et al., 1986].

negative samples, an approach based in ROC curves (non-parametric) was adopte. The ROC plot is a graph of sensitivity (probability of true positivity) versus 1 − specificity (probability of false positivity). A test with perfect discrimination (no overlap between positive and negative samples) has a ROC plot that passes through the upper left corner with an area under the curve of 1. Conversely, a test with no discrimination at all has a curve of 45° starting in the lower left corner with an area of 0.5 [Zweig and Campbell, 1993; Obuchowski et al., 2004]. In this study we included as "positives" all the samples that had their reactivity determined previously (at the INEVH, Pergamino) either by ELISA based on the viral antigen (Junín $XJCl_3$ virus infected Vero E6 cells) or by NT. This and the fact of only knowing the reactivity of each sample and not its titer, helps to avoid the so-called verification bias (selection of easily diagnosed samples). Thirty-six positive and one hundred twenty negative sera were used. As shown in Figure 3, the area under the ROC curve was 0.966 (95% confidence interval = 0.924–0.988), indicating that the assay has an excellent accuracy. The P value = 0.0001 confirms that this area is significantly different from 0.5. An area of 0.966 means that a randomly selected individual from the diseased group has a laboratory test value larger than that for a randomly chosen individual from the non-diseased group 96.6% of the times [Zweig and Campbell, 1993]. A similar AUC_{ROC} was obtained with a serum dilution of 1:10 (data not shown), but the higher dilution was selected to save sera.

Given the high accuracy of the assay, one might select a suitable threshold with high values of sensitivity and specificity. This cut-off value (COV) is dependent upon the use of the ELISA. In our case, the assay would be used for the etiologic confirmation of convalescent patients, which means that a high specificity is desired. Conversely, there are assays that require maximum sensitivity, for example, an RT-PCR developed in our laboratory for the acute phase, when the detection of the virus is important to make a decision on the administration of immune plasma [Lozano et al., 1993, 1995].

The $OD_{\times 1000}$ values obtained for all the sera are shown in the left panel of Figure 4. In the right panel the specificity and sensitivity from the ROC table are shown for every possible COV. A threshold was selected according to the maximum level of specificity + sensitivity (or positive maximum likelihood) at $OD_{\times 1000} \geq 81$, with a specificity of 99.16% and sensitivity of 89.19% (95% CI: 95.4–99.9 and 74.6–96.9, respectively). The high specificity at that COV suits the requirement for this assay. From twenty-eight sera studied by ELISA based on the natural antigen, five were classified as doubtful. From the remaining 23, 4 showed discrepancies with this ELISA and are indicated in Figure 4 with arrows

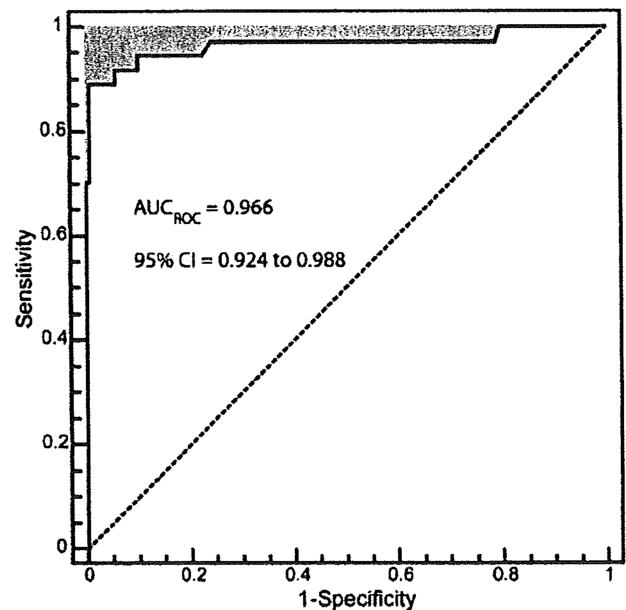

Fig. 3. ROC curve for the N JUNV IgG ELISA. ROC curve for the $OD_{\times 1000}$ values of 36 positive and 120 negative serum samples. AUC_{ROC}: area under the ROC curve. CI: confidence intervals.

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Development of Recombinant Nucleoprotein-Based Diagnostic Systems for Lassa Fever[▽]

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Diagnostic systems for Lassa fever (LF), a viral hemorrhagic fever caused by Lassa virus (LASV), such as enzyme immunoassays for the detection of LASV antibodies and LASV antigens, were developed using the recombinant nucleoprotein (rNP) of LASV (LASV-rNP). The LASV-rNP was expressed in a recombinant baculovirus system. LASV-rNP was used as an antigen in the detection of LASV-antibodies and as an immunogen for the production of monoclonal antibodies. The LASV-rNP was also expressed in HeLa cells by transfection with the expression vector encoding cDNA of the LASV-NP gene. An immunoglobulin G enzyme-linked immunosorbent assay (ELISA) using LASV-rNP and an indirect immunofluorescence assay using LASV-rNP-expressing HeLa cells were confirmed to have high sensitivity and specificity in the detection of LASV-antibodies. A novel monoclonal antibody to LASV-rNP, monoclonal antibody 4A5, was established. A sandwich antigen capture (Ag-capture) ELISA using the monoclonal antibody and an anti-LASV-rNP rabbit serum as capture and detection antibodies, respectively, was then developed. Authentic LASV nucleoprotein in serum samples collected from hamsters experimentally infected with LASV was detected by the Ag-capture ELISA. The Ag-capture ELISA specifically detected LASV-rNP but not the rNPs of lymphocytic choriomeningitis virus or Junin virus. The sensitivity of the Ag-capture ELISA in detecting LASV antigens was comparable to that of reverse transcription-PCR in detecting LASV RNA. These LASV rNP-based diagnostics were confirmed to be useful in the diagnosis of LF even in institutes without a high containment laboratory, since the antigens can be prepared without manipulation of the infectious viruses.

