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Analysis of the Properties of Neutralizing Monoclonal Antibodies against the Hemagglutinating Encephalomyelitis Virus and Inhibition of HEV Infection by Specific MAb

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ABSTRACT. Eleven monoclonal antibodies (MAbs) that were reactive against the hemagglutinating encephalomyelitis virus (HEV), as seen in the enzyme-linked immunosorbent assay, were obtained. All of these MAbs showed neutralizing activity (1:20,000 to 1:800,000) against the 67N strain of HEV. They also showed hemagglutination inhibition activity (1:400 to 1:409,600). Western blotting tests revealed that all of these 11 MAbs were specific for the S protein of HEV. All MAbs with neutralizing activity showed the same fluorescent staining pattern. Ten-day-old mice pups were immunized with MAb, inoculated with the 10^5 tissue culture-infective dose of HEV at 3 days after immunization, and then examined to determine the viral inhibition. The 1:800-diluted MAb120 inhibited the viral infection in mice pups, though the 1:1,000-diluted MAb120 failed to inhibit the viral infectivity. These MAbs would be a useful tool for rapid and specific diagnosis of HEV and also for antibody-based treatment of the disease.

KEY WORDS: HEV, monoclonal antibodies, neutralizing activity.

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The porcine hemagglutinating encephalomyelitis virus (HEV) is a member of the genus Coronavirus, (family Coronaviridae, orders Nidovirales), that causes encephalomyelitis, or vomiting and wasting disease in suckling piglets [1, 2, 10, 14]. In infected animals, the virus reaches the central nervous system (CNS) through the neural pathways from the peripheral nerves. Previous studies have demonstrated that HEV propagated through the neural route and its infection was restricted to neurons after inoculation into sciatic nerve or footpad of rats [6, 16]. There was no cross neutralizing reaction observed with porcine transmissible gastroenteritis virus (TGEV) or porcine epidemic diarrhea virus (PEDV) which is detected in the porcine population [13].

HEV was first isolated in 1962 in Canada from suckling piglets with encephalomyelitis [4]. Today HEV is widespread among swine worldwide and there are frequent subclinical infections. The virus has a strong tropism for the epithelial cells of the upper respiratory tract and the CNS, and it is transmitted through nasal secretions [1]. The clinical symptoms of vomiting and wasting are assumed to be centrally induced by the infection of the vagus nerve, but a possible further dissemination of the virus into the CNS may lead to centrally induced motor disorders. Hirai, *et al.* reported that approximately 50% of swine sera tested positive for HEV antibodies by the hemagglutination inhibition (HI) test in Japan, suggesting that the virus spreads in pigs [5]. Sasaki *et al.* developed the neutralizing activity test (NT) using the established cell line FS-L3, which supports *in vitro* replication of pigs, in our laboratory [17].

Japan has had no significant outbreaks of HEV. In an etiological survey of HEV, many neonatal piglets received maternal antibodies against HEV from the colostrums of sows that were resistant to HEV. Pigs exhibit an age-related resistance to HEV [12].

In this study, 11 neutralizing monoclonal antibodies (MAbs) to the HEV 67N strain were prepared and used to examine the characterizations of MAbs. The MAbs inhibited HEV growth in mice.

MATERIALS AND METHODS

Virus and cells: FS-L3 cells were cultured in Eagle's minimum essential medium (MEM), which contained 7.5% fetal calf serum (FCS), and used for the neutralization test (NT). P3-X63-Ag8-U1 (P3U1) cells were cultured in Dulbecco's modified Eagle's MEM (DMEM) containing 10% of FCS and cultured for production of hybridomas.

The hemagglutinating encephalomyelitis virus (HEV) 67N strain, isolated in 1972 in the United States [10], was propagated in FS-L3 cells [16] and purified by sedimentation with a sucrose density gradient (20% to 50%) in TNE buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA).

Hybridoma production and selection: In order to establish hybridomas that secrete specific MAbs against HEV,

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BALB/c mice were immunized with purified HEV that was inactivated with formalin. The immunizations were repeated 3 times at 2-week intervals using Freund's complete adjuvant. The fusion test was performed 3 days after the final immunization.

