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Identification of antigenic peptides derived from B-cell epitopes of nucleocapsid protein of mouse hepatitis virus for serological diagnosis

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A B S T R A C T

Mouse hepatitis virus (MHV) infection is found commonly in laboratory mice and this virus has been known to cause various diseases such as subclinical infection, enteritis, hepatitis, and encephalitis. Serological tests are used commonly to diagnose MHV infection. Complete MHV virions have been used primarily as antigens for serological diagnosis to date. To develop an antigen that is more specific, more sensitive, and easier to prepare for serological diagnosis, the antigenic sites in the MHV-nucleocapsid (N) protein were screened in this study. Sixteen antigenic linear sequences in the N protein were found using antisera obtained from mice infected naturally with MHV and a peptide array containing overlapping 10-mer peptides covering the entire N protein. From these antigenic sequences, two synthesized peptides, ILKKTTWADQTERGL and RFDSTLPGFETIMKVL, which were consistent with positions 24–38 and 357–372 of the N protein respectively, were used as antigens in ELISA. Evaluation of ELISA with these peptides revealed that both peptides were specific to anti-MHV antisera. Furthermore, ELISA performed using these peptides was more sensitive than commercial ELISA used for a screening sera from mice infected accidentally to MHV maintained in cages, suggesting that these peptides are useful for serological diagnosis of MHV infection.

1. Introduction

Mouse hepatitis virus (MHV) is an enveloped positive-stranded RNA virus, which belongs to the *Coronaviridae* (de Vries et al., 1997). MHV infection causes various diseases such as subclinical infection, enteritis, hepatitis, and encephalitis (Compton et al., 2004; Haring and Perlman, 2001; Matthews et al., 2002). MHV strains are classified as enterotropic or polytropic. Polytropic strains disseminate from the initial site of replication in the respiratory epithelium to neural, hepatic, vascular, and lymphatic tissues. Replication of enterotropic strains is limited generally to replication within the intestinal tract and these strains cause subclinical infection in adult mice. In contrast, polytropic strains are more likely to cause more severe diseases with clinical symptoms (Baker, 1998). Since MHV is one of the most prevalent viruses in laboratory mice (Jacoby and Lindsey, 1997), it is important to detect this virus in mice colonies by appropriate and sensitive screening tests during routine health surveillance programs.

Serological assays detecting antibodies against pathogens in sera are the most popular screening tests for diagnosis of infection (Compton and Riley, 2001). ELISA and the indirect immunofluorescent assay are the most common serological assays for diagnosis of MHV infection (La Regina et al., 1988; Peters et al., 1979). To date, infected cells or purified viruses are used usually as antigens in serological tests for detecting anti-MHV antibodies. Conversely, cross-reactivity between antibodies present in sera and nonspecific antigens used in the test may yield a false positive result. Therefore, the use of purified antigens such as recombinant proteins and synthesized antigenic peptides derived from pathogens will make serological assays more specific and sensitive. MHV comprises at least three proteins: the envelope glycoprotein designated as the spike (S) protein, the envelope membrane (M) protein, and the nucleocapsid (N) protein (de Vries et al., 1997). The S protein is involved in cellular receptor binding and membrane fusion (Gallagher and Buchmeier, 2001). The N protein is associated with genomic RNA and plays a role in virus assembly though interactions with the viral RNA and the M protein (Hurst et al., 2005; Verma et al., 2006). The N protein consists of three domains conserved domains among the strains, which are separated by two variable regions (Parker and Masters, 1990). Several monoclonal antibodies against the S, N, and M proteins were reported previously, indicating that these proteins are immunogenic (Fleming et al., 1983; Kubo et al., 1994; Nelson et al., 2000; Stohlman et al., 1994; Stühler

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et al., 1991). Therefore, these proteins are potential antigens for serological diagnosis.

In this study, the antigenicity of the peptides derived from the MHV-N protein was demonstrated by using anti-MHV antisera and peptide array membrane, on which a set of overlapping peptides covering the entire N protein was synthesized. The results showed that 16 antigenic linear sequences existed in the N protein. In these antigenic sequences, two sites coding amino acids 24–38 (ILKKTTWADQTERGL) and 357–372 (RFDSTLPGFETIMKVL) of the N protein are strongly antigenic to anti-MHV antisera derived from mice infected naturally. ELISA performed using these peptides was specific and could detect anti-MHV antisera with higher sensitivity, than a commercial ELISA. These results indicate that these two peptides are useful for serological diagnosis of MHV infection in laboratory mice colonies.

2. Materials and methods

2.1. Mouse sera

Sera from C57BL/6 mice infected accidentally with MHV were provided by Prof. Ichiro Miyoshi from the Center for Experimental Animal Science, Graduate School of Medical Sciences, Nagoya City University. As per the guidelines of the Institutional Animal Care and Use Committee (IACUC), Nagoya City University, for screening and eradication of MHV infection, the mice were euthanized and sera were obtained. Normal mice sera were acquired from BALB/c, C57BL/6N, and DBA/2 mice obtained from SLC (Shizuoka, Japan). These sera were derived from the samples obtained for routine screening during a health surveillance program in the animal facility as per the guidelines of IACUC of Graduate School of Veterinary Medicine, Hokkaido University.

