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Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies

J. Arikawa[1], J.-S. Yao[2], K. Yoshimatsu[1], I. Takashima[2], and N. Hashimoto[2]

[1] Institute of Immunological Science and [2] Department of Veterinary Public Health, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan

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Summary. To investigate the role of Hantaan virus envelope glycoprotein in infection, a panel of monoclonal antibodies (MAbs) was examined in vitro with several serological tests and in vivo by passive transfer experiments in mice. An antigenic site, specific for the inhibition of infected cell focus was detected with the focus inhibition neutralization test (FINT), in addition to the neutralization related antigenic sites, which were revealed by the ordinary focus reduction neutralization test (FRNT). Suckling mice were given the MAbs by passive transfer followed by lethal Hantaan virus challenge. All neutralizing MAbs detected by either FRNT or FINT protected all mice from lethal infection, confirming the importance of the antigenic sites as a protective antigen. Mice given non-neutralizing MAbs by passive transfer, however, began to die earlier than the control group; mean time to death (18.2 ± 2.1 to 21.5 ± 2.8 days) being significantly shorter than that of the control group (25.8 ± 1.8, $p < 0.01$, Mann-Whitney, *U* probability test). Virus titers in brains of mice which died early, were about 10 times higher than those of control mice. These results indicated the early death phenomenon of mice which was mediated by the anti-virus antibody.

Introduction

Haemorrhagic fever with renal syndrome (HFRS) is a rodent borne viral disease characterized by fever, renal disorder and hemorrhagic manifestations [34, 35]. HRFS patients have been reported throughout Eurasia and parts of Africa [12] particularly in China [8, 27], Korea [11], Northern [10], and Eastern Europe [15].

Hantaan virus is the causative agent of HFRS and classified in the family *Bunyaviridae*, genus *Hantavirus* [24]. This virus has two different glycoprotein projections (G1 and G2) on the surface of the virion [25]. In our previous

experiments, nine distinct antigenic sites, two on G1 and seven on G2, were demonstrated by competitive binding assay using a panel of monoclonal antibodies (MAb) to the G1 and G2 proteins. In addition, some of the MAbs to both the G1 and G2 proteins had neutralizing activity [1]. Therefore, similar to other enveloped viruses, the G1 and G2 proteins of the Hantaan virus have been considered to play an important role in virus infection.

Recently, it was found that the MAbs to the antigenic sites having hemagglutinating activity on G2 protein mediated the enhancement of hantavirus infection of macrophages in vitro [36]. This may be caused by the enhanced attachment of virus-MAb complex to the Fc receptor on the macrophage. Although the infected macrophage is thought to be responsible for the spread of infection in rodents and humans, the actual role of the antigenic sites on the viral envelope protein in the infection remains unclear.

To investigate the immunological role of antigenic sites in Hantaan virus infection in more detail, a panel of monoclonal antibodies (MAbs) was examined in vitro by two different neutralization tests, FRNT and FINT; and in vivo by passive transfer experiments in suckling mice.

Materials and methods

Cell culture and virus

The E6 clone of Vero cells (ATCC C1008, CRL 1586) [25] was grown in Eagle's minimum essential medium (Eagle's MEM, Nissui Co., Tokyo Japan) supplemented with 5% fetal calf serum and 0.292 g/l of L-glutamine. Hantaan virus, strain 76–118, isolated from the Korean field rodent *Apodemus agrarius coreae* [13], was used throughout this study. Virus was inoculated into the Vero E6 cell monolayer and maintained with the same medium described above. Four days after infection, culture fluid was collected and stored at −80 °C as stock virus. The infectivity titer of the virus was measured using the peroxidase-anti-peroxidase (PAP) method as described previously [30].