To obtain hybridomas secreting HEV-specific MAbs, cells from the mouse myeloma cell line P3U1 were fused with the spleen cells from the BALB/c mice that were immunized with purified HEV by an intensive immunization protocol described previously [8]. The cell fusions were carried out as described by Kida *et al.* [8].

Enzyme-linked immunosorbent assay (ELISA): ELISA was performed by a modification of the method described by Ruitenberg *et al.* [15]. The viral antigen was appropriately diluted with phosphate-buffered saline (PBS) and 50 μl of each dilution was added into the wells of 96-well ELISA plates (BD Falcon). The plates were allowed to stand for overnight at 4°C and the dilutions were replaced with 100 μl of 2% skim milk in PBS. After incubation at 37°C for 1 hr the plates were washed twice with PBS containing 0.05% Tween 20 (PBS-T) and 50 μl of the supernatant was added to each well to detect the antibody. For determination of the antibody titer in ascitic fluid, 50 μl of 2-fold serial dilutions of ascitic fluid were added and the plates were incubated at 37°C for 1 hr.

After washing with PBS-T 3 times, 50 μl of goat anti-mouse IgG peroxidase conjugate (Jackson ImmunoResearch) was added and further incubation was carried out at 37°C for 1 or 2 hr. Then, the plates were washed again with PBS-T and 50–100 μl of o-phenylenediamine (OPD) solution (40 mg OPD and 0.02% H_2O_2 in 100 m*l* of phosphate-citrate buffer, pH 5.0) were added. After incubation at 37°C for 15 min, the color that developed as a result of the enzyme-substrate reaction was measured at an optical density of 450 nm (optimal absorbance for OPD is 490 nm) with a microplate reader (MPR-A4iII, Tosoh). The dashed horizontal line indicates the cut-off value that was calculated by adding 2 standard deviations to the mean of the ascitic fluid.

Virus neutralization: Neutralization assays were carried out with the FS-L3 cell culture prepared in a 96-well of tissue culture plate (Nunc). A 250 μl of 200 $TCID_{50}$ virus and 250 μl of MAb were mixed in a 1.5 m*l* tube and incubated at 37°C for 1 hr. The virus-MAb mixtures were transferred into wells containing a monolayer of FS-L3 cell culture. After incubation at 37°C for 1 hr, 100 μl of MEM was added to the plates, and they were incubated in an atmosphere of 5% CO_2 in air at 37°C for 4 days. The antibody titer was expressed as the reciprocal of the highest antibody dilution that inhibited the cytopathic effect of the virus.

Hemagglutination (HA) test and hemagglutination inhibition (HI) test of MAbs: The HA test was carried out by the microtiter method as described by Noda *et al.* [11]. One unit of the HA titer of HEV was determined using 1% chicken erythrocytes (ChRBC). Four units of HEV in a volume of 25 μl was mixed with 25 μl of 2-fold serially diluted MAb, and added along with 50 μl of 1% ChRBC to a 96-well plate. After the agitation the plate was allowed to stand at 4°C for 30–45 min. The HI titer of MAb was calculated as the reciprocal of the highest antibody dilution that showed HI.

Protease inhibitor: Protease inhibition mixture-DMSO solution for mammalian cell and tissue extracts (Wako) (1 vial) diluted at 10^{-3} was added to virus purification including ultracentrifugation.

Immunoblotting analysis: The binding of MAbs to the viral proteins and transfer onto a Polyvinylidene Difluoride (PVDF) membrane (Millipore Immobilon P) were carried out essentially as described by Burnette [3]. Briefly, the proteins were separated by 6% polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli [9]. The proteins were transferred to PVDF membranes, and the membranes were incubated with skim milk at 37°C for 1 hr. The membranes were incubated with MAb at a dilution of 1:100, rinsed three times in PBS-T, and incubated with goat anti-mouse IgG peroxidase conjugate (Jackson ImmunoResearch). Subsequently, after 3 more 20-min rinses, the membranes were incubated with BCIP/NBT substrate solution (Sigma).

