Epitope Mapping of the Nucleocapsid Protein of Sendai Virus and Application of Antigenic Epitopes for the ELISA-Based Diagnosis of Sendai Virus Infection

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ABSTRACT. Sendai virus (SeV) is one of the most prevalent viral pathogens infecting laboratory mice and rats. To date, mature SeV virions have been used as antigens for serological diagnosis. To develop antigens that are more specific and easier to prepare for diagnosis, we examined the antigenic sites in the nucleocapsid protein (NP) of SeV with antisera from experimentally SeV-infected mice and a peptide array membrane containing overlapping 10-mer peptides covering the entire NP. We found antigenic linear sequences in two regions, amino acids 120–160 and 420–500, of the SeV-NP. From these antigenic sequences, we applied two synthesized peptides, IVKTRDMEYERTTEWL and FVTLHGAERLEEETNDE, which correspond to positions 119–134 and 458–474 of the SeV-NP, respectively, as antigens in an enzyme-linked immunosorbent assay (ELISA). Evaluation of the ELISAs using these peptides revealed that they were specific to anti-SeV antisera. Furthermore, the ELISAs using these peptides were able to distinguish between SeV-positive and SeV-negative mouse sera to the same extent as a commercial ELISA kit. These results indicate that these peptides are useful for the serological diagnosis of SeV infection.

KEY WORDS: ELISA, epitope mapping, nucleocapsid, Sendai virus.

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Sendai virus (SeV) is a single-stranded RNA virus belonging to the family *Paramyxoviridae* [12]. SeV infection occurs in various rodents, such as mice, rats, hamsters and guinea pigs [26], and is one of the most prevalent pathogens for laboratory mice and rats [17]. Although natural SeV infection in rats is generally subclinical, natural infections of mice are either enzootic or epizootic in nature. Epizootic infections occur upon the first introduction of the virus to a colony. It is known that there is a considerable difference in susceptibility to SeV between different strains of rats and mice [2, 11, 27, 32]. This difference in susceptibility to SeV yields variations in the severity of pathologic lesions. SeV causes acute respiratory tract infections and bronchopneumonia, leading to teeth chattering, dyspnea, prolonged gestation, poor growth and death in young mice [2]. Therefore, it is important for the health of laboratory animal colonies that the presence of SeV be detected by an appropriate and sensitive screening test in a routine health surveillance program.

Serological assays detecting antibodies against pathogens in sera represent the most popular screening test for the diagnosis of infection [9]. The enzyme linked-immunosorbent assay (ELISA) is the most common serological assay for the detection of SeV-infection [9, 26]. To date, mature virions are usually used as antigens in ELISAs for the detection

of anti-SeV antibodies [18, 19]. However, a false-positive result may be obtained, because of cross-reactivity between antibodies present in sera and non-specific antigens derived from contaminated microorganisms used in the ELISA. Therefore, the use of purified antigens, such as recombinant protein or antigenic synthesized peptides derived from pathogens, will allow serological assays to be more specific and sensitive. The SeV genome contains six genes encoding the following 6 structural proteins: the nucleoprotein (NP), the phosphoprotein (P), the matrix (M) protein, the viral fusion (F) protein, the hemagglutinin/neuraminidase (HN) protein and the large (L) protein [12]. The SeV-NP is known to be antigenic on the basis of epitope mapping of monoclonal antibodies against SeV reported previously [10, 14]. Therefore, this protein is expected to be a candidate antigen for use in the serological diagnosis of SeV infection [34].

To identify the B-cell epitopes in the SeV-NP, in this study, we used antisera from SeV-infected mice and a SPOT peptide array, which contains a set of overlapping peptides covering the entire region of the SeV-NP protein. In addition, we attempted to develop an ELISA using synthesized peptides derived from B-cell epitopes in SeV-NP as antigens.