Indirect immunofluorescenct antibody (IFA) test

Vero E6 cells infected with Hantaan virus, strain 76–118 were monodispersed in culture medium and dropped onto spot slide glasses. They were incubated at 37 °C in a CO_2 incubator for four hours to extend the cells on the glass, then washed with PBS. The cells were fixed with cold acetone and used as antigen. Fluorescent isothiocyanate (FITC) conjugated anti-mouse immunoglobulins or anti mouse IgM (μ chain specific) (goat; Cappel Laboratories, Cochranvilla, Pa.) were used as the second antibody. IFA titers were expressed as the reciprocals of the highest dilution giving specific immunofluorescence in the infected cell cytoplasm. IFA titers of 1 : 10 or more were regarded as positive [3].

Focus reduction neutralization test (FRNT) and focus inhibition neutralization test (FINT)

FRNT was described previously [20]. Briefly, a serial 10-fold dilution of mouse ascitic fluid or immune serum was mixed with an equal volume of virus suspension. The mixture was kept at 37 °C for 1 hr, then inoculated onto Vero E6 cell monolayers in 96 well plates. After incubation for 1 hr, the inoculum was removed and the monolayers were covered with overlay medium (MEM containing 1.5% carboxymethylcelullose and 5% FCS). After

incubation for 7 days at 37 °C in a CO_2 incubator, the cell monolayer was washed with phosphate buffered saline (PBS) and fixed with methanol for 10 min at room temperature. Infected cell foci were stained by the peroxidase-anti-peroxidase (PAP) method with rabbit immune sera and the number of infected cell foci was counted. FRNT titer was regarded as the highest dilution of ascitic fluid causing 80% or more reduction in the number of infected foci formed in the presence of control ascitic fluid instead of antibody. In the focus inhibition neutralization test (FINT), a virus suspension containing 30 to 50 focus-forming units (FFU) of virus per 50 µl was inoculated onto Vero cell monolayers in 96 well plates. After incubation for 1 hr, the inoculum was removed and the cells were covered with overlay medium containing serial 10-fold dilutions of ascitic fluid. The FINT titer was defined by the 80% reduction method as described for the FRNT titer.

Membrane FA

Membrane FA was performed according to the method of Franko et al. [4]. Briefly, a Vero E6 cell monolayer infected with Hantaan virus, strain 76–118 was monodispersed by PBS containing 0.1% trypsin and 0.02% EDTA. The cells were washed twice with PBS by low speed centrifugation. The cell pellet was resuspened with a 1 : 100 dilution of ascitic fluid or control ascitic fluid, then incubated for 1 hr at 37 °C. After 3 PBS washes, the cells were mixed with FITC labeled anti-mouse Ig(G,M,A) (goat; Cappel Laboratories, Cochranville, Pa.). After incubation for 1 hr at 37 °C, the cells were washed as above and finally mixed with 50% (v/v) glycerin in PBS. Cells were examined under the fluorescence microscope.

Passive protection studies with MAbs to Hantaan virus

Outbred ICR mice were obtained from Shizuoka Laboratories (Shizuoka, Japan) and mated to produce suckling mice. A group of 8 to 10 suckling mice (less than 24 hr after birth) were inoculated subcutaneously (s.c.) with 50 µl of undiluted ascitic fluid containing a MAb specific for Hantaan virus, strain 76–118 (Table 1) or normal ascitic fluid. Four hours after the adoptive transfer of the ascitic fluid, mice were challenged with a s.c. injection of 5×10^3 FFU (10 LD_{50}) of Hantaan virus, strain 76–118. Survival rates were recorded for 5 weeks after the challenge. Serum specimens of surviving mice were obtained by cardiac puncture under ether anesthesia. The filter paper method [3] was used for collecting blood from dead or moribund mice. The passive protection study was carried out one time to each MAb clones, except to clone 6D4. All animals were treated according to the Laboratory Animal Control Guidelines in our institute which was basically in conformity to National Institutes of Health-American Association of Laboratory Animal Control Guidelines. All the animal experiments were carried out in a class P3 facility.