Immunofluorescence assay (IFA): The FS-L3 cells monolayer on 8-well heavy teflon-coated glass slide (Bokusui Brown) was infected with the HEV in 25 μl of MEM at a multiplicity of infection of 1. After 5, 10, and 24 hr incubation at 37°C, the cultures were rinsed with PBS, dried, and fixed with cold acetone. For the IFA the infected cells on the glass slide were inoculated with 25 μl of 1:100 dilutions of the MAbs and incubated at 37°C for 1 hr. The cells were then rinsed with PBS-T for 30 min, and anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Jackson ImmunoResearch) that was diluted to 1:500 was added. After incubation at 37°C for 1 hr, the cultures were washed in PBS-T for 30 min. The infected cells were examined under a fluorescence microscope (Olympus BX52), equipped with 10X objective lens, and the images were captured using an attached digital camera (Magnifier).

Isotype and subtype of MAb: The isotype and subtype of MAbs were determined with Immunoglobulin Typing, kits, Mouse (Wako Pure Chemical Industries Ltd). A 100 μl solution of each MAb (1:500) was added to each respective well corresponding to IgG1, IgG2a, IgG2b, IgG3, and IgM.

Inhibition or neutralization of viral infection by MAbs in mice: HEV was propagated and assayed for infectivity in FS-L3 cells. Ten-day-old ICR mice pups were used in this experiment. Each different dilution of MAb120 (1:10, 1:100, 1:400, 1:800, 1:1,000, and 1:1,500) was inoculated at a dose of 50 μl/mouse intraperitonealy. After 3 days HEV (50 μl of the 10^5 $TCID_{50}$) was inoculated intraperitonealy and examined for 5 days. Three mice were inoculated with myeloma ascites fluid as a control group.

The infected brains of the dead mice were collected and the virus was titered in the FS-L3 cells. The mouse inoculated with 1:800-diluted MAb120 survived and was sacrificed 5 days after viral inoculation. One dead mouse with the myeloma ascites and another from the group inoculated with 1:1,500-diluted MAb were titered. The viral titer was

assayed by $TCID_{50}$ in the FS-L3 cells. A 0.5 g brain was homogenized in 5 m*l* of PBS and centrifuged at 2,500 rpm for 15 min. The supernatant was passed through a Millipore HA filter and inoculated to a 96-well plate (0.1 m*l*/well).

RESULTS

Hybridomas secreting antibody specific to HEV were cloned by the limiting dilution assay, and 11 clones secreting MAbs were finally obtained. The antibody titers determined by the NT, HI activity, ELISA, and immunoglobulin isotype of these clones against the 67N strain of HEV are shown in Table 1. Immunofluorescence staining patterns are shown in Fig. 4. MAb22 and MAb42 were representative of all MAbs.

NT and HI activity of MAbs against HEV: Neutralization studies showed that the titers of all the MAbs ranged between 1:20,000 and 1:800,000. HI activities revealed that the titers ranged between 1:400 and 1:409,600 (Table 1).

ELISA titers of MAbs against HEV: A wide range of ELISA titers ranging from 1:4,450 to 1:250,000 were found among the different MAbs (Table 1).

Polypeptide specificity: The HEV hybridomas were also examined by western blotting tests. Purified virus was ultracentrifuged without protease inhibitor. The purified virus was treated only with 2-mercaptoethanol (2-ME) and did not react with any MAbs in the western blotting tests (SDS-PAGE) (Fig. 1A). However, all the MAbs reacted with approximately 150 kDa protein of HEV obtained by treatment with protease inhibitor alone (Fig. 1B). The purified