MATERIALS AND METHODS

Sendai virus infection to mice: BALB/c, C57BL/6, and AKR mice were obtained from SLC (Shizuoka, Japan). Mice used in the experiments reported in Figs. 2–4 and Table 2 were maintained in the animal breeding rooms in the Graduate School of Veterinary Medicine, Hokkaido University until SeV infection. The animal breeding rooms were kept at $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity, with a 12-hr

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Fig. 1. Detection of antigenic sites in the SeV-NP using a peptide array membrane. Sera from BALB/c, C57BL/6 and AKR mice on 0 d (Cont) or 14 d (SeV-infected) after inoculation with SeV were used. Antibodies in sera bound to each peptide on a peptide array membrane were visualized by chemiluminescence. Typical images from representative sera from each strain of mice are shown.

light-dark cycle. For virus infection, mice were inoculated intranasally with 1×10^2 TCID₅₀ of SeV (MN strain, a gift from Dr. Hiroshi Iwai, Rakuno Gakuen University, Japan). Virus inoculation and serum sampling from the tail vein were performed under ketamine/xylazine anesthesia. Virus-inoculated mice were maintained in ventilated isolator cages placed in infected animal facility. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Veterinary Medicine, Hokkaido University. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University.

SPOT peptide array: A set of overlapping 10-mer peptides, which shift in 1-amino acid increments from the amino-terminus of the NP from SeV-Z strain (accession

no. M30202), were synthesized on derivatized cellulose membranes (INTAVIS Bioanalytical Instruments AG, Köln, Germany) using an ASP-222 Auto Spot Peptide Synthesizer (INTAVIS Bioanalytical Instruments AG) according to the manufacturer's protocol [13]. We synthesized a total 515 spots for the SPOT peptide array corresponding to the peptides from 1–10 to 515–524 of the NP.

Detection of peptide-binding antibodies in mouse sera on the SPOT peptide array: Sera were diluted at 1:1,000 by 5% skim milk in PBS-Tween 20 (137 mM NaCl, 81 mM Na₂HPO₄, 15 mM KH₂PO₄, 27 mM KCl and 0.1% Tween 20) and incubated on the SPOT peptide array for 2 hr at room temperature. The SPOT peptide array was washed three times with PBS-Tween 20 and incubated with HRP-conjugated anti-mouse IgG (GE Healthcare Bio-Sciences, Uppsala, Sweden) diluted to 1:10,000 with 5% skim milk

Fig. 2. Antigenicity of the SeV-NP. Sera were collected from BALB/c (n=6), C57BL/6 (n=5) and AKR (n=5) mice before and at 14 d after SeV infection and were used for the detection of antibodies bound to peptides shown in Fig. 1. The intensity of the chemiluminescence at each spot was quantified. The values are the relative intensity of each spot using sera from mice collected after infection for 14 d to those using sera from the same mice collected before infection and are presented as means $\pm$ SEM. The values on the x-axis indicate the spot number.

in PBS-Tween 20 for 2 hr at room temperature. The SPOT peptide array was then washed a further three times with PBS-Tween 20, and the antibodies bound specifically to the peptides on the SPOT array were detected using an ECL Advance Western blotting detection kit (GE Healthcare Biosciences). Visualization and calculation of the chemiluminescence of each spot were performed with an LAS-3000 imaging system (Fuji Film, Tokyo, Japan).

ELISA: A comparison of the sensitivity of detection of anti-SeV antibodies in mouse sera by ELISA with that using SeV peptides was performed using a commercial ELISA, MONILISA HVJ (Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan), in accordance with the manufacturer's protocol. For the commercial ELISA, 200 μ l of sera diluted to 1:40 was used. The positive/negative determination of samples was performed by macroscopically comparing the coloring of the wells to that of positive control serum within the ELISA kit. The positive control serum within the commercial ELISA kit shows an OD value of 0.3–0.4 at 492 nm [19].