Titration of virus in brain, lung, and spleen

Organs were removed from mice when they were moribund, dead, or 35 days post challenge (surviving mice). Each organ was homogenized as a 10% suspension in Eagle's MEM containing 5% fetal calf serum, 60 µg/ml of kanamycin, 400 units/ml of penicillin, and 400 µg/ml of streptomycin. Ten-fold dilutions of the suspension were inoculated onto Vero E6 cell monolayers in 96 well plates. After incubation for 1 hr at 37 °C, the inoculum was removed and replaced with overlay medium. After incubation for 7 days, infectivity titers were measured by the PAP method as described previously [30].

Results

Characterizations of antigenic sites using monoclonal antibodies

Table 1 shows the list of MAbs used in this study. Names of MAb clones, antigenic sites recognized by the clones and HAI activities have been described

Table 1. Characterization of polyclonal and monoclonal antibodies by an in vitro assay

Antigenic site	MAb	HAI activity	Neutralization by[a]		Membrane FA
			focus reduction (FRNT)	focus inhibition (FINT)	
G1-a-(1)	8B6	−	−	−	+
G1-a-(2)	6D4	−	−	−	+
	10F11	−	−	−	+
G1-b	2D5	+ + +	+ +	+ +	+
	3D5	+ + +	+ + +	+ + +	+
	16D2	+ + +	+ + +	+ + +	+
G2-a-(1)	HCO2	+	+ +	+ +	+
G2-a-(2)	16E6	+	+ +	+ +	+
G2-b	EBO6	+ + +	−	−	+
G2-c	11E10	+ + +	+ +	+ + +	+
G2-d	17G6	+ +	−	−	+
	3D7	+ + +	−	−	+
	5B7	+ + +	−	−	+
G2-e	20D3	+ + +	−	−	+
G2-f-(1)	8E10	+ + +	−	+ +	+
	1C6	+ + +	−	+	+
	1G8	+ + + +	−	−	+
	23G10-2	+ + +	−	−	+
	3B6	+ + +	−	−	+
G2-f-(2)	23G10-1	+ +	−	−	+
	7G6	+ +	−	−	+
	18F5	+	−	−	+
G2-f-(3)	GDO5	−	−	−	+
Anti Hantaan mouse immunesera		+ +	+ + +	+ +	+

[a] − Less than 80% focus reduction or inhibition at 1 : 10 dilution; more than 80% focus reduction or inhibition at 1 : 10 (+), 1 : 100 (+ +), 1 : 1,000 (+ + +) dilution

in our previous report [1]. The immunoglobulin isotypes of clones HCO2 and 18F5 were IgG2b and IgG2a, respectively. The rest of the clones possessed IgG1 isotype. Neutralizing activities of the MAbs to Hantaan virus, strain 76–118 were examined using two different neutralization tests; FRNT and FINT. As shown in Table 1, antigenic sites G1-b, G2-a and G2-c related to virus neutralization in vitro and these results were the same as those obtained by plaque reduction neutralization test in our previous report [1]. All the MAb clones positive in FRNT also inhibited focus formation (FINT). Clones 8E10

and 1C6 to antigenic site G2-f however, were negative by FRNT, but inhibited focus formation. Thus, additional neutralizing antigenic sites were found out by FINT.

Expression of viral antigens on the surface of infected cells was examined by the membrane FA test. As listed in Table 1, all the MAb clones reacted with unfixed infected cells. The intensity of FA was similar regardless of the MAb clones used.