Table.1. Characterization of MAbs produced against HEV 67N strain

MAb	Neutralization test (NT)	Hemagglutination inhibition test (HI)	ELISA test	Viral protein[a)]	Isotype[b)]
3	40,000	12,800	12,800	S	IgG2b
17	20,000	800	4,450	S	IgG2b
22	400,000	12,800	12,800	S	IgG2b
37	40,000	38,400	75,000	S	IgG2b
42	200,000	12,800	6,400	S	IgG1
61	160,000	6,400	150,000	S	IgG2b
97	80,000	400	104,200	S	IgG1
120	800,000	409,600	12,800	S	IgG2b
124	40,000	400	12,800	S	IgG2b
151	400,000	102,400	12,800	S	IgG2b
1F1	200,000	51,200	250,000	S	IgG1
Myeloma	<10	20			

a) Determined by western blotting test.
b) Determined by Immunoglobulin Typing Kit, Mouse (WAKO).

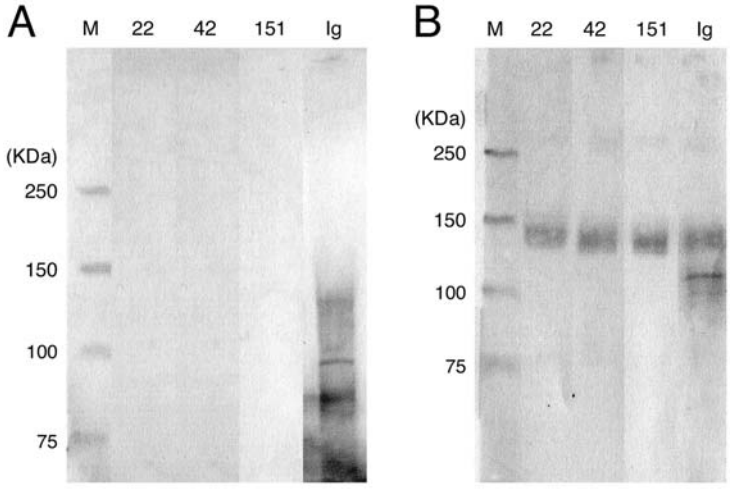

Fig. 1. Polypeptide specificity of anti-HEV monoclonal antibodies by western blotting tests. (A) Purified HEV in the absence of protease inhibitor was mixed with 2-ME (+) sample buffer, (B) purified HEV in the presence of protease inhibitor was mixed with 2-ME (–) sample buffer. They were boiled for 2 min, separated by 6% SDS-PAGE, and transferred onto the PVDF membrane. M: size maker (Precision Plus Protein Standards), 22: MAb22, 42: MAb42. 151: MAb151, Ig: immunoglobulin (mouse polyclonal antibody).

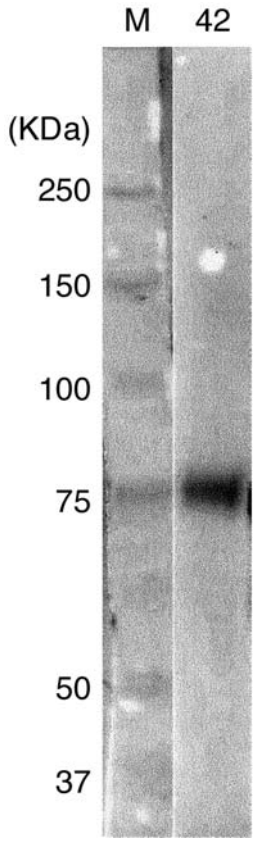

Fig. 2. Polypeptide specificity anti-HEV monoclonal antibody against the S protein cleaved by protease, Protease in cells worked and 2-ME did not destroy the S-S bond. Purified HEV was separated by 6% SD-PAGE and transferred onto the membrane. M: size marker (Precision Plus Protein Standards, Bio-Rad), 42; MAb42.

virus that was not treated with either protease inhibitor during purification and not treated with 2-ME in SDS-PAGE showed a band of 75 kDa (Fig. 2).

Immunofluorescence staining patterns and reaction of MAbs: The reactivity of MAbs against HEV was examined by IFA. The staining patterns were detected by the immunofluorescence reaction with MAb22 and MAb42 (Fig. 3). MAb22 and MAb42 appeared at 5 hr post infection (p.i.) and were strongly diffused in the cytoplasm, and they appeared around the cytoplasmic membrane at about 10 and 24 hr p.i. In the case of the other MAbs, the same pattern was observed (data not shown).