Lassa fever (LF) is a viral hemorrhagic fever caused by Lassa virus (LASV), an Old World arenavirus. Many cases of LF occur in western Africa in countries such as Guinea, Sierra Leone, and Nigeria (7, 23, 27, 29–31). It is thought that LASV infects tens of thousands of humans annually and causes hundreds to thousands of deaths (34). Humans become infected through contact with infected excreta, tissue, or blood from the peridomestic rodent, *Mastomys natalensis*, the reservoir host of LASV (34). LASV can be transmitted to other humans via mucosal or cutaneous contact or through nosocomial contamination (27). More than 20 imported cases of LF have been reported outside the endemic region in areas such as the United States, Canada, Europe, and Japan (1, 2, 13, 15, 18, 24, 25). Recently, the potential for the use of hemorrhagic fever viruses, including LASV, as a biological weapon has been emphasized (5, 6). Therefore, the development of diagnostic systems for LF is important even in countries free from LF outbreaks to date.

Manipulation of infectious LASV is necessary for the detection of specific antibodies. However, a high-containment laboratory (biosafety level 4 [BSL-4]) is required for handling infectious LASV and, therefore, the preparation of LASV antigens cannot be implemented in institutes without a BSL-4 facility. Within this framework, it is important to develop sensitive and specific diagnostic systems for LF that eliminate the need for the manipulation of infectious LASV. In the present study, the recombinant nucleoprotein (rNP) of LASV (LASV-rNP) was expressed and evaluated for its ability to detect LASV antibodies. LASV-rNP-based enzyme-linked immunosorbent and indirect immunofluorescence assays (ELISA and IIFA) were developed. Furthermore, novel monoclonal antibodies to LASV-rNP were generated and used in combination with the recombinant antigen to develop an LASV antigen (nucleoprotein) capture ELISA. The present study presents an alternative strategy to develop diagnostic systems without handling infectious LASV.

MATERIALS AND METHODS

Cells. A HeLa cell line was cultured in the Eagle minimum essential medium supplemented with 10% fetal bovine serum and the antibiotics penicillin G and streptomycin (MEM-10FBS). Tn*5* insect cells were used for the expression of the rNPs of arenaviruses (LASV, lymphocytic choriomeningitis virus [LCMV], and Junin virus [JUNV]) in a baculovirus system. The Tn*5* insect cells were cultured as reported previously (38).

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Viruses. LASV (strain AV), which was isolated from an imported case of LF to Germany from West Africa, was used (13). The experimental process that required manipulation of infectious LASV was carried out in the BSL-4 laboratory in the P4 laboratory, INSERM, Lyon, France. Mopeia virus (MOPV), which belongs to the family *Arenaviridae*, genus *Arenavirus*, was also used. Recombinant NPs of LCMV (26) and JUNV (11), designated LCMV-rNP and JUNV-rNP, respectively, were also expressed in a baculovirus system and used in the study. A baculovirus (Ac-ΔP), which lacks polyhedrin expression, was used as a control virus (26). The virus titer of LASV in serum samples was determined by using a focus-forming unit (FFU) assay as described previously (3).

Sera. Four human serum samples—three samples serially collected from one patient with LF and one additional sample from another patient with LF—and ninety-six human sera collected from Japanese subjects with no history of travel to areas where LF is endemic were used as positive and negative controls, respectively. The patient with LF, from whom three serial serum samples were collected, was the first case of LF to be imported in Japan in 1987 (15). The other human serum sample was provided from the Special Pathogens Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.

Serum samples collected from five monkeys (*Macaca fascicularis*) subcutaneously infected with LASV strain AV at 10^3 FFU (two monkeys) or 10^7 FFU (three monkeys) and those collected from four monkeys with mock infection were also used. The serum samples used in the study were collected at 4 to 5 weeks postchallenge.

Five hamsters were subcutaneously infected with 10^3 FFU of LASV, strain AV, and blood was drawn on days 0, 4, 11, and 16 postinfection, taking the day on which the virus was inoculated as day 0. Serum fractions of the collected blood specimens were separated and tested for LASV antigen by antigen capture (Ag-capture) ELISA and reverse transcription-PCR (RT-PCR).

Rabbit sera (polyclonal antibodies) were raised against LASV-rNP, LCMV-rNP, and JUNV-rNP by immunization of rabbits with the purified LASV-rNP, LCMV-rNP, and JUNV-rNP, respectively, in the form of a mixture with the adjuvant, Inject Alum (Pierce). Rabbits were immunized with sufficient amount of the purified nucleoproteins of each virus three times with an interval of 2 weeks. After confirmation of the increased titer, >10,000 times as determined by indirect immunofluorescence assay, which was developed in the present study, blood was drawn from the rabbits, and the serum fraction was used in the present study.

Recombinant baculovirus. In order to construct the transfer vector, a cDNA clone of NP from LASV strain Josiah was used. The cDNA was kindly provided by J. B. McCormick, former Director of the Special Pathogens Branch, National Centers for Infectious Diseases, Centers for Disease Control and Prevention. The complete nucleotide sequence of the NP gene is registered in GenBank under the accession number NC_004296. The DNA of the LASV-NP was amplified by PCR from the source using the primers LAS-NfB (5′-GTGGATCCA ACACAACAATCTGG-3′; the BamHI restriction site is underlined) and LAS-NrB (5′-CCGGATCCATTTACAGAACGACTC-3′). The PCR conditions were the same as previously reported (38). The 1,743-bp amplification product was digested with BamHI and subcloned into the BamHI site of pQE32 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE32-LASV-NP. The inserted LASV-NP DNA was sequenced by using appropriate primers with an ABI Prism 310 genetic analyzer (PE Applied Biosystems, Foster City, CA) and confirmed to be in proper orientation downstream the promoter and identical to the original sequence. The DNA fragment of LASV-NP with a histidine (His) tag was isolated from the plasmid, pQE32-LASV-NP, by digestion with EcoRI and HindIII. It was then blunt repaired with Klenow enzyme and ligated into the blunt-ended BamHI site of pAcYM1 (26). The resulting recombinant transfer vector with the correct orientation with respect to the polyhedrin promoter was constructed (pACYM1-His-LASV-NP). Tn5 insect cells were transfected with mixtures of purified *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA and pAcYM1-His-LASV-NP according to the procedures described by Kitts et al. (20), with the modification of Matsuura et al. (26). Recombinant baculovirus was then isolated. The baculovirus, which expressed His-tagged LASV-rNP (His-LASV-rNP), was designated Ac-His-LASV-NP.