The peptides coding 119–134 (IVKTRDMEYERTTEWL) and 458–474 (FVTLHGAERLEEETNDE) of the SeV-NP were synthesized by Life Technologies (Carlsbad, CA, U.S.A.). Briefly, 200 μ l of 0.5 μ M peptide in 0.1 M sodium

carbonate-bicarbonate buffer (pH 9.6) was added to the wells of a 96-well plate, and the plate was incubated overnight at 4 $^{\circ}$ C. The wells were washed three times with PBS-Tween 20 and incubated with 200 μ l of 1% BSA in PBS-Tween 20 (blocking buffer) at 37 $^{\circ}$ C for 1 hr. The wells were again washed three times with PBS-Tween 20, and 200 μ l of sera diluted to 1:200 by blocking buffer was added prior to further incubation at 37 $^{\circ}$ C for 1 hr. After washing the wells three times with PBS-Tween 20, 200 μ l of HRP-conjugated anti-mouse IgG, diluted to 1:10,000, was added to the wells and the well were incubated at 37 $^{\circ}$ C for 1 hr. After again washing the well three times with PBS-Tween 20, 200 μ l of 1.5 mg/ml *o*-phenylenediamine with 0.01% H₂O₂ was added to each well, and the wells were further incubated at 37 $^{\circ}$ C for 10 min. Next, 50 μ l of 6 N H₂SO₄ was added, and the OD was measured at 450 nm using a plate reader.

RESULTS

Epitope mapping of the nucleocapsid protein of Sendai virus using the SPOT peptide array: To determine antigens for the detection of anti-SeV antibodies, we performed B-cell epitope mapping of the NP of SeV using a SPOT peptide

Fig. 3. ELISAs using the SeV-NP peptides. SeV-positive or SeV-negative sera were diluted and used for ELISA as described in Materials and Methods. The upper graph is a plot of the OD₄₅₀ values from the ELISA using the SeV-NP 119–134 peptide. The lower graph is a plot of the OD₄₅₀ values from the ELISA using the SeV-NP 458–474 peptide. Open circles indicate SeV-negative sera, and closed squares indicate SeV-positive sera. (–) indicates a sample without serum. The plots are representative of two independent experiments.

array membrane. The 10-mer peptides, overlapping by nine amino acids and covering the entire region of the NP, were synthesized on the cellulose membrane as described in Materials and Methods. Sera collected from BALB/c, C57BL/6 and AKR mice on 0 d or 14 d after inoculation with SeV were used for hybridization to the peptide array membrane. The sera IgG antibodies that specifically bound to the peptides on the membrane were detected by chemiluminescence as shown in Fig. 1. No significant chemiluminescence signals were observed for sera samples from all three strains of mice that were collected before infection with SeV, as shown in Fig. 1. In contrast, several sequential spots were detected using sera samples from mice collected post-SeV infection. We measured the chemiluminescent intensity of each spot

and calculated the normalized intensity values by dividing the intensity value obtained from the serum of the SeV-infected mouse by that from the serum sample from the same mouse collected before SeV-inoculation. The means of the normalized intensity values are shown in Fig. 2. Two high-intensity sites were found in spots positioned at 122–124 and 145–148, which corresponded to amino acids 122–133 and 145–157 of the SeV-NP, respectively. In addition, high-intensity spots were also observed between 420 and 500. In particular, the intensities of the spots positioned at 420–421 and 463–466 (corresponding to amino acids 420–430 and 463–475, respectively) were the highest on the array. We determined the peptides on those spots with a mean normalized intensity above 2.0 to be antigenic sites. The positions of spots, amino acids and peptide sequences of the antigenic sites in the SeV-NP are listed in Table 1. Although some variations in sequences were observed among the mouse strains, the high-intensity sites were localized in two regions corresponding to amino acids 120–160 and 420–500 of the SeV-NP, and overlapping each other. Therefore, we considered these peptides to antigenic B-cell epitopes.