Isotype of MAbs: Isotypes of MAbs against HEV were determined with the mouse Immunoglobulin Typing kit, Mouse. As summarized in Table 1, they were either IgG2b or IgG1.

Inhibition of virus infection by MAb in mouse: All the mice pups survived after inoculation of MAb120 dilution at 1:10, 1:100, 1:400, and 1:800 after 5 days. Two of three mice inoculated with 1:1,000 diluted MAb120 died after HEV injection. All the mice inoculated with a 1:1,500 dilution of MAb120 died after HEV injection (Fig. 4).

The mice were inoculated with MAb120, which had the highest neutralizing titer. The brain of one of the mice that survived after inoculation with 1:800-diluted MAb120 was checked. One dead mouse inoculated with myeloma ascites fluid and another from the group inoculated with 1:1,500-diluted MAb were titered and found to contain 10^6 $TCID_{50}$/m*l* and 10^5 $TCID_{50}$/m*l*, respectively. There was no detectable viral titer in the brain of the mouse inoculated with 1:800-diluted MAb.

DISCUSSION

Eleven hybridomas producing neutralizing antibodies were obtained by screening ELISA against the HEV 67N strain (1:20,000 to 1:80,000), and exhibited HI activity (1:400 to 1:409,600). This is the first report on monoclonal antibodies against HEV. Fluorescent microscopy, virus antigen was observed as fine granulation that was essentially limited to the perinuclear area (5 hr p.i.), and uniformly but faintly distributed in the cytoplasm (24 hr p.i.). All MAbs showed to the same staining pattern.

Western blotting revealed that all MAbs recognized approximately 150 kDa protein. When the purified viral sample was treated with 2-ME in SDS-PAGE and without protease inhibitor during viral purification, the MAbs did not recognize the 150 kDa protein (Fig. 1A). On the other hand, MAbs recognized the 150 kDa protein when the viral sample was treated with the protease inhibitor alone during ultracentrifugation (Fig. 1B). The spike glycoprotein (S protein) of HEV is translated as a large polypeptide that is subsequently cleaved by virus-encoded or host-encoded proteases to produce 2 functional subunits, namely, S1 and S2, similar to other coronaviruses [18]. The molecular weight of S, S1, and S2 of HEV 67N strain (Gen Bank accession no.Q8BB25) were estimated respective 149 kDa, 84 kDa, and 65 kDa by using a GENETYX-MAC v10.1 (Software Development). When the purified viral sample was not treated with either protease inhibitor or 2-ME, MAb 42 identified a 75 kDa protein (Fig. 2). We could not estimate whether the 75 kDa protein was corresponding to S1, S2 or an artificial protein of S protein.

Hirano *et al.* reported that HEV infection occurred 48 hr after the treated rats were inoculated with HEV in the hind leg [7]. However, in the case of our experiment, MAb120 was inoculated before HEV injection and the complete immunization was achieved (1:800 of MAb120). MAbs inoculated in the interior of body may inhibit viral invasion of the nerve cells.

The highlight of the present study is production of monoclonal antibodies of neutralizing MAbs. Second, the neutralizing MAbs serve as probes for studying the immunogenicity and antigenicity of the HEV S protein. Third, the neutralizing MAbs may aid in the development of immunotherapeutic treatment of HEV.