The baculovirus, Ac-LCMV-NP, which expressed LCMV-rNP, was used in the study (26).

The recombinant baculovirus that expressed JUNV-rNP, Ac-JUNV-NP, was generated as follows. The gene encoding the NP of JUNV (strain MC2) was reconstructed from cloned cDNA. The nucleotide sequence of the interest gene was deposited in GenBank under accession number D10072 (12). A complete NP gene with the initiation and stop codons amplified by PCR using appropriate primers, which possessed BamHI restriction sites. The entire DNA product of JUNV-NP was digested with BamHI and ligated into the transfer vector pAcUW2B (28). Clones containing the insert in the correct orientation were selected, and the plasmid DNA was used for cotransfection in Sf21 cells with a polyhedrin-positive AcMNPV DNA, and the supernatant culture was screened for a polyhedrin-negative phenotype by plaque assay (19). Finally, recombinant baculovirus clones overexpressing JUNV-rNP were obtained after three successive plaque purifications. One of them, designated AcNMPV-Jun-N122, was used in the present study and is referred to hereafter as Ac-JUNV-NP.

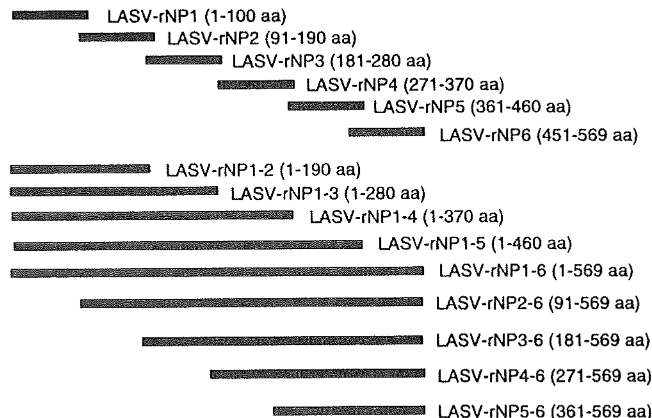

FIG. 1. Schematic representation of truncated LASV-rNP expressed as a form of GST fusion protein in *E. coli* transformed with the corresponding expression vector. The description "LASV-NP1-6" in the middle portion of the figure indicates full-length LASV-rNP.

Expression and purification of His-LASV-rNP, LCMV-rNP, and JUNV-rNP. Tn5 cells infected with Ac-His-LASV-NP were incubated at 26°C for 72 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution. A preliminary study demonstrated that most of the Tn5 cellular proteins were solubilized in PBS containing 2 M urea (PBS–2 M urea) but that the His-LASV-rNP was insoluble and that the LASV-rNP could be solubilized in PBS containing 8 M urea (PBS–8 M urea). Therefore, the Tn5 cells infected with Ac-His-LASV-NP were first suspended in PBS–2 M urea. After the centrifugation of the cell suspensions at 15,000 × *g* for 10 min, the pellet fractions were collected and then were solubilized in PBS–8 M urea. After the centrifugation of the samples, the supernatant fractions were used as the purified antigens. LCMV-rNP and JUNV-rNP showed dissolution characteristics in urea similar to those of His-LASV-rNP; therefore, LCMV-rNP and JUNV-rNP were also fractioned in the same way as the His-LASV-rNP. The control antigen was produced from Tn5 cells infected with Ac-ΔP in the same manner as that for the positive antigens. The His-LASV-rNP was also purified by using the Ni^{2+} column purification method as reported previously (38). The source for His-LASV-rNP-purification was the supernatant fraction of the PBS–8 M urea-treated Tn5 cells infected with Ac-His-LASV-NP after sufficient dilution with PBS in order to reduce the urea concentration.

SDS-PAGE. The expression and purification efficiency of His-LASV-rNP, LCMV-rNP, and JUNV-rNP were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12% polyacrylamide) after staining with Coomassie blue.

Establishment of MAbs. Monoclonal antibodies (MAbs) were generated as previously described (32, 41). BALB/c mice were immunized with purified His-LASV-rNP in the present study. Isotypes of the MAbs were determined by using a mouse MAb isotyping kit (Life Technologies).

Expression of truncated NPs of LASV. In order to determine the epitope of the MAbs to the His-LASV-rNP, truncated LASV-rNPs were expressed as a form of fusion protein with glutathione *S*-transferase (GST) as shown in Fig. 1. The DNA corresponding to each of the truncated NP fragments was amplified with the designed primers. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). Each insert was sequenced and confirmed to be in the correct frame and identical to the original sequence. The GST-tagged nucleoprotein fragments were expressed in an *Escherichia coli* BL21 system.

Western blotting. The MAbs were tested for reactivity to His-LASV-rNP and its fragments by Western blotting as reported previously (17, 32, 41).

Pepscan analyses. ELISA was performed as reported previously with the purified rNP or partial nucleoprotein peptides as the antigen (33). The peptides

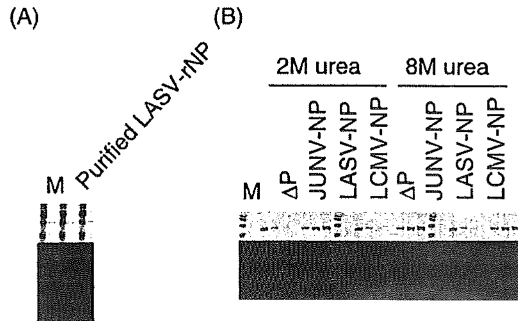

FIG. 2. SDS-PAGE analyses of the purification of His-LASV-rNP using the Ni^{2+} column purification method (A) and of the semipurification strategy based on the hydrophobic property of arenavirus nucleoproteins (B). The supernatant fractions of the Ac-ΔP-, Ac-His-LASV-NP-, Ac-LCMV-NP-, or Ac-JUNV-NP-infected Tn*5* cells treated with PBS–2 M urea (B, left part) are shown. The pellet fractions of these cells treated with PBS–2 M urea were further solubilized with PBS–8 M urea (B, right part).