Development of ELISAs for the detection of anti-Sendai virus antibodies using antigenic peptides derived from the nucleocapsid protein: The B-cell epitopes of the SeV-NP were expected to be antigens for the serological diagnosis of SeV infection. We found that amino acids 120–160 and 420–500 of the SeV-NP were antigenic B-cell epitopes for all three strains of mice (Fig. 2 and Table 1). Next, we examined whether these epitopes are suitable for diagnostic use by developing ELISAs using synthesized peptides corresponding to the B-cell epitopes of the SeV-NP. We synthesized two peptides for use as ELISA antigens: a 16-mer peptide corresponding to amino acids 119–134 (SeV-NP 119–134: IVKTRDMYERTTEWL) and a 17-mer peptide corresponding to amino acids 458–474 (SeV-NP 458–474: FVTLHGAERLEEETNDE) of the SeV-NP. The results when the SeV-NP 119–134 peptide was used as an antigen for the ELISA are shown in the upper panel of Fig. 3. When 1:50-diluted serum from a SeV-infected mouse was used, the OD value was higher than for other diluted samples, and the values decreased with increases in the dilution rate. In contrast, the OD values using normal serum did not change with dilution rate, and were almost the same as that obtained in the absence of serum. Similar results were obtained in the case of the ELISA using the SeV-NP 458–474 peptide (Fig. 3, lower graph). These results indicate that the ELISAs using the antigenic SeV-NP peptides could detect antibodies specifically, suggesting that these peptides may be suitable for use in the serological diagnosis of SeV infection.

Evaluation of the ELISAs using the SeV-NP peptides for the serological diagnosis of SeV infection: We examined the immunoreactivity of the ELISAs using the synthesized SeV-NP peptides with sera from experimentally SeV-infected mice and from non-infected control mice. The OD values of the ELISAs using the SeV-NP peptides obtained from 14 SeV-infected mice and 12 control mice are plotted in Fig. 4. When using the SeV-NP 119–134 peptide, the OD values ranged between 0.121 and 0.187 for sera from the control

Fig. 4. Evaluation of the ELISAs using the SeV-NP peptides. Sera from mice that were not infected with SeV (Cont, n=12) and that were infected with SeV for 14 d (SeV-infected, n=14) were used for the ELISAs. The OD₄₅₀ values were plotted on the graph. Horizontal lines in the plots indicate the means of the OD values. * $P < 0.05$ vs. Cont.

Table 1. Antigenic sites in the Sendai virus nucleoprotein for infected mouse

Mouse strain	Position of spots (Intensity>2.0)	Position of amino acids	Peptide sequences
BALB/c	121-125	121-134	VKTRDMEYERTTEWL
	130, 131	130-140	TTEWLFGPMVN
	145-149	145-158	FQGQRDAADPDTLL
	420, 421	420-430	ALDNADIDLET
	460, 461, 463-466	460-475	TLHGAERLEEETNDE
	473-475	473-484	DEDVSDIERRIA
	488, 489	488-498	AERRQEDSATH
C57BL/6	38-40	38-49	FVLGPSVTDDAD
	123, 125, 126	123-135	RDMEYERTTEWLF
	148, 149	148-158	QRDAADPDTLL
	420-423	420-432	ALDNADIDLETKA
	432-437	432-446	AHADQDARGWGDSG
	439-441	439-450	RGWGGDSGERWA
	456	456-465	GHFVTLHGAE
	459-466	459-475	VTLHGAERLEEETNDED
	472, 473, 475-478	472-487	NDEDVSDIERRIAMRL
	488, 489	488-498	AERRQEDSATH
	495-500	495-509	SATHGDEGRNNGVDH
AKR	513	513-522	DDTAAVAGVG
	122, 123	122-132	TRDMEYERTTE
	146-149	146-158	QGQRDAADPDTLL
	420	420-429	ALDNADIDLE
	441	441-450	WGGDSGERWA
	456-466	456-475	GHFVTLHGAERLEEETNDED
	473, 476-478	473-487	DEDVSDIERRIAMRL
	488, 489, 491	488-500	AERRQEDSATHGD

mice. In contrast, the OD values ranged between 0.296 and 0.577 for sera from the SeV-infected mice (left panel). The means $\pm$ standard deviation (SD) of the OD values were

as follows: 0.157 ± 0.022 for the control mice and 0.412 ± 0.091 for infected mice. When using the SeV-NP 458-474 peptide, the OD values of the ELISA ranged between 0.149

Table 2. Evaluation of the ELISA using SeV-NP peptides for the detection of SeV infection

	Positive samples/total samples		
	Commercial ELISA	ELISA using the SeV-NP 119–134 peptide	ELISA using the SeV-NP 458–474 peptide
Control mice	0/12	0/12 ^{a)}	0/12 ^{b)}
SeV-infected mice	14/14	14/14 ^{a)}	14/14 ^{b)}

a) Positive samples showed an OD value above the cutoff value (0.201).