Passive protection of suckling mice with MAbs

To examine the antigenic sites related to protective immunity in suckling mice, at least one MAb clone representative of each of the different antigenic sites was selected and passively transferred to suckling mice followed by a lethal

Table 2. Passive protection of suckling mice with monoclonal antibodies to Hantaan virus, strain 76–118

Antigenic site	MAb	No. mice						Mean time to death[b] (Day- ± SD)
		Tested	Died	Sero-positive				
				IgG	(GMT)[a]	IgM	(GMT)	
G1-a-(1)	8B6	10	10	10	(970)	0	(< 10)	18.2 ± 2.1[c]
G1-a-(2)	6D4	10	0	10	(1,016)	0	(< 10)	survived
	6D4	10	0	10	(676)	0	(< 10)	survived
G1-b	16D2	10	0	0	(< 10)	0	(< 10)	survived
G2-a-(1)	HCO2	10	0	0	(< 10)	0	(< 10)	survived
G2-a-(2)	16E6	10	0	10	(520)	0	(< 10)	survived
G2-b	EBO6	10	5	10	(4,064)	0	(< 10)	20.6 ± 3.2[c]
G2-c	11E10	10	0	5	(226)	1	(160)	survived
G2-d	17G6	10	4	10	(4,188)	1	(40)	20.3 ± 5.6[c]
G2-e	20D3	10	10	10	(2,048)	0	(< 100)	20.0 ± 1.6[c]
G2f-(1)	8E10	10	0	10	(279)	0	(< 10)	survived
G2-f-(2)	23G10-1	10	8	10	(761)	1	(160)	21.5 ± 2.8[c]
G2-f-(3)	GD05	8	7	8	(1,076)	1	(40)	18.9 ± 3.2[c]
Anti Hantaan mouse immunesera		10	0	3	(2,037)	2	(40)	survived
Normal ascitic fluid		10	9	10	(549)	0	(< 10)	25.8 ± 1.8

[a] Geometric mean IFA titer

[b] Mean time to death was calculated among the mice that died within observation period (35 days)

[c] $p < 0.01$

Table 3. Hantaan virus titers in tissues of challenged mice that passively received polyclonal or monoclonal antibodies

Antigenic site	MAb	Mean virus titers (log 10)[a] in		
		brain	lung	spleen
G1-a-(1)	8B6	5.94	4.87	4.38
G1-a-(2)	6D4	–[b]	–	–
G1-b	16D2	–	–	–
G2-a-(1)	HCO2	–	–	–
G2-a-(2)	16E6	–	–	–
G2-b	EBO6	5.94	5.26	–
G2-c	11E10	–	–	–
G2-d	17G6	6.15	5.12	–
G2-e	20D3	5.88	5.86	–
G2-f-(1)	8E10	–	–	–
G2-f-(2)	23G10-1	6.08	4.64	–
G2-f-(3)	GDO5	6.18	5.49	3.52
Anti Hantaan mouse immunesera		–	–	–
Medium		5.08	4.90	–

[a] log10 FFU/g tissue
[b] Less than 200 FFU/g tissue

challenge with Hantaan virus, strain 76–118 (Table 2). All the FRNT MAbs (clones 16D2, HCO2, 16E6 and 11E10) completely prevented lethal infection. In addition, clones 16D2, HCO2 and 11E10 protected all or half of the mice from infection since they had no sero-conversion after the challenge. Clone 8E10, positive in FINT but negative in FRNT, protected all the mice from lethality but failed to protect from infection. Thus, either G1 or G2 envelope proteins were responsible for induction of protective immunity. Interestingly, clone 6D4 to antigenic site G1-a-2, which had no neutralizing activity in either FRNT or FINT, protected all the mice from lethal infection. The experiment was repeated and the same result was obtained. The rest of the non-neutralizing clones were divided into two groups depending on the protective activity from lethal infection. Most of the mice which received MAb clones 8B6, 20D3, 23G10-1 and GDO5 died, but clones EBO6 and 17G6 partially protected the mice from lethal infection.

Early death of the mice passively transferred with MAb

As described above, non-neutralizing MAbs failed to protect the mice from lethal infection. Mean time to death for mice are listed in the right hand column

of Table 2. Although the survival rates differed between clones transferred, the mean time to death among groups of mice ranged from 18.2 ± 2.1 (clone 8B6) to 21.5 ± 2.8 days (clone 23G10-1) and were significantly shorter than those of the control group (25.8 ± 1.8, $p < 0.01$, Mann-Whitney U probability test). Survival curves for the mice transferred with the non-protective antibodies (clones GDO5, 8B6 and 20D3) are shown in Fig. 1 a. In all groups, mice began to die 4 to 5 days earlier than control mice. On the other hand, though clone 17G6 or EBO6 partially protected the mice from lethal infection, they began to die earlier than the controls (Fig. 1 b).