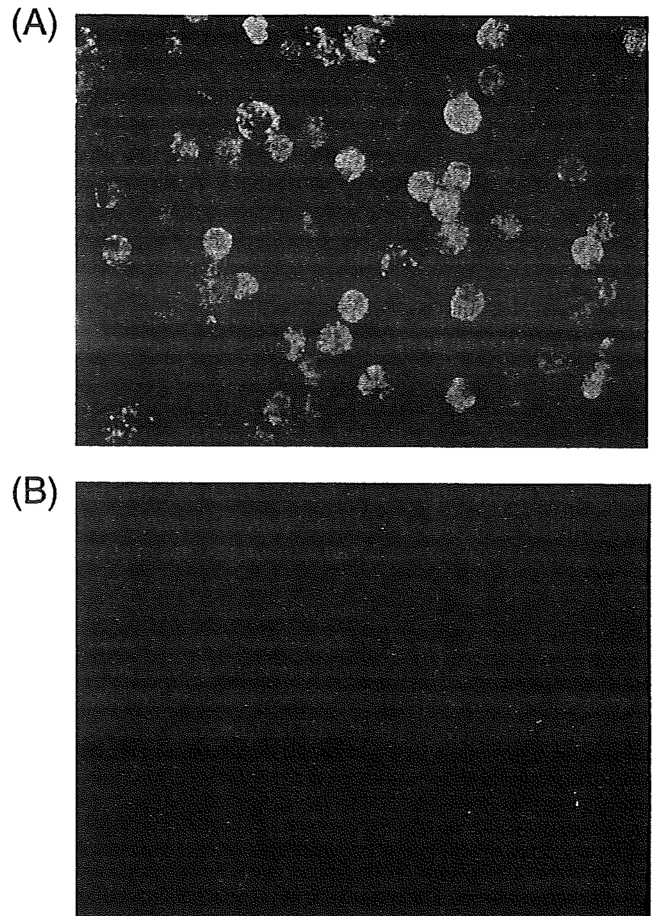

FIG. 3. Staining patterns of LASV-rNP-expressing HeLa cells by sera from an LF patient (A) and a healthy control (B) in an IIFA.

were shifted by 1 amino acid (aa), with a consecutive overlap of 9 aa to cover the entire LASV-NP1 (aa 1 to 100) and LASV-NP5 (aa 361 to 460) fragments. Linear epitopes on the NP were determined by using Pepscan (Chiron Technologies, Clayton, Australia) according to the manufacturer's instructions. Ninety-six peptides were prepared as 14-aa biotinylated peptides, including a 4-aa spacer sequence (SGSG) at the amino-terminal end, according to each of the amino acid sequences of the LASV-rNP1 and LASV-rNP5 of the LASV Josiah strain. The methods were previously described in detail (33).

IgG-ELISA. Immunoglobulin G (IgG)-ELISA was performed as previously described except for the antigen preparation (38, 39). Briefly, ELISA plates (96-well type plate, Pro-Bind; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified His-LASV-rNP, LCMV-rNP, or JUNV-rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (M-T-PBS), followed by incubation for 1 h for blocking. The plates were washed three times with T-PBS and then inoculated with the test samples (100 μl/well), which were diluted fourfold from 1:100 to 1:6,400 with M-T-PBS. After a 1-h incubation period, the plates were washed three times with T-PBS, and then the plates were inoculated with goat anti-human IgG antibody labeled with HRPO (1:1,000 dilution; Zymed Laboratory). After a further 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and optical density was at 405 nm (OD_{405}) was measured against a reference of 490 nm. The adjusted OD_{405} was calculated by subtracting the OD of the negative antigen-coated wells from that of the corresponding wells. The means and standard deviations were calculated from the 96 control sera. The cutoff value for the assay was defined as the mean plus 3 standard deviations.

Immunofluorescence. The pQE32-LASV-NP was digested with BamHI, and the insert was subcloned into the BamHI site of the pKS336 vector (40). The LASV-NP gene that was inserted into the pKS336 vector, pKS336-LASV-NP, was confirmed to be in the correct orientation to the promoter, tested for nucleotide sequencing as described above, and the nucleotide sequence of the gene was confirmed to be identical to the original sequence. HeLa cells were then transfected with pKS336-LASV-NP by using a FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. The cells transfected with the plasmid were selected with 3 μg of blasticidin *S*-hydrochloride/ml in MEM-10FBS. The HeLa cell clones were analyzed for the expression of LASV-rNP by IIFA using the rabbit serum raised against His-LASV-rNP. The cells expressing LASV-rNP were subcloned and used as IIFA antigens.

Ag-capture ELISA. Ag-capture ELISA was performed as previously described (32, 41). The purified MAb to His-LASV-rNP, MAb 4A5, produced in the present study was diluted in PBS solution, and 100 μl was adsorbed overnight at 4°C onto the immunoplates (96-well type plate, Pro-Bind, Falcon; Becton Dickinson Labware). Purified MAb 4A5 was coated onto the immunoplates at a concentration of approximately 100 ng/well in 100 μl of PBS. The difference in the procedures between the Ag-capture ELISA in the present study and those in previous studies (32, 37, 41) is that the MAb, MAb 4A5, and rabbit serum raised to His-LASV-rNP were used as capture and detector antibodies, respectively. The procedure for the Ag-capture ELISA was performed as follows. The ELISA plate was coated with capture MAb, followed by blocking of the plate with M-T-PBS, addition of the samples to the ELISA plate, detection of the captured LASV-NP with rabbit serum raised to His-LASV-rNP, detection of rabbit IgG antibody that reacted with the captured antigen with goat anti-rabbit IgG antibodies conjugated with HRPO (Zymed Laboratories), and substrate reaction. In each run of the Ag-capture ELISA, the negative control antigen (M-T-PBS) was also tested. Serially diluted samples were added to the MAb-coated wells. The OD_{405} values of each well were adjusted by subtracting the OD_{405} value of the negative control antigen from the corresponding well. The adjusted OD_{405} was taken as a measure of the amount of antigen specifically bound. All samples were treated with 1% Nonidet-P40 (NP-40) in PBS to destroy the LASV virion and expose the nucleoprotein in the LASV virion.