b) Positive samples showed an OD value above the cutoff value (0.246).

and 0.229 for sera from the control mice and between 0.302 and 0.555 for sera from the SeV-infected mice (right panel). The means $\pm$ SD were as follows: 0.192 ± 0.027 for the control mice and 0.420 ± 0.087 for the SeV-infected mice. In both cases, the means of the OD values from the infected mice were significantly higher than those from the control mice. To evaluate the ELISAs using the SeV-NP peptides, we determined a cutoff OD value for SeV-positive to be 0.201 for that using the SeV-NP 119–134 peptide and to be 0.246 for that using the SeV-NP 458–474 peptide, which equates to the mean + 2 SD of the value for the control mice. As shown in Table 2, the values from all samples from the control mice were below the cutoff value for each ELISA, indicating that the samples were SeV-negative. In contrast, all sera from the SeV-infected mice were above the cutoff values, indicating that these samples were SeV-positive. These results corresponded to those obtained using a commercial ELISA for the diagnosis of SeV infection in mice, and indicate that the ELISAs using antigenic peptides from SeV-NP show similar specificity to the commercial ELISA for the diagnosis of SeV-infection.

DISCUSSION

SeV-NP was shown to be an immunogenic protein in previous studies using monoclonal antibodies [10, 14]. Therefore, we examined the antigenic peptide sequences in SeV-NP. SPOT peptide arrays have been successfully used for the screening of antibody epitopes [13, 28, 29]. We have also identified B-cell epitopes in the nucleoprotein of mouse hepatitis virus (MHV) using a SPOT peptide array and developed an ELISA using antigenic peptides derived from the nucleoprotein of MHV for serological diagnosis [1]. In this study, we prepared a peptide array membrane containing a set of 10-mer peptides, each with a 9-aa overlap, covering the entire sequence of the SeV-NP. The use of a peptide array membrane together with sera from experimentally SeV-infected mice was considered a feasible approach for identifying the linear sequences of B-cell epitopes in the SeV-NP. As expected, the SPOT peptide array was reactive to the sera from various strains of mice experimentally infected with SeV. We detected several rows of spots demonstrating IgG antibody-specific binding. The present results clearly indicate the linear B-cell epitopes in the NP of mature SeV. Although various antigenic sites were found, these sites overlapped each other and were restricted to two regions: amino acids 120–160 and 420–500. Our present results differed in

part from those of a previous study on epitope mapping of monoclonal antibodies (mAbs) produced by the immunization of mice with denatured SeV virions [14]. That report showed that it was possible to produce mAbs that recognized sites in the amino-terminal half (amino acids 1–289) of the SeV-NP, but one mAb was obtained that recognized epitopes in amino acids 289–295. However, they also demonstrated that mAbs were obtained that recognized epitopes in amino acids 295–425, 425–455 and 455–524, respectively. These previous observations are consistent with our present results. In contrast, mAbs that recognized epitopes in the amino-terminal half or carboxy-terminal half of the NP from other paramyxoviruses, such as human parainfluenza virus type 1, mumps virus and measles virus, were isolated previously [7, 30, 33]. In particular, the report using anti-measles mAbs showed the existence of three antigenic sites in amino acids 122–150, 457–476 and 519–525 of the NP [7]. The antigenic site in amino acids 120–160 of the NP from measles virus is also predicted to form a loop exposed to solvents [20]. This site and the equivalent site in the SeV-NP are involved in self-assembly and in RNA-binding necessary for it to function as a template for viral RNA-dependent RNA polymerase [3, 22]. In addition, hydrophobicity plots of the SeV-NP revealed that the region in amino acids 120–160 is hydrophilic, suggesting the region is antigenic (data not shown). The carboxy-terminal region of the NP is known to contain the binding sites for the viral phosphoprotein, which is a co-factor for binding viral RNA-dependent RNA polymerase [5, 6, 8, 22–24]. This site is highly hydrophilic throughout [20], suggesting strong antigenicity. The present results in terms of the antigenicity of the SeV-NP are in agreement with those obtained from its structural properties. Furthermore, the approach using a peptide array membrane and sera from virus-infected animals is considered to be suitable for the comprehensive screening of linear B-cell epitopes in viral antigenic proteins.