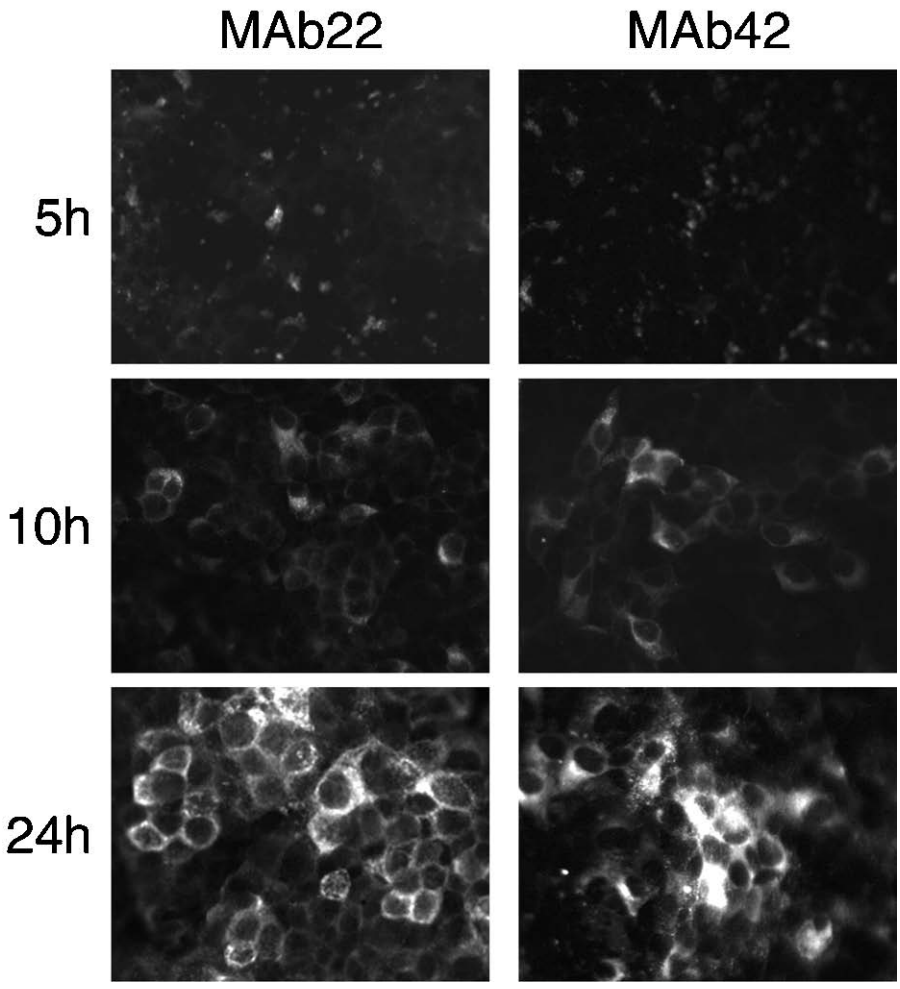

Fig. 3. Immunofluorescence staining of HEV in FS-L3 cells by MAb22 and MAb42 at 5, 10 and 24 hr p.i. Cells were fixed and labeled with HEV S specific MAbs followed by FITC-labeled goat anti-mouse IgG. Incubation time-dependent fluorescence increment was observed in the cytoplasm.

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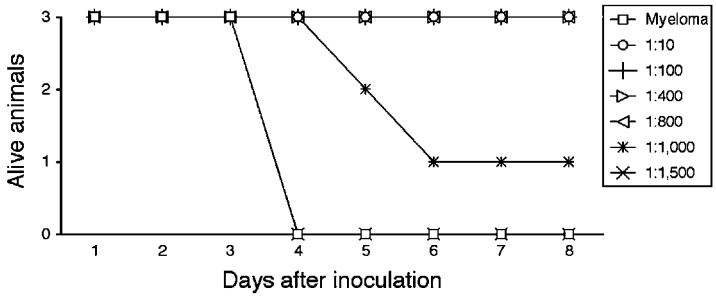

Fig. 4. Inhibition of HEV infection by MAb120. More than 1:800 (1:10, 1:100 and 1:400) diluted: not dead, 1:1,000 diluted: 2/3 dead, Myeloma and 1:1,500 diluted: all dead. After the inoculation of HEV, mice lived days. MAb120 (1:10, 1:100, 1:400, 1:800, 1:1,000, 1:1,500 and myeloma) were inoculated into 3 mice (BALB/c). After 3 days, virus were injected into the same mice.

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