RT-PCR. RT-PCR was performed as previously described (10). The primers used in the RT-PCR were 36E2 (5′-ACCGGGGATCCTAGGCATT-3′) and 80F2 (5′-ATATAATGATGACTGTTGTTCTTTGTGCA-3′). The RT-PCR was carried out with a Ready-to-Go RT-PCR tube (Pharmacia). The amplified PCR products were visualized with ethidium bromide in 2% agarose gel after electrophoresis.

RESULTS

Expression of His-LASV-rNP. Tn*5* cells infected with each of the recombinant baculoviruses—Ac-His-LASV-NP, Ac-LCMV-rNP, and Ac-JUNV-rNP—were suspended in PBS–2 M urea. Most of the cell proteins were solubilized by this

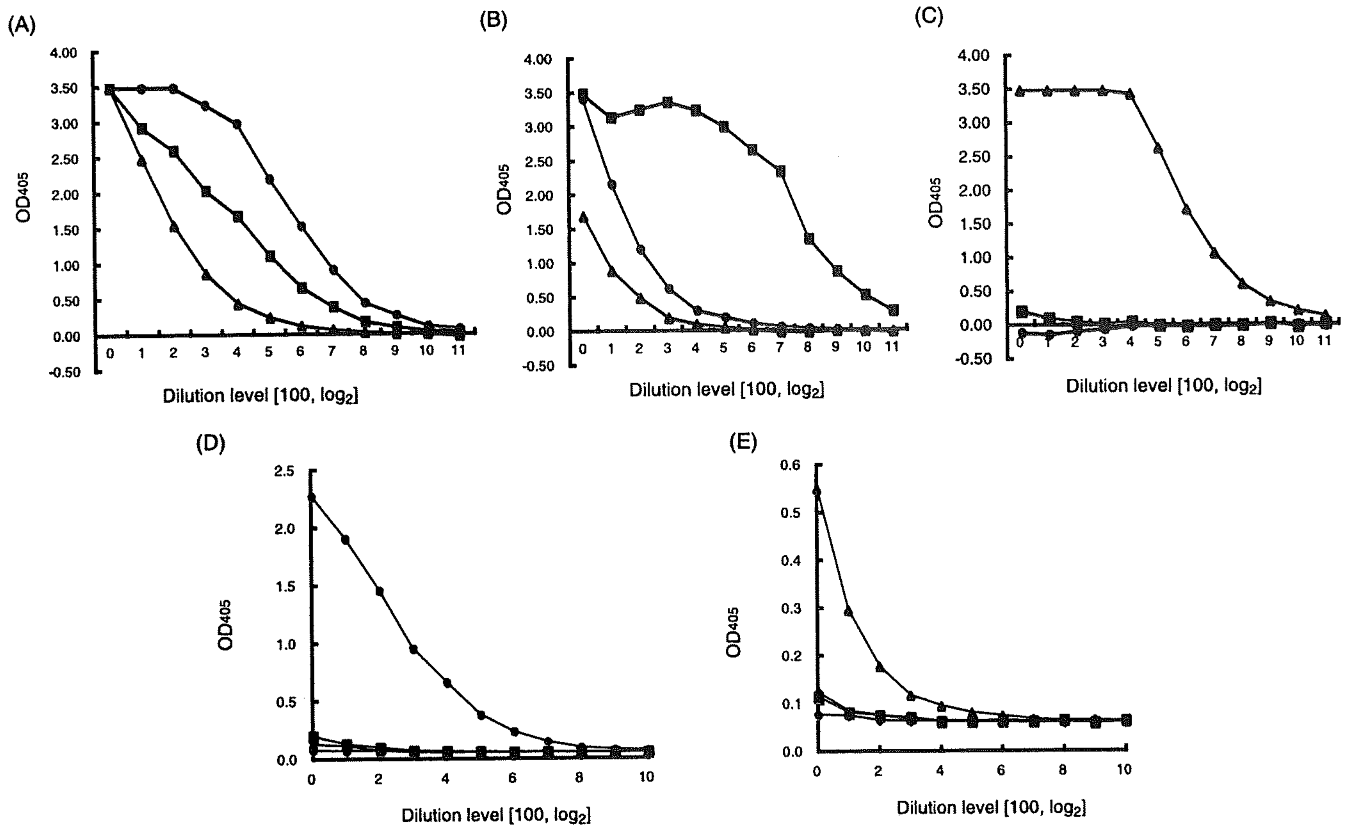

FIG. 4. Reactivity of antibodies to arenaviruses (LASV, LCMV, and JUNV) to the rNPs of these viruses. The reactivities of rabbit sera raised to LASV-rNP (●), LCMV-rNP (■), or JUNV-rNP (▲) with the antigens His-LASV-rNP (A), LCMV-rNP (B), and JUNV-rNP (C) in an IgG-ELISA are shown. The reactivities of the sera collected from patients with LF (D) and AHF (E) with the antigens LASV-rNP (●), LCMV-rNP (■), and JUNV-rNP (▲) and negative control antigen (◆) in an IgG-ELISA are also shown.

treatment, whereas the rNPs of these viruses remained insoluble. After centrifugation at 15,000 × *g* for 10 min, pellet fractions were collected. The rNPs, which were still present in the pellet fractions, were completely solubilized in PBS–8 M urea. The samples were then centrifuged at 15,000 × *g* for 10 min, and the supernatant fractions of the PBS–8 M urea were confirmed to contain highly purified recombinant rNPs of arenaviruses (Fig. 2).