Virus titers in tissues of challenged mice that received passively transferred polyclonal and monoclonal antibodies

Virus titers in brain, lung and spleen tissues of immunized mice were measured and compared with those of the control group. Organs were collected when mice were moribund, dead, or 35 days after the challenge (surviving mice). Virus was recovered from the organs from dead mice, but not from those of the survivors. Higher virus titers for dead mice were detected in brain tissues

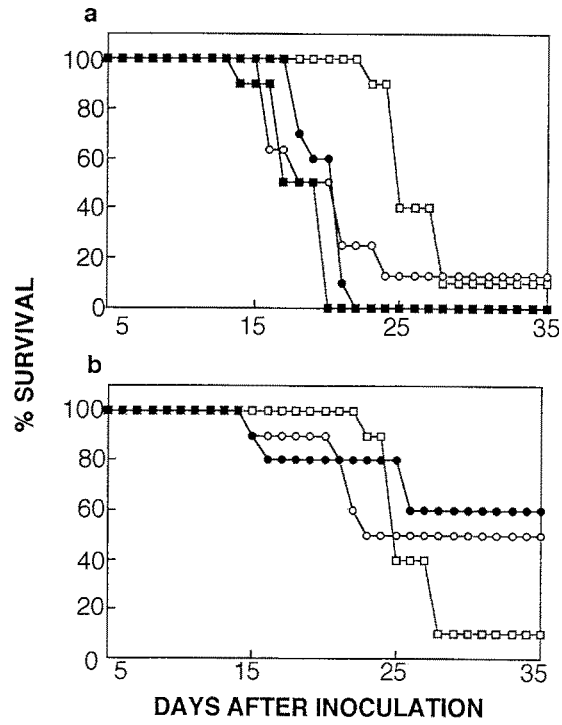

Fig. 1. Passive protection study of suckling mice. Outbred ICR suckling mice (less than 24 hr old) were inoculated s.c. with 50 µl of normal ascitic fluid or MAbs to Hantaan virus envelope protein. Four hours after the transfer, mice were challenged by s.c. infection with 5×10^3 FFU (10 LD50) of Hantaan virus, strain 76–118. **a** Transferred with normal ascitic fluid (□) or MAb clones GDO5 (○), 8B6 (■), 20D3 (●). **b** Transferred with normal ascitic fluid (□) or MAb clones 17G6 (●) and EBO6 (○)

rather than in the lung or spleen. Virus titers obtained from brain tissues were about ten times higher than those from control mouse brain, although the virus titers in lung tissues were similar for control and immunized mice.

Discussion

One of the G1 antigenic sites and two of the G2 sites of Hantaan virus envelope protein are involved in virus neutralization, as demonstrated by plaque reduction neutralization with a panel of MAbs [1]. In the present study, an additional antigenic site related to virus neutralization was identified with MAb clones 8E10 and 1C6, which were positive in FINT but negative in FRNT (Table 1). In paramyxoviruses, when the MAb to fusion (F) protein was contained in the agar overlay medium, plaque formation was effectively inhibited comparing to its plaque reduction neutralization activity [31]. The inhibition of plaque formation is considered to be caused by the inhibition of cell to cell spread of virus infection, since the (fusion) F protein mediated the cell to cell fusion but not relate to the virus attachment to cell. We have reported that Hantaan virus has cell fusion activity under the low pH condition [2]. Therefore, to examine the neutralization mechanism related to the newly recognized antigenic site, attachment blocking activity as well as fusion inhibition activity of clones 8E10 and 1C6 will be required.