2.2. MHV-N peptide array

A set of overlapping 10-mer peptides, obtained by shifting one amino acid from the amino terminus to the carboxy terminus of the N protein that was derived from MHV-JHM strain (GenBank ID: X00990), were synthesized on derivatized cellulose membranes (INTAVIS Bioanalytical Instruments AG, Köln, Germany) using the Auto Spot Peptide Synthesizer ASP-222 (INTAVIS Bioanalytical Instruments AG) according to the manufacturer's protocol (Frank, 2002). Four hundred forty-six peptides were synthesized on the membrane, which are consistent with the peptides coding amino acids from 1–10 to 446–455 of the N protein.

2.3. Detection of antibodies in mouse sera binding to peptides on the peptide array membrane

Sera were diluted to 1:1000 with 5% skim milk in PBS-Tween 20 (137 mM NaCl, 81 mM Na_2HPO_4, 15 mM KH_2PO_4, 27 mM KCl, and 0.1% Tween 20) and incubated with the peptide array membrane for 2 h at room temperature. The peptide array membrane was washed with PBS-Tween 20 three times and incubated with the HRP-conjugated anti-mouse IgG (GE Healthcare Bio-Sciences, Uppsala, Sweden) diluted to 1:10,000 with 5% skim milk in PBS-Tween 20 for 2 h at room temperature. After reaction with the HRP-conjugated anti-mouse IgG, the membrane was washed with PBS-Tween 20 three times. The antibodies bound specifically to the peptides on the membrane were detected by the ECL Advance Western Blotting Detection Kit (GE Healthcare Biosciences). Visualization and calculation of chemiluminescence of each spot were performed using LAS-3000 imaging system (Fuji film, Tokyo, Japan).

2.4. ELISA

A commercial ELISA, MONILISA MHV (Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan), was used in this study. Sera used in this ELISA were diluted to 1:40. Evaluation of whether sera were MHV positive or negative was confirmed by comparison with immunoreactivity of positive control sera provided by the manufacture. The peptides coding amino acids 24–38 (ILKKTTWADQTERGL) and 357–372 (RFDSTLPGFETIMKVL) of the MHV-N protein were synthesized by Invitrogen (Carlsbad, CA, USA). In a 96-well plate, 200 μl of 0.5 μM peptide in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) was seeded, and the plate was incubated overnight at 4 °C. The wells were washed three times with PBS-Tween 20 and incubated with 200 μl of 1% BSA in PBS-Tween 20 (blocking buffer) for 1 h at 37 °C. The wells were washed three times with PBS-Tween 20, and 200 μl of sera diluted to 1:200 with blocking buffer was added and the plate was incubated for 1 h at 37 °C. After washing the well three times with PBS-Tween 20, 200 μl of the HRP-conjugated anti-mouse IgG diluted to 1:10,000 was added to the wells and incubated for 1 h at 37 °C. After washing the well three times with PBS-Tween 20, 200 μl of 1.5 mg/ml *o*-phenylenediamine with 0.01% H_2O_2 was added to each well and incubated for 10 min at 37 °C. Following this, 50 μl of 6 N H_2SO_4 was added and OD was measured at 450 nm using a plate reader.

2.5. Statistics

For statistical analyses, Student's *t*-test, Fisher's exact test, and chi square test were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Epitope mapping of the MHV-N protein

To determine antigenic peptides for detecting anti-MHV antibodies, B-cell epitope mapping of the MHV-N protein was performed using the peptide array membrane. This array membrane comprised 446 spots, which consisted of 10-mer peptides obtained by a single amino acid shift, to produce a complete overlapping set of the N protein. Sera from mice that were infected accidentally with MHV were incubated with the peptide array membrane. These mice were confirmed to be positive using commercial ELISA for detecting anti-MHV antibodies. As shown in Fig. 1, the IgG antibodies in the sera, bound specifically to the peptides on the spots, were detected. The serum used in Fig. 1 contained antibodies that recognized at least four rows of spots, positioned at 27–29, 84–86, 183–187, and 362–366. This result indicates the presence of four antigenic sites in the MHV-N protein that are B-cell linear epitopes. In contrast, no significant intensity was observed when sera from control mice were used (data not shown). Sera from seven mice infected with MHV were then analyzed with the same method. The chemiluminescence intensity of each spot was measured, and the mean values are shown in the graph (Fig. 2). Highest intensity was observed around the 28th spot, on which a peptide consistent with amino acids 28–37 of the MHV-N protein was synthesized. A similar intensity was observed at the 362nd spot consistent with the intensity observed at the sequence position 362–371. The peptides on the spots with an intensity higher than 2.0 in Fig. 2 were defined as antigenic, and therefore, as B-cell linear epitopes. Sixteen B-cell epitopes in the MHV-N protein were found, and their position and sequence are shown in Table 1.