Development of indirect immunofluorescence. The LASV-rNP was expressed in HeLa cells by transfection with the expression vector, pKS336-LASV-NP. The transfected cells were stained by anti-His-LASV-rNP rabbit serum and human serum samples from LF patients (Fig. 3). All 4 serum samples collected from two LF patients showed a positive staining, but 96 control serum samples did not. The LASV-rNP-based IIFA was also evaluated using serum samples collected from monkeys experimentally infected with LASV. All of the sera collected from five LASV-infected monkeys showed a positive staining, but those from four mock-infected monkeys did not.

Development of His-LASV-rNP-based IgG-ELISA. Four serum samples collected from LF patients were determined to be positive by His-LASV-rNP-based IgG-ELISA, whereas 94 of the 96 control serum samples were determined to be negative. Thus, the sensitivity and specificity of the ELISA were 100 and 96%, respectively. All serum samples collected from five LASV-infected monkeys were determined to be positive, whereas those from four mock-infected monkeys were negative.

In order to examine cross-reactivity among arenaviruses in the LASV-rNP-based IgG-ELISA, antisera against LASV-rNP, LCMV-rNP, or JUNV-rNP were examined (Fig. 4). The anti-LASV-rNP serum showed a strongly positive reaction, and anti-LCMV rNP and anti-JUNV-rNP sera showed strongly positive reactions in the IgG ELISA using the respective antigens (Fig. 4A, B, and C). Anti-LCMV-rNP and anti-JUNV-rNP sera showed a less strongly positive reaction in the His-LASV-rNP-based IgG-ELISA than anti-LASV-rNP serum (Fig. 4A). Anti-LASV-rNP and anti-JUNV-rNP also showed a less strongly positive reaction in the His-LCMV-rNP-based IgG-ELISA than anti-LCMV-rNP serum (Fig. 4B). However, anti-LASV-rNP and anti-LCMV-rNP sera showed a negative reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4C). Human sera from LF patients showed a highly positive reaction in the LASV-rNP-based IgG-ELISA, but sera from patients with Argentine hemorrhagic fever (AHF), which is caused by JUNV, did not (Fig. 4D). Serum from an AHF patient showed a highly positive reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4E). These results suggest that cross-reactive antibody among arenaviruses may be detected by the newly developed LASV-rNP-based IgG-ELISA.

Development of LASV Ag-capture ELISA. Three clones of a hybridoma that excreted an MAb to His-LASV-rNP were es-

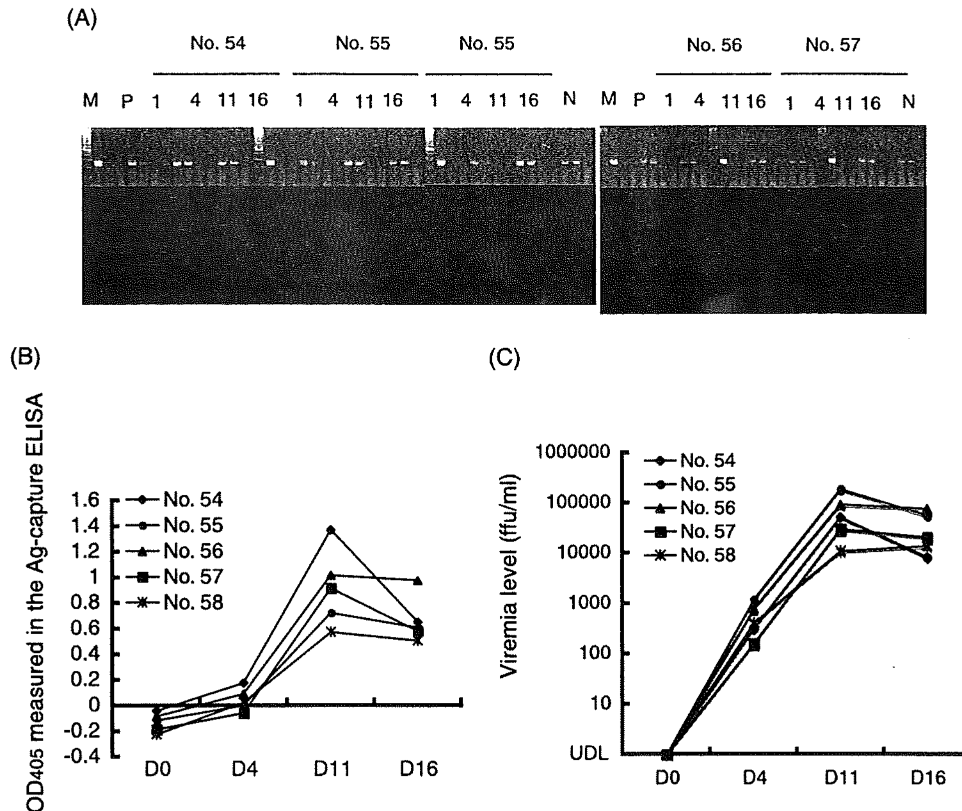

FIG. 5. Detection of the LASV genome by the RT-PCR (A), LASV-NP by the LASV-NP-Ag-capture ELISA (B), and the infectious dose of LASV (C) in serially collected sera of hamsters experimentally infected with LASV. The OD_{405} values in panel B were obtained at a dilution of 1:40.

tablished. The isotype of the three MAbs were identified as IgG1. These MAbs were designated MAb 4A5, MAb 6C11, and MAb 2-11. Of these MAbs, MAb 4A5 was the most efficient in capturing His-LASV-rNP in the Ag-capture ELISA format. The Ag-capture ELISA with MAb 4A5 detected His-LASV-rNP concentrations as low as 800 pg/ml (data not shown). Furthermore, the Ag-capture ELISA detected the MOPV-NP but not the rNPs of LCMV and JUNV (data not shown).