Since the sites corresponding to amino acids 119–134 and 458–474 of the SeV-NP were highly antigenic for all three strains of mice examined (Table 1), we selected these two sites as ELISA antigens to examine whether the antigenic sites found in this study are suitable for serological diagnosis. In the ELISAs, both the SeV-NP 119–134 and SeV-NP 458–474 peptides were able to detect anti-SeV antibodies without any nonspecificity (Fig. 3). In the ELISAs using these synthesized peptides, the OD values of sera from SeV-infected mice were significantly higher than those of sera from the control mice (Fig. 4). Furthermore, we analyzed the same

sera using both a commercial ELISA and our ELISAs (Table 2). The serological determination of positive/negative in the ELISAs using the SeV-NP peptides was identical to that in commercial SeV ELISA used in this study. For the ELISAs using SeV-NP peptides, the dilution ratio of sera was 1:200, which was higher than that of the commercial ELISA. The antigen in the commercial ELISA was ether-treated entire SeV virions [18, 19]. These results indicate that the SeV-NP 119–134 and SeV-NP 458–474 peptides have comparable sensitivity to entire SeV virions for serological diagnosis. Several serological surveys of Sendai virus (SeV) infection among wild rodents have reported previously. Baker *et al.* surveyed wild house mice (*Mus musculus domesticus*) using a commercial ELISA [4]. We considered that the ELISAs using the SeV-NP peptides might be suitable for the wild house mouse, which is the same species as laboratory mice. However, further evaluation may be necessary, before these ELISAs can be applied to wild rodents belonging to other species.

We used several strains of mice (C57BL/6, BALB/c and AKR) in the present study. These mice have different H-2 haplotypes and levels of susceptibility to SeV infection [16, 27]. Therefore, the ELISAs using the SeV-NP peptides appear suitable for use in the diagnosis of SeV-infection in colonies of various strains of mice. In addition, the amino-acid sequence between 119 and 134 of the NP is identical among various strains of SeV [15, 21, 25]. In the case of the sequence from 458–474 of the SeV-NP, a substitution in the amino-terminal residue (I458V) is seen in the Hamamatsu and Ohita M-1 strains, but not in other strains [15, 21, 25]. Therefore, the antigenic sites used in the ELISAs presented herein are highly conserved among various strains of SeV, suggesting their suitability for the serological diagnosis of SeV infection.

The NP of human parainfluenza virus type 1 (PIV-1) contains sequences that are highly homologous with those of the SeV-NP peptides, sharing 100% amino acid identity to SeV-NP 119–134 and 71% amino acid identity to SeV-NP 458–474, respectively. Indeed, Sangster *et al.* previously reported that human PIV-1 was serologically cross-reactive to SeV [31]. Therefore, it is suggested that the ELISAs using the SeV-NP peptides are not able to distinguish between human PIV-1 and SeV infections. The NP of human PIV-3 has low homology to the SeV-NP peptides, sharing just 40% amino acid identity to SeV-NP 119–134. In particular, human PIV-3 NP does not contain any site that was homologous to SeV-NP 458–474. These results suggest that the ELISAs using the SeV-NP peptides have the potential to distinguish between SeV and PIV-3 infections. In addition, we could not find any amino acid sequences homologous to the SeV-NP peptides in other viruses, including the mouse *Pneumonia virus* that infects mice and belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae*. Therefore, we consider that the ELISAs using the SeV-NP peptides may not be cross-reactive to sera from mice infected with viruses other than SeV and human PIV-1.

In summary, we identified linear B-cell epitopes in the SeV-NP. The SPOT peptide array is a powerful tool for the

detection of antigenic sites in proteins derived from pathogenic agents. Our present results indicate that two peptides, IVKTRDMEYERTTEWL and FVTLHGAERLEEETNDE, are useful for the ELISA-based serological diagnosis of SeV infection.

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