To investigate the role of Hantaan virus envelope protein in infection in vivo, suckling mice were passively given MAbs followed by Hantaan virus challenge. All the animals transferred with FRNT MAbs either to G1 or G2 protein were completely protected from Hantaan virus challenge because all the mice survived and most of them showed no sero-conversion (Table 2). These results were consistent with a recent report by Schmaljohn et al. [23], in which hamsters were passively protected from infection only with MAbs having plaque reduction neutralizing activity. In addition, MAb clone 8E10, which recognized the antigenic site related to neutralization discovered here, also protected suckling mice against lethal infection, although it did not protect the mice from infection (Table 2). The MAb may retard the spread of viral infection in mice. The fatal outcome of experimentally inoculated mice or rats was strictly dependent on the age of the animals. Complete resistance from lethal Hantaan virus challenge was gained at two weeks of age in mice [9, 18] and at one week in the rat [37]. Therefore, the delay in viral spread at an early stage of infection may result in survival at a later stage due to age-dependent resistance. These results confirmed the important role of envelope protein as protective antigen.

Clone 6D4 to antigenic site G1-a-2 protected all the mice from lethal infection, although the antibody had no neutralizing activity in either FRNT or FINT and also had no HAI activity. The protective effect of non-neutralizing antibody in passively transferred mice has been reported in various viruses [7, 14, 20–22, 26, 29]. All the reports ascribed the protective mechanisms to cell mediated immune responses against infected cells, such as complement-mediated lysis of infected cells, or antibody dependent cell-mediated cytotoxicity (ADCC).

Since this MAb clone was positive in the membrane FA test but lacked complement binding activity (subclass IgG1), ADCC rather than complement-mediated cytolysis of infected cells was suggested. No protective activity was detected in the remaining non-neutralizing clones for reasons which remain to be determined. To examine relationship between this phenomenon and antigenic site (G1-a-2) in more detail, we are now planing to carry out similar experiments with the other MAb clone 10F11, which binds to the same antigenic site as clone 6D4.

As shown in Fig. 1 and Table 2, mice began to die earlier than the control group and the mean time to death were significantly shorter than those of control mice. Virus titers in the brains of mice that died early (7.5×10^5 to 1.5×10^6 FFU/g), were apparently higher than those of control mice (1.2×10^5 FFU/g), although the virus titers in the lungs were similar. Several animal experiments have indicated that the death of challenged mice is closely related to virus titers in brain tissues and virus titers of a lethal threshold are around 10^5 FFU per gram [17, 18, 28]. Recently, the antibody dependent enhancement (ADE) of hantavirus infection to Fc-receptor bearing cells such as macrophages, caused by enhanced binding of virus and antibody complex via Fc receptors on the cell has been reported [36]. Since macrophages are susceptible to hantavirus infection and are believed to be responsible for the spread of infection in mice [16], ADE of infection to macrophages is considered a plausible mechanism for the rapid growth of virus in the brain. To examine if the early death presented here is related to high virus titers in the brain, studies on the kinetics of virus growth in various organs of mice with or without antibody transfer are now in progress. Similar phenomena have been reported in mice challenged with yellow fever and JE [5], Langat [32] and rabies viruses [19], as well as cat coronavirus [33], but few reports have discussed the mechanisms involved. Gould et al. [6] reported that enhanced neurovirulence in mice infected with yellow fever virus is not mediated through Fc and complement receptor-bearing macrophages, but that the cytotoxicity to infected brain tissues is caused by antibodies. There were found to be severe pathologic changes in the brain tissue of antibody transferred mice compared with controls, however virus titers in the brain were similar regardless of pre-existing antibody. In the present study, however, the high virus titers in brain tissues without histological changes suggest a different mechanism from that shown in yellow fever virus infection.

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Authors' address: Dr. J. Arikawa, Institute of Immunological Science, Hokkaido University, Sapporo 060, Japan.

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