All of the sera collected from five LASV-infected hamsters on days 11 and 16 postinfection were antigen positive in the Ag-capture ELISA using MAb 4A5 as a capture antibody, whereas the sera collected on days 0 and 4 were antigen negative. The OD_{405} values in the ELISA were highest on day 11. The reactivity patterns in each hamster in the ELISA were similar to the viremia levels (Fig. 5). The sera collected on days 11 and 16 were found to be LASV genome positive by RT-PCR (10). Thus, the sensitivity of the Ag-capture ELISA was similar to that of the RT-PCR.

Determination of the epitope recognized by the monoclonal antibodies. The epitope recognized by MAbs was determined. MAb-4A5 reacted in Western blots with GST-LASV-rNP1-6 (full-length LASV-rNP), GST-LASV-rNP1-5, and GST-LASV-rNP1-4 but not with the other truncated LASV-rNPs shown in Table 1, suggesting that MAb 4A5 reacted with a conformational epitope located on the amino-terminal portion of LASV-rNP. The epitope was maintained when the extreme amino-terminal portion, LASV-rNP1, was present but was lost when LASV-rNP1 was removed. These results suggest that the extreme amino-terminal portion, LASV-rNP1, is essential for the maintenance of the conformational epitope. MAbs 6C11 and 2-11 reacted in Western blots with GST-LASV-rNP1 and GST-LASV-rNP5, respectively (Table 1).

The Pepscan analyses indicated that MAbs 6C11 and 2-11

TABLE 1. Reactivities of the MAbs developed in the present study with the GST-tagged truncated LASV-rNP in Western blot analyses

Truncated LASV-rNP	Reactivity with MAb[a]:		
	6C11	4A5	2-11
LASV-rNP1	+	–	–
LASV-rNP2	–	–	–
LASV-rNP3	–	–	–
LASV-rNP4	–	–	–
LASV-rNP5	–	–	+
LASV-rNP6	–	–	–
LASV-rNP1-2	ND	–	ND
LASV-rNP1-3	ND	–	ND
LASV-rNP1-4	ND	+	ND
LASV-rNP1-5	ND	+	ND
LASV-rNP1-6[b]	ND	+	ND
LASV-rNP2-7	ND	–	ND
LASV-rNP3-6	ND	–	ND
LASV-rNP4-6	ND	–	ND
LASV-rNP5-6	ND	–	ND

[a] "+" and "–" indicate positive and negative reactions, respectively. ND, not determined.
[b] LASV-rNP1-6 indicates LASV-rNP.

TABLE 2. Reactivities of the MAbs developed in the present study with the NPs of LASV, MOPV, LCMV, and JUNV in Western blot analyses

MAb	Reactivity of MAb[a] with NP of:			
	LASV	MOPV	LCMV	JUNV
4A5	+	+	–	–
6C11	+	ND	+	–
2-11	+	ND	–	–

[a] "+" and "–" indicate positive and negative reactions, respectively. ND, not determined. The reactivities of MAb 6C11 and MAb 2-11 were not evaluated with MOPV-NP. However, theoretically, MAb 6C11 should be reactive with MOPV-NP due to the presence of the amino acid residues that can react with MAb 6C11, but MAb 2-11 should not react with MOPV-NP due to the absence of the amino acid residues that can react with MAb 2-11.

recognized linear epitopes. MAbs 6C-11 and 2-11 recognized GLDFSEV (aa 41 to 47) within LASV-rNP1 and FATQP (aa 439 to 443) within LASV-rNP5, respectively (Fig. 6). The reactivity patterns of these MAbs with NPs of LASV, MOPV, LCMV, and JUNV are summarized in Table 2.

DISCUSSION

We report here the development of diagnostic systems (antibody and antigen detection systems) for LF using LASV-rNP. The LASV-rNP-based IgG-ELISA was sensitive and specific in detecting anti-LASV-IgG. Although the data were not shown, an IgM-capture ELISA using purified LASV-rNP as an antigen was developed in the same way as that shown in previous reports and detected LASV-IgM antibody (42, 43). All sera collected from LF patients and monkeys infected with LASV showed positive reactions in the LASV-rNP-based IIFA. The staining patterns of the rNP with these sera were granular in the IIFA (Fig. 3), making it easy to distinguish positives from negatives. IIFA using LASV-rNP-expressing HeLa cells was also highly sensitive and specific in detecting LASV-IgG. In the preliminary study, ca. 15% of the sera collected from 334 Ghanaians and only less than 1% of 280 Zambians showed positive reactions in the LASV-rNP-based IgG ELISA (our data). The results are considered to be compatible with the fact that LF is endemic to the western African region, including Ghana, but not to the eastern African region. The LASV-rNP-based antibody detection systems such as ELISA and IIFA were suggested to be useful not only in the diagnosis of but also in the seroepidemiological study of LF.

The LASV-rNPs were expressed by a transformation system in *E. coli* or by recombinant baculovirus systems and have already been applied as antigens in ELISA, Western blotting, and IIFA for the detection of antibodies to LASV (4, 14, 16, 22, 23, 44). In the present study, an Ag-capture ELISA using

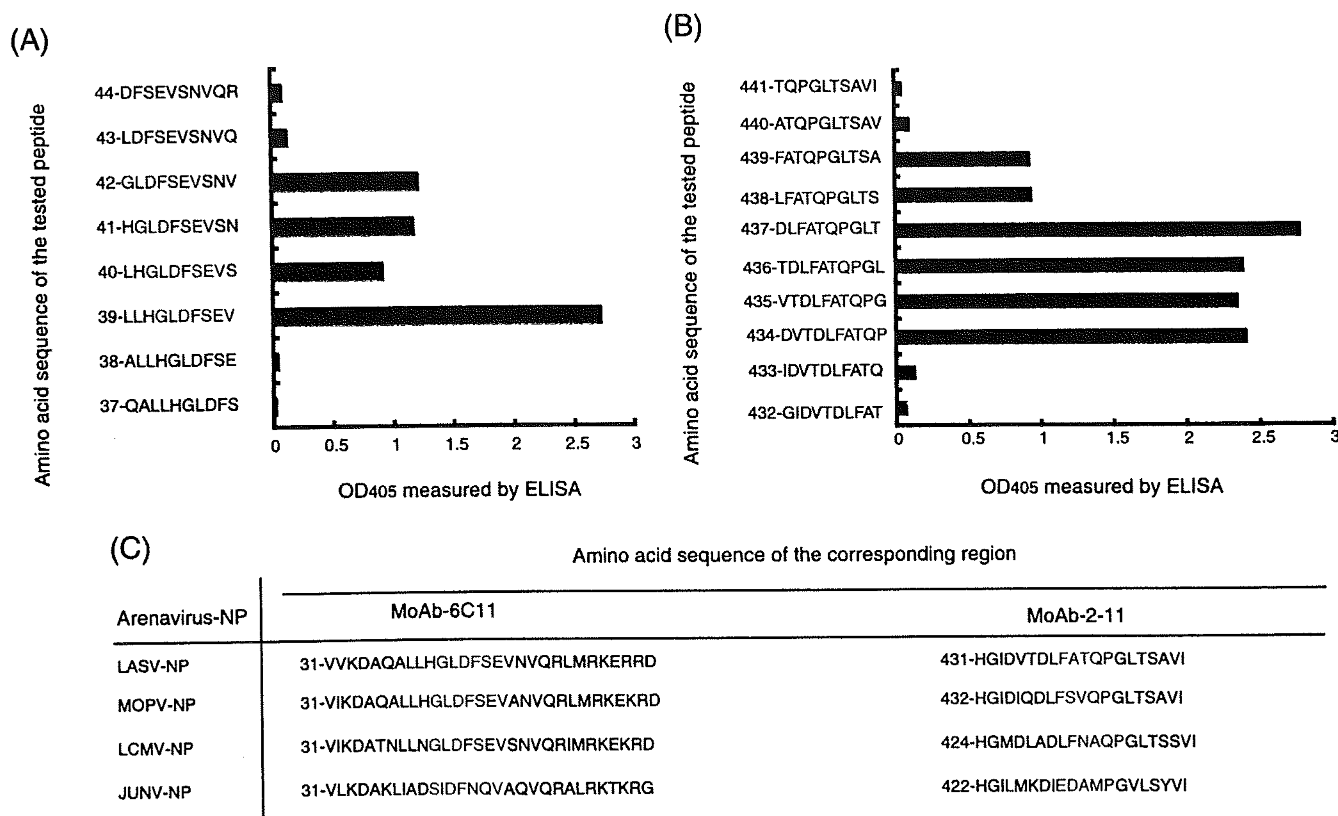

FIG. 6. Pepscan analyses to determine the epitopes of MAb 6C11 (A) and MAb 2-11 (B). The vertical bar indicates the amino acid residues with an amino acid position within the LASV-NP. MAb 6C11 was confirmed to react with 7 aa residues positioned from aa 42 to 48 (GLDFSEV) within LASV-NP1. MoAb-2-11 was confirmed to react with 5 aa residues positioned from aa 439 to 443 (FATQP) within LASV-NP5. (C) The corresponding amino acid residues to the epitope of the MAb 6C11 and MAb 2-11 among MOPV, LCMV, and JUNV are shown. The GenBank accession numbers for the S genes of LASV, MOPV, LCMV, and JUNV are NC_004296, AY772170, AY847350, and DQ272266, respectively. The epitope of the MAb 6C11 is present not only in the nucleoprotein of LASV but also in those of MOPV and LCMV—but not in that of JUNV.

MAbs to LASV-rNP was also developed. Furthermore, detection of the cross-reactive antibody by LASV-rNP-based IgG-ELISA was examined. The results for cross-reactivity indicate that the LASV-rNP-based IgG-ELISA detects not only antibodies to LASV but also those to LCMV.

The Ag-capture ELISA using MAb 4A5 was confirmed to be useful in the detection of authentic LASV antigen in sera serially collected from hamsters infected with LASV. The sensitivity of the MAb 4A5-based Ag-ELISA was similar to that of conventional RT-PCR, the efficiency of which in the diagnosis of LF was previously reported (10). Therefore, the MAb 4A5-based Ag-capture ELISA is regarded as useful in the diagnosis of LF. Unfortunately, the efficacy of the MAb 4A5-based Ag-capture ELISA in the diagnosis of LF was not evaluated using serum samples from patients. Thus, further study is still required. The three MAbs, including MAb 4A5, were characterized, and the corresponding amino acid residues within the nucleoproteins of LASV, MOPV, LCMV, and JUNV to the epitopes of MAb 6C11 and MAb 2-11 are summarized in Fig. 6C. It was of interest that LASV, MOPV, LCMV, and JUNV might be identified by analyses of the reactivity patterns of MAbs 4A5, 6C11, and 2-11 to the nucleoproteins of each virus. The nucleoproteins of all LASV strains circulating in the western and central parts of Africa would be detected by the MAb 4A5-based Ag-capture ELISA, since this ELISA was able to detect MOPV-NP that was different from LASV in terms of genetic and evolutional characteristics.

We have thus far reported the development of antibody and antigen detection systems using the recombinant nucleoproteins of the viruses for Ebola hemorrhagic fever, Marburg hemorrhagic fever, and Crimean-Congo hemorrhagic fever (32, 33, 36–42). Recently, a number of highly pathogenic emerging virus infections in humans appeared, such as Nipah virus encephalitis (8), SARS-coronavirus infections (21, 35), and highly pathogenic avian influenza virus infections (9, 45, 46). The strategy shown here might be applicable to the development of diagnostic systems for severe viral infections whose etiologic agents are highly pathogenic to humans as an alternative method to methods using infectious viruses.

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